From THE DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

ASPECTS ON THE PSYCHOPHARMACOLOGY OF CHOLECYSTOKININ

Diana Radu MD

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Cover: CCK mRNA expression in monkey, rat and human brain.

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ABSTRACT

This thesis includes studies of the effect of mild stress on brain cholecystokinin (CCK), anatomy of markers for CCK-ergic transmission in the brain and the systemic effect of CCK receptor stimulation in a clinical test for anxiety and fear. CCK is a brain-gut peptide acting as a neurotransmitter in mammalian brain via CCK_A (CCK_1) and CCK_B (CCK_2) receptors. In the brain, CCK is widely distributed to both cortical and sub-cortical structures, coexisting and interacting with "classical" neurotransmitters such as dopamine (DA), glutamate and GABA. Brain CCK seems to be involved in regulation of various physiological and cognitive functions. The prefrontal cortex (PFC) is an important association area in the psychopathology of stress, anxiety disorders and schizophrenia. To date there are several models involving DA and glutamate-systems in mimicking aspects of psychosis in humans and different paradigms for studying stress, and human and animal data suggest possible CCK-ergic involvement in these conditions.

In rat PFC, mild stress such as an intraperitoneal (i.p.) saline injection leads to acute region-specific decrease in CCK peptide levels within 20 minutes followed by increased levels at 8 hours. The decrease in CCK 20 min following i.p. saline injection was more accentuated in ketamine (glutamate NMDA receptor antagonist) pre-treated rats and the increase at 8 hours was absent in those animals. However, no changes in CCK mRNA in rat PFC up to 8 hours following i.p. saline injection were observed.

Tripeptidyl peptidase II (TPP II) is a putative CCK-degrading enzyme and the brain distribution of its mRNA was examined. The mRNA encoding TPP II is broadly distributed to regions rich in CCK in monkey brain. Distribution of the mRNA encoding the CCK_A receptor in monkey brain suggests a broader localization than previously reported using receptor binding autoradiography. CCK_A mRNA was localized to the cerebral cortex, ventral striatum, amygdala region, hippocampus and substantia nigra. TPP II and CCK_A receptor mRNAs in Brodmann area (BA) 10 of human PFC show tendencies to decrease in subjects having had a schizophrenia diagnosis as compared to controls.

In anxiety research, CCK_B receptor stimulation has been used to simulate panic attacks. In addition, gastrin/CCK_B receptor stimulation is known to induce insulin/C-peptide release from the pancreas. In an effort to develop a bioassay for phenotypes relevant for anxiety sensitivity and anxiety disorders, serum levels of C-peptide were measured following CCK_B receptor stimulation by pentagastrin. Pentagastrin administration in doses up to 0.2 µg/kg leads to acute transient increased serum levels of C-peptide, elevated physiological parameters such as heart rate and skin conductivity and to increases in subjective discomfort as rated on the subjective anxiety and discomfort scale (SADS) within the same time frame (2-4 minutes following i.v. pentagastrin injection). Pretest ratings on the anxiety sensitivity index (ASI) and Hamilton anxiety scale (HAS) as well as baseline levels of C-peptide correlated to ratings on SADS following pentagastrin.

In conclusion, CCK-ergic mechanisms in the PFC may be involved in the neurobiology of mild stress. Brain distribution of TPP II is in agreement with a CCK-degrading function of this enzyme. TPP II and CCK_A receptors in BA 10 may be involved in the pathophysiology of schizophrenia. C-peptide measurements along with psychophysiological, psychological and personality characterization in a "pentagastrin test for anxiety and fear" may be a useful bioassay for anxiety sensitivity and anxiety disorders.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to by their roman numerals:

I. Radu D, Brodin E, Weber G, Lindefors N. Delayed stress-induced increase in tissue level of cholecystokinin in rat prefrontal cortex: modulation by microdialysis probe implantation and systemic ketamine.
Brain Res. 2001 Jul 27;908(2):197-203

II. Radu D, Tomkinson B, Zachrisson O, Weber G, de Belleroche J, Hirsh S, Lindefors N. Overlapping gene expression for CCK and TPP II in rat and primate brain: tendency to decreases in TPP II mRNA in human Brodmann Area (BA) 10 following schizophrenia.
Manuscript

III. Radu D, Zachrisson O, de Belleroche J, Hirsh S, Lindefors N. Human CCK_A receptor mRNA distribution in human and primate brain - alterations following schizophrenia.
Manuscript

IV. Radu D, Åhlin A, Svanborg P, Lindefors N. Anxiogenic effects of the CCK_B agonist pentagastrin in humans and dose-dependent increase in plasma C-peptide levels.
Psychopharmacology (Berl). 2002 Jun;161(4):396-403.

V. Radu D, Åhlin A, Svanborg P, Lindefors N. Pentagastrin test for anxiety--psychophysiology and personality.
Psychopharmacology (Berl). 2003 Mar;166(2):139-45.

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List of abbreviations

µS	micro Siemens
aa	Amino acid
ACTH	Corticotropin
ASI	Anxiety Sensitivity Index
BA	Broadmann area
BP	Blood pressure
bp	Base pair
BPM	Beats per Minute
BSA	Bovine serum albumin
CCK	Cholecystokinin
CCK_A	Cholecystokinin type A receptor
CCK_B	Cholecystokinin type B receptor
CCK-LI	CCK-like immunoreactivity
CO_2	Carbon dioxide
C-peptide	Connecting peptide
cpm	Counts per minute
DA	Dopamine
DARPP 32	Dopamine and cAMP regulated phosphoprotein of 32 kDa
DG	Dentate gyrus
DSM	Diagnostic Statistical Manual for Mental Disorders
GABA	Gamma-amino-butyric acid
GSR	Galvanic Skin Response
HAS	Hamilton Anxiety Scale
HR	Heart Rate
i.p.	Intraperitoneal
i.v.	Intravenous
IP_3	Inositol-3-phosphate
ket	ketamine
KSP	Karolinska Scale of Personality
LTP	Long-term potentiation
mRNA	Messenger RNA
NMDA	N-metyl-D-aspartate
PFC	Prefrontal cortex
RT	Room temperature
SADS	State Anxiety and Discomfort Scale
SCID	Structured Clinical Interview for DSMIV
TPP	Tripeptidyl peptidase
VTA	Ventral tegmental area

Abbreviations used for different brain regions are explained in the respective figure texts.

1 AIMS

The neurobiology of CCK displays implications for psychopharmacology of psychiatric disorders. This thesis addresses issues related to stress, anxiety and schizophrenia, including studies of the effect of mild stress on brain CCK, anatomy of markers for CCK-ergic transmission in the brain and the effect of CCK receptor stimulation in a clinical test for anxiety and fear.

The specific aims were to study:

- effects of mild stress and glutamate NMDA receptor blockade by ketamine on CCK peptide levels and CCK mRNA in rat PFC
- suitable probes and subsequently study distribution of the mRNA encoding the enzyme TPP II and the CCK_A receptor as relative to the distribution of CCK
- possible changes in TPP II and CCK_A receptor mRNA levels and CCK_A receptor binding in *post mortem* PFC from subjects having had schizophrenia as compared to controls
- the possible link between C-peptide release and subjective discomfort following CCK_B receptor stimulation by the panicogenic agent pentagastrin as a bioassay for anxiety and fear
- associations between CCK_B receptor sensitivity and anxiety related personality traits

2 BACKGROUND

The neuropsychopharmacology of human brain and psychopathological states imply the involvement of various neurotransmitters. Along with small molecules such as amino acids (aa) or monoamines, neuropeptides are an expanding family of neurotransmitters and/or modulators serving different functions in the brain.

2.1 CHOLECYSTOKININ

Cholecystokinin (CCK) is a peptide first discovered in the gut. After its sequencing [128] CCK was found in mammalian brain as a peptide crossreacting with antigastrin antibodies [178]. The human CCK-gene is located on the third chromosome [102] and the sequence is highly conserved among different species. The gene transcript is translated into a 115 aa pre-pro-CCK molecule [134]. Al molecular forms of CCK present in the brain and gut are carboxy-terminal fragments of different lengths generated from this common precursor. Pro-CCK is processed in the endoplasmic reticulum and seems to involve the proteases PC1 and PC2 [10]. Finally, CCK is stored in dense core vesicles until release. The predominant form of CCK in the brain is the sulfated octapeptide (CCK8S) accounting for about 80% of the immunoreactive material [94]. Other molecular forms of CCK found in mammalian brain are CCK7, unsulphated CCK8, CCK5, CCK4 and also larger forms such as CCK33 are present [44, 97].
CCK and gastrin share the same C-terminal pentapeptide, considered necessary for biological activity (Fig. 1).

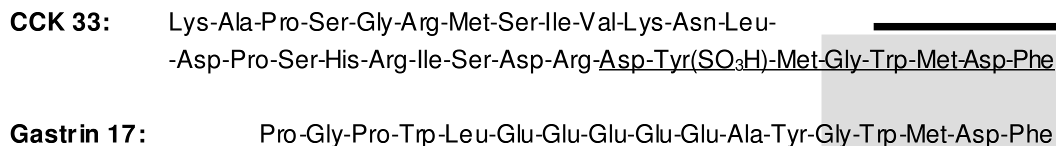

Fig. 1. Amino acid sequence of CCK33, CCK8 (underlined), CCK4 (black bar) and CCK5 (gray area indicating the common terminal five aa), as compared to the major form of gastrin [134].

CCK is very abundant in the brain (up to 500 ng CCK/g wet weight in cortical areas,[12]). CCK-like immunoreactivity (CCK-LI) is found in high levels in cerebral cortex, caudate-putamen, hippocampus and amygdala, lower levels in the thalamus, hypothalamus and very low to undetectable levels in the cerebellum [12, 98]. CCK mRNA is found in high levels in cortical areas especially in the PFC, claustrum, amygdala and hippocampus, while low to undetectable levels are seen in caudate-putamen and cerebellum [21, 36, 98, 155].
In this thesis, mRNA encoding pre-pro-CCK will be referred to as CCK mRNA.

2.2 CCK RECEPTORS

CCK acts via two receptors, the CCK_A (CCK_1) and CCK_B (CCK_2) receptors. Both receptor subtypes have been cloned [38, 145] showing about 50% homology. Both receptor proteins are about 450 aa long 7-transmembrane domain receptors belonging to the G-protein coupled receptor family[134]. In human, the genes for CCK_A and $_B$ receptors are found on chromosome 4 and 11, respectively (see review [134] p758 for further details). For the CCK_A receptor it has been shown that internalization follows ligand binding, the receptor being recycled to the surface within one hour, whilst the ligand is degraded in the endosomal system [150, 171]. Both CCK_A and CCK_B receptors can form homodimers that may dissociate after ligand binding [24, 25]. Heterodimers have also been found in cell lines coexpressing CCK_A and CCK_B receptors [24]. These heterodimers have normal agonist binding capacity but display enhanced agonist-stimulated cellular signaling and delayed agonist-induced internalization in cell cultures [24]. Both CCK receptors may after stimulation lead to formation of inositol-3-phosphate (IP_3) and increase in intracellular Ca2+ as second-messenger mechanisms. The CCK_A receptor seems also to be able to activate adenylyl cyclase (Fig 2.)(reviewed in [134]).
CCK_A and $_B$ receptors can be differentiated by their affinities for different ligands, as summarized in table 1.

Table 1. Affinities for some endogenous and synthetic CCK receptor ligands (from [10, 134]):

Ligand	CCK_A	CCK_B
CCK8S	Ki = 0.9 nM	Ki = 1.1 nM
CCK8	Ki = 311 nM	Ki = 5.9 nM
CCK4	Ki = 5113 nM	Ki = 18.6 nM
Gastrin		Ki = 10 nM
L-364 718, devazepide	IC50 = 1 nM	IC50 = 300 nM
L-365 260	IC50 = 300 nM	IC50 = 1 nM
SR146131	IC50 = 0.56 nM	IC50 = 162 nM

The CCK_A (CCK_1) receptors

This receptor subtype is primarily found in pancreatic acinary cells, but is also localized to discrete brain regions such as the nucleus tractus solitarius, area postrema, interpeduncular nucleus, nucleus accumbens, ventral tegmental area (VTA), substantia nigra, caudatus and ventral pallidum, as shown by ligand binding studies (reviewed in [10, 134]). Species differences are seen in the distribution of CCK_A receptor with wider distribution in primates [70] and bovine brain [9]as compared to rodents [9, 22, 71, 101, 188]. However, ligandbinding studies may be limited by the availability of selective labeled ligands.
Using *in situ* hybridization [75] and immunolocalization studies [113, 114], a wider distribution of CCK_A receptor was suggested, as CCK_A receptor mRNA was found in cerebral cortex, hippocampus, olfactory regions, septum and hypothalamic nuclei and immunoreactivity for the CCK_A receptor protein was found in the forebrain, substantia nigra, VTA, hippocampus, and other structures.

The CCK_B (CCK_2) receptors

The CCK_B receptor is the predominant CCK receptor subtype in the brain. The CCK_B receptor seems to be identical to the gastrin receptor located in the periphery. In the brain, the CCK_B receptors are abundant in cerebral cortex, caudate-putamen, amygdala and cerebellum, although the distribution pattern differs between the species studied [31, 42, 43, 57, 85, 115]. In the cerebellum, CCK_B receptors are found in human [31, 42, 43, 85], primates[42, 115], guinea pig [42, 105] and mouse [42] but not in rat [42, 176]. Alternative splicing of CCK_B receptor gene has been described [168] and the resulting isoforms have been found both in rat and human brain studies [80, 122, 187]. These CCK_B receptor variants do not appear to differ much in their binding characteristics [134]. There are however reports of differences between CCK_B binding sites corresponding to different activation states of the receptor, with possible relevance in anxiety and memory research [33].

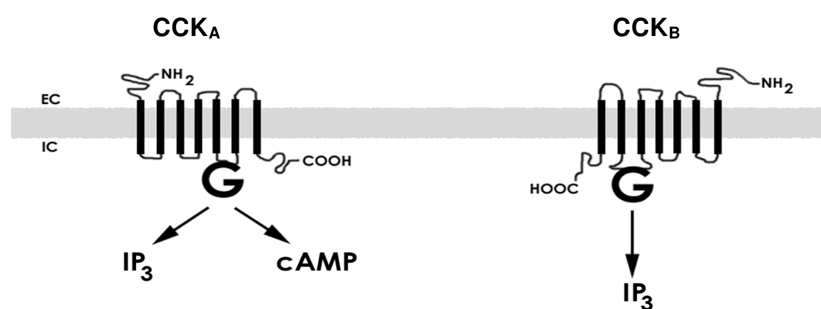

Fig. 2: Schematic representation of CCK receptors and the possible second messenger pathways activated following agonist binding. EC = extra-cellular space; IC = intra-cellular space. G = G-protein.

2.3 REGULATION OF CCK RELEASE

Neuropeptides such as CCK are stored in large dense core vesicles and released upon strong stimuli requiring general elevation of intracellular Ca^{2+} in contrast to small molecule neurotransmitters such as DA, synthesized and stored in small clear vesicles in the terminals that can be released by low frequency stimuli causing transient local alterations in Ca^{2+} concentration [61]. CCK release requires 40-60 mM potassium and higher frequency stimulation or burst impulses for release as compared to DA [10, 61].

CCK release from cortex, caudate and hippocampus increases after activation of the DA D1 receptor [20]. GABA inhibits CCK release in the cortex [147] while excitatory amino acids stimulate CCK release through the NMDA receptor [8, 60, 184]. NMDA receptor antagonists inhibit CCK release [4]. Serotonin $5HT_3$ receptor mediated increase in CCK release have been reported in cortex and nucleus accumbens [139]. CCK release can be detected in rat nucleus accumbens using *in vivo* microdialysis and transiently elevated by amphetamine [77]. This amphetamine induced elevation of ectracellular CCK is attenuated following pre-treatment with amphetamine daily for 7 days [77].

CCK mRNA expression and CCK peptide release are stimulated by estrogen in limbic-hypothalamic reproductive circuits [118, 119].

2.4 CCK TURNOVER; TRIPEPTIDYL PEPTIDASE II

The turnover of CCK in the brain is not fully characterized. In rat brain, overall halflife of labeled CCK8 is about 16 hours [112]. Receptor bound CCK8 is degraded in the lyzozomal-endosomal system after internalization of receptor-ligand complex [150, 171], essentially by the lyzozomal enzyme tripeptidyl peptidase I (TPP I) [179].
Tripeptidyl-peptidase II (TPP II) is a putative CCK inactivating peptidase [151]. It is a serine exopeptidase of high molecular weight, which removes tripeptides from the N-terminus of longer peptides. It has been purified from liver and red blood cells from different species and the amino acid sequence of TPP II is highly conserved [172]. Two isoforms of TPP II have been identified, a cytosolic form and a membrane-bound form, the latter accounting for 30-40 % of brain TPP II [151, 172]. The membrane-bound variant of TPP II has been purified from rat brain and is involved in the degradation of CCK [151, 173]. This is evident since butabindide, a specific inhibitor of TPP II, decreased food intake in rodents by a CCK-dependent mechanism [151]. TPP II can inactivate both extracellular CCK8 and CCK5. In rat brain TPP II mRNA and immunoreactivity are localized to cortex, striatum, hypothalamus, hippocampus [46, 151, 173], regions rich in CCK. Beside TPP II, aminopeptidases seem to be involved in extracellular CCK8 degradation [88, 120].

2.5 FUNCTIONAL ANATOMY OF CCK

CCK and its receptors are found in all cortical gray areas, in most sub-cortical gray structures, peri-aqueductal gray and all along the dorsal horn, co-localized and/or interacting with many of the "classical" neurotransmitters.
In cortical areas, there is strong evidence for co-localization of CCK with GABA and glutamate, most of the cells in this areas expressing CCK mRNA [21, 68]. CCK neurons projecting from cortical areas, especially the from PFC [116, 126], account for most of the CCK immunoreactivity found in the striatum (where expression of CCK mRNA is low to undetectable [97, 155]). Some of the CCK immunoreactivity in the striatum originates in the substantia nigra [72]. There is a large amount of evidence for co-localization of CCK and DA in some cells in substantia nigra and VTA [73, 163], the degree of this co-localization being higher in rodents as compared to primates [135] and humans [137]. In schizophrenia, there seems to be higher degree of co-localization of DA and CCK in substantia nigra [157, 158]. Moreover, from the substantia nigra and VTA there is a CCK-ergic projection to the amygdala and the PFC [47, 135, 186].
Thalamic cells expressing CCK mRNA project to the cortex and striatum [21]. From cortical areas there is a CCK-ergic projection to the thalamus [162]. CCK-containing neurons in the hypothalamic regions project to the pituitary [47]. In these neurons, CCK is expressed together with oxytocin, vasopressin or CRF [117, 138].
Ascending pathways from different brainstem regions containing CCK have been found. CCK has been found in locus coerulleus[28, 53]. It is also found in raphe nucleus, but not in cells expressing 5HT, projecting to the forebrain [175]. There are descending CCK-ergic pathways from brainstem regions to the spinal cord (for a review, see [10]).

2.6 FUNCTIONS OF CCK

CCK is involved in regulation of many physiological functions such as satiety [39, 59], body temperature [170], sleep [83, 104] and pain [109, 182] as well as in higher functions such as attention [159], learning and memory [63], motivation [153]and stress [164, 165].

CCK acts as an excitatory neurotransmitter, potentiating glutamate effect on striatal neurons and antagonizing DA, affecting DARPP32 levels in the striatum [167]. CCK influences brain monoamines, region specifically decreasing striatal DA [50] and DA in frontal regions [5, 55]. CCK increases the firing rate of midbrain DA neurons [65, 166]. In the nucleus accumbens, CCK acts differentially on DA, increasing DA in the posterior parts via CCK_A receptors while inhibiting DA release by CCK_B receptor mediated mechanisms in the anterior part of nucleus accumbens [30]. CCK seems to affect release of other monoamines in rat brain, stimulating release of both norepinephrine and serotonin in different regions of rat brain [50].

CCK increases glutamate and aspartate release [58] but also protects cortical neurons against NMDA receptor-mediated glutamate neurotoxicity [3] via CCK_B receptors.

In hippocampus, CCK and CCK_B receptor agonists augument long-term potentiation (LTP) [7, 185]. CCK has effects on the pituitary-adrenal axis [1]. CCK8 inhibits prolactin release while CCK 58 increases prolactin secretion and NA synthesis in hypothalamic areas [54]. Peripherally, CCK leads to gallbladder contraction and pancreatic enzyme secretion (CCK was first named cholecystokinin-pancreozimin). CCK stimulates pancreatic B-cells leading to insulin[149] and, consequently, C-peptide release.

2.7 THE PREFRONTAL CORTEX

The PFC is an association area involved in higher functions, interconnected both afferently and efferently [181, 90] with many cortical and sub-cortical brain structures such as the amygdala, striatum, thalamus, hypothalamus, hippocampus and brain stem nucleii, also a region rich in CCK. Brodmann area (BA) 10 in humans (Fig. 3) is part of the PFC [181, 90, 141]. Experimental and lesion data from primates and human data from patients with lesions in this region suggest its involvement in cognitive control of behavior, attention, emotion, reward/motivation and social interaction [90]. Dysfunction of the PFC may lay behind cognitive deficits in schizophrenia [103]. The adverse effects of stress in causing relapses in psychiatric disorders such as schizophrenia may be mediated by mechanisms involving the PFC [123]. In animal studies, a link between high trait anxiety and hyporeactivity to stress also involve the PFC [82].

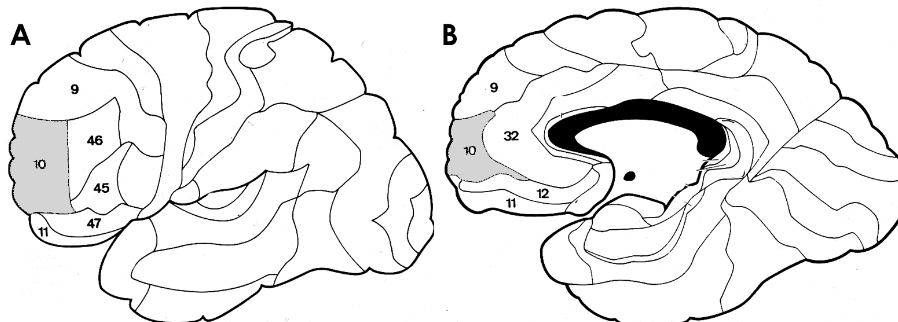

Fig. 3. Localization of BA 10 (shaded) and adjacent areas according to Brodmann in the human brain, at the frontal pole. (**A**) lateral view, (**B**) medial view. Modified after [141].

2.8 PSYCHOPHARMACOLOGY OF CCK

In the brain, the widely distributed CCK seems to be involved in the neurobiology of several psychiatric disorders such as anxiety, schizophrenia, depression, chronic pain, anorexia, drug addiction. The present thesis emphasizes the role of CCK in the context of stress, anxiety and schizophrenia with focus on the PFC, a region important for stress adaptability [15, 78, 121] and also a region implicated in the psychopathology of anxiety and schizophrenia.

2.8.1 CCK and stress

Hans Selye, a pioneer in stress research, defined stress as the nonspecific response of the body to any demand [161]. The definition was refined over the years describing stress as selective pressure from the environment eliciting adaptive responses specific to the stressors [26]. Stress may be defined as the response to aversive stimuli or hostile conditions (= stressors), resulting in neuroendocrine, cardiovascular, immune and gastrointestinal functional changes [23]. Stressors can be grouped in psychological stressors (fear, anxiety, exposure to novel or uncontrollable environment), physical stressors with strong psychological component (pain, immobilization) and cardiovascular stressors (hemorrhage, heat, exercise) [23].
Besides genetic factors, stress is considered a major cause influencing incidence, course and treatment outcome of psychiatric disorders. Stress may affect cognitive processes depending on the type and intensity of the stress. Memory processes may be enhanced by brief stress while prolonged exposure to stress may induce cognitive deficits [110]. Animal studies suggest that stress induced glutamate release in the PFC may regulate neuroendocrine and monoaminergic responses to stress [123].
The role of CCK in adaptive behavior and reaction to stress has been documented in human and animal studies [33, 34, 66]. The CCK-ergic system in animals seems sensitive to various forms of stress, even mild stress such as experimental handling inducing changes in extracellular levels and tissue levels of CCK or CCK mRNA [18, 87]. Different stressors such as restraint, exposure to diethyl-ether or yohimbine administration lead to acute increases in CCK-release as studied by microdialysis [100, 132]. Exposure to foot shock or smell of a predator (cat) lead to increased tissue levels of CCK-LI in frontal regions [140]. Saline i.p. [146] or s.c. [152] injections or isolation [66] do not seem to have an acute effect on tissue levels of CCK in PFC. Long-term isolation or repeated restraint stress do not seem to affect CCK mRNA in cortical regions, but lead to increases in sub cortical areas [40, 62]. Surgical stress with minimal damage of the cerebral cortex leads to ipsilateral increased mRNA levels of CCK within 24 hours followed by normalization within 6 days, a response diminished by the NMDA antagonist MK801 [79]. Increases in CCK release in the PFC are seen after NMDA administration [60, 100] and blocked by the NMDA antagonist MK801 (dizocilpine) [8].

2.8.2 CCK and anxiety

Using CCK analogs as panicogenic agents started with Rehfeld's anecdotic experience of injecting himself with CCK4 and experienced discomfort reminding of panic attacks [37]. CCK analogues such as pentagastrin and CCK-4 have proven to be a reliable model for anxiety and panic attacks in animal and human studies [67]. Patients suffering from panic attacks experience similar or identical sensations after administration of CCK analogues as in spontaneous panic attacks, including both physical and cognitive

symptoms [2, 89]. Normal subjects are less prone to experience panic attacks after CCK analogues administration as compared to patients with anxiety disorders [108, 177] and the panicogenic effects of CCK analogues can be blocked by anxiolytics [16, 37]. Moreover, some CCK_B antagonists may have anxiolytic properties but seem to lack the withdrawal effect seen after benodiazepine treatment [27, 76, 92].

2.8.3 CCK and schizophrenia

Schizophrenia is characterized by the presence of positive symptoms (e g hallucinations, delusions, disorganized speech and thoughts) and negative symptoms (emotional flattening, anhedonia, cognitive deficits). Dopaminergic dysfunction has dominated schizophrenia research as the potential of antipsychotic drugs is related to their DA D2 receptor occupancy [160]. Moreover, drugs such as amphetamine release DA and cause psychotic symptoms such as hallucinations. DA D2 receptor antagonists have little effect on negative symptoms. Blockade of the glutamate NMDA receptor causes a syndrome very similar to schizophrenia in healthy individuals, including both positive and negative symptoms [81, 91]. Moreover, in patients with schizophrenia, NMDA receptor antagonists exacerbate psychosis, reproducing symptoms identical to the ones experienced in the active phase of the disease [93]. Compared to amphetamine, the NMDA receptor blockade model adds negative symptoms and disorganized thoughts to the symptomatology seen. The possible implication of other classical transmitters such as serotonin (reviewed in [180]) and GABA [95] have also been discussed in relation to schizophrenia. CCK coexists with both glutamate and dopamine in corticostriatal and mesolimbic pathways, respectively (see 2.5).

In striatum, CCK potentiates glutamate effect and counteracts DA D2 effects on DAPRR32 phosphorylation [167], a signaling pathway that may be involved in the psychopathology of schizophrenia [169]. Animal studies have demonstrated that CCK and DA modulate each other [30]. CCK has a neuroleptic-like effect on prepulse inhibition in animal studies [49]. *Post mortem* human data revealed decreases in CCK-ergic function, as there are findings of decreases in both CCK mRNA, CCK_B receptor binding and expression of CCK_B receptor mRNA in frontal, temporal or entorhinal cortices from patients with schizophrenia [6, 84, 183, 187]. The decreases in CCK mRNA are not confirmed in all studies, e g upregulation of CCK mRNA in the ventral midbrain has been described in schizophrenia[158]. CCK in cerebrospinal fluid is decreased in schizophrenia [11], particularly in non-responders to antipsychotic treatment [56]. CCK appears to be required for neuroleptic-induced depolarization-inactivation of dopamine neurons and associated antipsychotic response and it is concluded therefore that patients having a schizophrenia diagnosis and low CCK may be resistant to the antipsychotic effects of neuroleptics [56]. In clinical studies, some CCK agonists seem to have antipsychotic properties [127, 129, 130], but negative findings have also been encountered [74, 107, 131, 144]. More recent studies have shown beneficial effect of CCK agonists added to ongoing antipsychotic therapy [125, 143], implying the possible beneficial effects of CCK agonists in a subgroup of non-responders to antipsychotic therapy probably due to low brain CCK-function. CCK analogs have also been shown beneficial in treatment of tardive diskinesia [133].

3 MATERIALS AND METHODS

3.1 ANIMALS (PAPERS I, II AND III)

Male Sprague-Dawley rats (ALAB Sweden) weighing 250-300 g at delivery were housed 5 per cage at constant room temperature of 21°C with unlimited access to food and water and a 12 hours light-dark cycle. Animals were allowed to accommodate for 5 days before the experiments. Animals were anaesthetized for 2 minutes in a CO_2 chamber prior to decapitation. After decapitation, brains were immediately removed and frozen on dry ice for *in situ* hybridization use or microdissected on ice prior to freezing for radioimmunoassay (RIA) use.

Mature normal *Cynomolgus* monkeys (housed at Swedish Institute for Infectious Disease Control, Sweden) were decapitated following a lethal dose barbiturate. The tissue was rapidly frozen and stored at -70°C.

3.2 MICRODIALYSIS PROBE IMPLANTATION (PAPER I)

Rats were anaesthetized with halothane and placed in a stereotaxic frame. The skull was fixed with blunt ear bars. After exposing the skull, a burr hole (0.8 mm in diameter) was drilled at the following co-ordinates: AP + 2.7, L –2.3[142]. A guide cannula containing a "dummy probe" was lowered into the right PFC DV –3.4 at an angle of 30°. The guide cannula was secured in place with two anchor screws and dental cement. 1% lidocain spray was administrated locally and the wound was closed with 1 stitch of 4.0 Ethicon. Animals were allowed to recover for two days in individual cages. On the third postoperative day, the dummy probe was removed under halothane anesthesia and replaced with a microdialysis probe (CMA 12, CMA Microdialys AB, Solna, Sweden). Animals were allowed to recover for 1 hour or 24 hours after probe implantation before receiving an i.p. saline injection.

3.3 MILD STRESS

Saline injection administrated i.p. was used as mild stress in this study. Prior to the experiments, rats were handled daily and administrated saline i.p. once every day for 7 days.

3.4 NMDA ANTAGONIST KETAMINE

Animals received ketamine in subanesthaetic doses (25 mg/kg administrated i.p.). Within minutes following ketamine i.p. injection, most animals displayed head weaving, rearing, and hind limb ataxia and were awake during the duration of the experiment.

3.5 ANIMALS USED FOR CCK-LI ANALYZES (PAPER I)

A total number of 175 rats were used, 7 rats in each of the 24 groups as described in paper I. For an overview of the groups, see table 2.

Following removal of brains, a brain blocker was used to obtain 1 mm slices in a standardized fashion. A circular micro punch was used to dissect nucleus accumbens and amygdala, and a scalpel for the PFC, caudatus-putamen, hippocampus and entorhinal cortex, according to Paxinos and Watson brain atlas [142] and rapidly frozen in pre-weighted 2 ml Eppendorf tubes on dry ice.

Table 2: Overview of rat groups used for CCK-LI analyzes; S = i.p. saline injection, K = i.p. ketamine injection; time points indicated represent minutes from injection to decapitation. Control animals were decapitated during the same time interval as injected and/or operated animals.

Control	5 min	10 min	20 min	Control 480 min	480 min
Non-injected	S	S	S	Non-injected	S
	K	K	K		K
Animals with a microdialysis probe implant in the right PFC:					
Non-injected non-operated				Non-injected non-operated	
Operated	S	S	S	Operated	S
Operated 24h	S	S	S	Operated 24h	S

3.6 ANIMALS USED FOR *IN SITU* HYBRIDIZATION (PAPER I)

A total number of 30 rats were used. Rats were decapitated 4 respectively 8 hours after i.p. saline or ketamine administration. Rats receiving no treatment served as controls.

3.7 TISSUE EXTRACTS (PAPER I)

Tissue extracts were performed according to method B as described in [19]. Extraction was first performed in 10 volumes of distilled water. Samples were homogenized using a Polytron PT 1200, incubated at 100°C for 10 minutes and centrifuged at 3000 rpm. The pellets were then resuspended in 1 M acetic acid at 100°C. The supernatants from the distilled water and acid extraction were pooled and dried in a Speed-Vac, resuspended in 1 ml 0.15 M NaCl containing 0.2% BSA and used for radioimmunoassay, diluted 1:10.

3.8 RADIOIMMUNOASSAY (PAPER I)

Samples were analyzed using antiserum 2609 [148], ^{125}I-gastrin as radioligand and synthetic CCK8 as standard. The total incubation volume was 1.1 ml and the sample volume 0.1 ml. The radioligand and diluted antiserum were added to the samples in 0.5 ml barbital buffer (0.02 M, pH 8.6) containing 0.8% BSA. Samples were incubated at 4°C for 72 hours and separated by adding 0.5 ml of sheep anti-rabbit IgG coupled to sepharose solution (Decanting suspension NR 3 Pharmacia), followed by incubation at 4°C for 30 minutes and centrifugation (1000 x g, 10 minutes, 4°C). The supernatants were discarded and pellets were counted 10 minutes each in a gamma counter.

3.9 *IN SITU* HYBRIDIZATION (PAPERS I, II AND III)

Frozen brains were cut at -14°C in 14 µ coronal sections using a Frigocut 2800 E (Leica, Germany) cryostat and thawed onto slides pre-treated with 2 % 3-aminopropyl-triethoxy-silane.

Sections were fixed in 4 % formaldehyde for 5 minutes, deproteinated for 15 minutes in 0.2 M HCl, treated in 0.25 % acetic anhydride solution for 20 minutes and dehydrated in a series of ethanol including a 5 minutes chloroform step prior to hybridization. The sections were incubated at 55°C for 16 hours with a hybridization buffer containing 50 % formamide, 20 % 50 X Dextran sulphate, 6.7 % 5 M sodium chloride, 4 % 5 M DTT, 2 % 1 M Tris-HCl pH 7.6, 2 % 50 X Denhardt's solution, 2 % yeast tRNA (25 mg/ml), 13.3 µl H_2O containing $10x10^6$ cpm probe per ml hybridization buffer. After hybridization, the sections were washed in 4 X SSC, treated with RNase 10 µg/ml at 37°C for 30 minutes and washed in 2 X SSC, 1 X SSC and 0.5 X SSC for 10 minutes each, 0.1 X SSC at 60°C, and 0.1 X SSC at room temperature for 5 minutes. The sections were dehydrated in graded series of ethanol, air dried and exposed to X-ray film (B-Max, Amersham). The developed films were scanned using a flat bed scanner, ScanJet 6100 C/T or ScanJet 3970 (Hewlett Packard) and analyzed using Scion Image for Windows, Beta 4.02 version (Scion Corporation) or Image Gauge 4.0 (FujiFilm).

3.10 RIBOPROBES FOR HYBRIDIZATIONS (PAPERS I, II AND III)

The DNA fragments described below were cloned into different plasmids; sense and antisense probes were synthesized using ^{35}S-UTP. For details see papers I – III.

CCK probes

Rat CCK: bp 263-535, acc nr X01032
Cynomolgus monkey CCK: bp 247 – 650, acc nr M60458
Human CCK: bp 195-344, acc nr L29400

CCK receptor probes

Human CCK_A receptor: bp 907-1056 acc nr L19315
Human CCK_B receptor: bp 816-1001 acc nr L04473

TPPII probes

A: non-coding 3'end of human TPPII, bp 1008 – 1319 acc nr M73047
B: probe complementary to the catalytic site, murine TPP II, bp 847-1314 acc nr X81323
C: murine probe containing the 39 bp of the splicing variant of TPP II, bp 3033 – 3084, acc nr X81323

3.11 RECEPTOR BINDING AUTORADIOGRAPHY (PAPER III)

Slides used for ligand binding assays were brought to room temperature (RT) and preincubated 2 x 15 minutes at RT in buffer A containing 50 mM Tris-HCl, 5 mM $MgCl_2$ 5 nM DTT at a pH of 7,4 . The incubation was performed for 60 minutes at RT using buffer A containing 0.25 nM 3H-devazepide (specific CCK_A receptor antagonist [69] available until 2003 from PerkinElmer Life Sciences) with or without 10 µM CCK8S

used as a blocker. The slides were then rinsed twice in RT buffer A, dipped shortly in distilled water to eliminates salt traces and exposed after air-drying to phosphoimager plates (BAS-IP TR2040, Fuji Photo Film, Japan) for 2-3 weeks. The phosphoimager plates were then scanned using a phosphoimager (BAS 1500, Fujifilm, Tokyo, Japan.) and analyzed using Image Gauge 4.0 (FujiFilm, Science Lab 2001). A calibration stripe (Amersham) exposed together with the slides was used to check the linearity of the signal.

3.12 STUDY DESIGN FOR HUMAN STUDIES (PAPERS IV AND V)

The subjects were 20 volunteer healthy adults, 14 men and 6 women, recruited by advertising, provided written informed consent to participate in this study and were paid 500 SEK for the participation in each pentagastrin trial. The mean age was for the males 29 ± 3 (range 22 - 38) and for the females 24 ± 2 (range 19 - 28). The subjects were medically healthy as determined by physical examination and medical history. Details about the subjects are given in paper IV.
During the first visit, the subjects underwent physical examination, psychiatric interview and filled in screening questionnaires for psychiatric disorders. At the following four consecutive sessions performed between 8.00 - 11.00 a.m., the subjects received intravenously (i.v.) pentagastrin in increasing doses, one dose per trial. Two consecutive trials for the same subject were performed allowing a washout period of at least 24 hours. Blood samples and physiological data as well as subjective discomfort after pentagastrin administration were recorded as described below. (Table 3)

Table 3. Overview of time points for different blood samples and physiological data collection in relation to pentagastrin administration. BP = blood pressure, ECG = electrocardiogram, GSR = galvanic skin response, min = minutes preceding/following bolus pentagastrin injection.

Min	-1	0	2	4	5	6	8	10	15	20	30
BP	X	Pentagastrin administration			X			X	X	X	X
Cortisol	X								X		
Lactate	X								X		
Glucose	X								X		
C-peptide	X		X	X		X	X	X	X		X
ECG	Continuous registration										
GSR	Continuous registration										

3.12.1 Assessments of psychiatric diagnoses

A psychiatric diagnosis on DSM-IV Axis I or II was an exclusion criterion for enrollment in the present study. Therefore, at the first visit, 21 potential subjects filled in screening questionnaires for psychiatric disorders according to the DSM-IV[51, 52]. Both instruments are constructed to be over-inclusive, i.e. to have a high sensitivity but

low specificity for a diagnosis. For subjects screened positive, a complete SCID-I and/or SCID-II interview was performed [51, 52]. None of the subjects was screened positive for an axis-I diagnosis; however, one subject was excluded due to fulfilled criteria for an axis-II disorder.

3.12.2 Pentagastrin dose

The subjects received 0.003, 0.012, 0.05 and 0.2 µg pentagastrin (Peptavlon, Cambridge Laboratories Ltd., UK) per kg body weight in 1 ml saline vehicle i.v. at four consecutive sessions. The highest dose of pentagastrin to be administrated was chosen within the lower range shown in previous studies to give psychotropic effects in healthy subjects [99, 177].

3.12.3 Instruments used for assessment of personality and anxiety

Assessment of subjective discomfort

The subjects were instructed to self-assess their current feeling of unease, tension, anxiety or panic following pentagastrin administration rating their subjective experience on a simple Likert rating-scale (Fig. 4), ranging from 0 (no discomfort) to 5 (worst imaginable discomfort). Intermediate levels were 1 (slight discomfort), 2 (moderate discomfort), 3 (severe discomfort), and 4 (very severe discomfort). This scale will be referred to as the State Anxiety and Discomfort Scale (SADS).

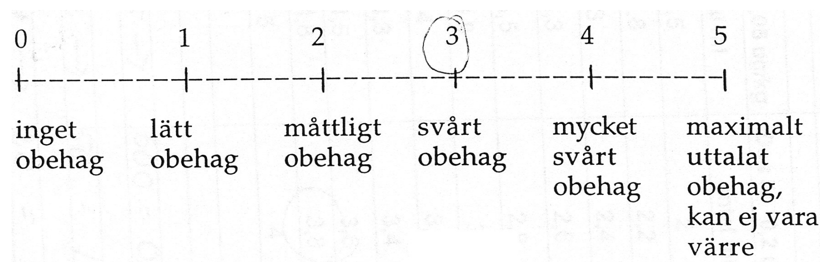

Fig. 4: The SADS scale; in this case, a subject rated severe discomfort following penatagstrin administration

Assessments of trait anxiety and other personality traits

Anxiety sensitivity was measured using the Anxiety Sensitivity Index (ASI).
Personality traits were assessed with the Karolinska Scale of Personality (KSP) [156].

Assessment of state anxiety

Before the pentagastrin trial series, we administered the Hamilton Anxiety Scale (HAS) [64] to control for differences in current anxiety baseline. The HAS consists of 14 items, each defined by a series of symptoms. Each item is rated on a 5-point scale, ranging from 0 (not present) to 4 (severe).

3.13 STATISTICAL ANALYZES

For all statistical analysis we used Statistica 5.5, StatSoft Scandinavia AB Uppsala, Sweden.

Paper I

We used one way ANOVA followed by Tukey's HSD test for the statistical analysis of data.

Papers II and III

Mann-Whitney U-test, correlation statistics and Bonferroni corrections were used when necessary.

Papers IV and V

Pre-injection values for C-peptide from the four test occasions were averaged to give a base-line level. Values at 2, 4, 6, 8, 10, 15 and 30 minutes after pentagastrin administration were analyzed as relative to the individual baseline levels.
Correlations for normally distributed variables were performed using parametric and ordinal variables using non-parametric statistics.

3.14 ETHICAL APPROVALS

Animal experiments: Nr N188/98, approved 1998-08-27
Human *post mortem* studies: Nr 00-167, approved 2001-01-08
Pentagastrin studies: Nr 97-272, approved 1997-11-03

4 RESULTS AND DISCUSSION

4.1 STRESS EFFECTS ON CCK IN RAT PFC (PAPER I)

4.1.1 Non operated animals

As suggested from earlier studies (see 2.8.1), CCK in the PFC is susceptible to even mild stressors such as i.p. saline injection. Within 20 minutes from the time point of the injection, a significant decrease of CCK-LI levels could be seen in this area ($p < 0.05$, Tukey's HSD test), more pronounced when the i.p. injection contained ketamine ($p < 0.05$ at 5 minutes and $p < 0.001$ at 20 minutes following injection, Tukey's HSD test). This decrease was followed by an increase in CCK-LI in the PFC at 8 hours after the injection in animals receiving saline ($p < 0.001$, Tukey's HSD test) but not in animals receiving ketamine. In other studied areas such as nucleus accumbens, caudate/putamen, amygdala, hippocampus and the entorhinal cortex, the increase seen at 8 hours following the i.p. saline injection was modest or absent and did not reach significance at $p< 0.05$ level (Fig. 5).

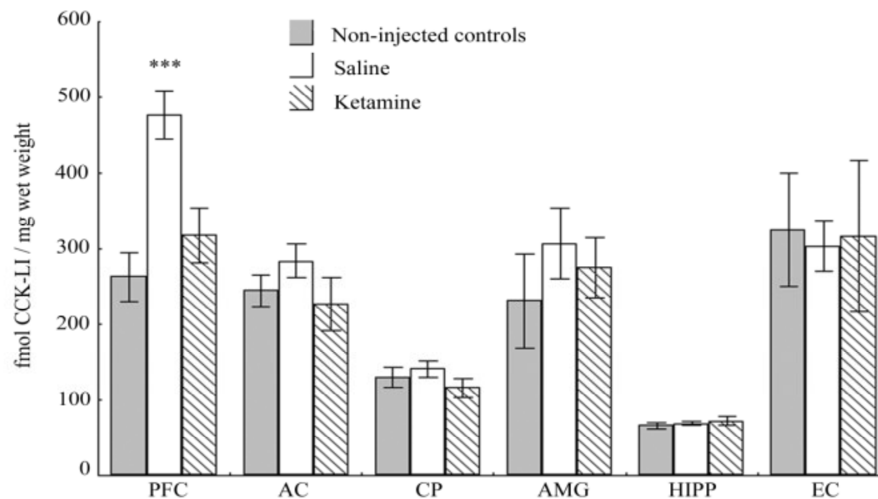

Fig. 5. Effect of saline and ketamine 25 mg/kg i.p. injections on tissue levels of CCK-LI in selected regions of the rat brain at 8 hours following administration: PFC = prefrontal cortex, AC = nucleus accumbens, CP = caudatus-putamen, AMG = amygdala, HIPP = hippocampus, EC = entorhinal cortex; *** $p<0.001$ as compared to non injected controls, Tukey's HSD test.

The changes in CCK-LI seen in this study were not followed by increases in mRNA for CCK in the PFC within the time frame studied (8 hours after i.p. saline injection, see table 4). The increase seen in CCK-LI at 8 hours could be the result of post-translational processing of pre-pro-peptide pools into immunoreactive material in local cortical neurons and CCK mRNA upregulation in other cortical regions or afferent systems. However, at a rate of axonal transport of 1 mm/day, CCK newly synthesized from afferent systems could probably not account for the total increase seen.

Table 4. Results from measurement of CCK mRNA levels in rat PFC on autoradiogram from *in situ* hybridization histochemistry. Mean values ± SEM of CCK mRNA in rat PFC (calibrated optical density, arbitrary units).

Control	Saline 240 min	Saline 480 min	Ketamine 240 min	Ketamine 480 min
330.14±26.57	352.65±28.01	333.70±24.90	312.34±11.34	297.20±18.23

The effects of ketamine in this study are difficult to interpret. Ketamine affects glutamate transmission differently depending on the administrated doses. At subanesthaetic doses, as the dose used in this study, ketamine increases glutamate release in the PFC [123, 124]. Excitatory aa such as glutamate stimulate CCK release [8, 60, 184]. Considering these mechanisms, decreased levels of CCK in the PFC as seen within 20 minutes after ketamine administration in our study could be expected.
The differences seen at 8 hours between animals receiving i.p. saline and the ones injected i.p. with ketamine is not as easy to explain. Ketamine is a drug with a short half-life (about 30 min). The behavioral effects disappear during this period. However, there is evidence for long-lasting effects of intermittent ketamine administration on other neurotransmitters such as DA and serotonin in rat PFC [96]. The present set of results suggests long-lasting effects of ketamine on CCK in rat PFC.

In an effort to evaluate the possible use of microdialysis to study *in vivo* effects of mild stress on CCK in rat PFC, we examined the effect of microdialysis probe implantation on tissue levels of CCK as a methodological pilot study.

4.1.2 Operated animals

In animals having a microdialysis probe operated in the right PFC, the effects of i.p. saline injection were diminished when experiments were performed the same day as the dummy-probe was replaced with a microdialysis probe, allowing the animals to recover one hour following this replacement. In this group of animals, there was no significant decrease within 20 minutes following i.p. saline injection and the increase seen at 8 hours was lower, and also not significant. However, in the non-injected group of animals receiving a microdialysis probe replacement the same day had a significant increase at 8 hours after the one hour recovery allowed after probe-replacement (i e 9 hours after probe implantation) ($p < 0.05$, Tukey's HSD test).
In animals allowed to recover 24 hours after the replacement of the dummy-probe with a microdialysis probe, no changes in CCK-LI in PFC at 5, 10, 20 minutes could be detected and a significant decrease at 8 hours after i.p. saline injection was seen ($p < 0.01$, Tukey's HSD test) (see table 5).

Microdialysis is a widely used method to study in vivo dynamics of neurotransmitter release in the brain. Interpretation of such results should be approached with caution as microdialysis probe implantation in itself may cause changes in neurotransmission. In our studies, the stress-induced changes seen in non-operated animals were attenuated both in the group studied one hour after probe implantation and in animals allowed to recover for 24 hours after probe implantation. The presence of an implant in the PFC seems to interfere with CCK-ergic mechanisms in rat PFC.

Table 5: Overview of rat groups used for CCK-LI analyzes; S = i.p. saline injection, K = i.p. ketamine injection; time points indicated represent minutes from injection to decapitation. Bold style indicates groups where significant changes in CCK-LI were found as compared to non-injected non-operated control group; *p<0.05; **p<0.01

Control	5 min	10 min	20 min	Control 480 min	480 min
Non-injected	S	S	**S ↓ ***	Non-injected	**S ↑ ****
Non-operated	**K ↓ ***	K	**K ↓ ****		K
Animals with a microdialysis probe implant in the right PFC:					
Operated	S	S	S	**Operated ↑***	S
Operated 24h	S	S	S	Operated 24h	**S ↓ ****

4.2 DISTRIBUTION STUDIES OF CCK AND RELATED MARKERS IN PRIMATE BRAIN; HUMAN BA 10 IN SCHIZOPHRENIA (PAPERS II, III)

4.2.1 Distribution studies

Choice of TPP II mRNA probe

We tested three different probes for TPP II as described in the "methods" section. Homology to human sequence for the murine probes was 95 % for the probe designed to recognize the catalytic site (B) and 97% for the spicing fragment (C). For distribution studies, we used the human probe (A, Fig. 6), with high signal after hybridization of the antisense probe and very low/undetectable signal in hybridization of the sense control. Two murine probes (B and C, see Fig. 6) proved to be unsuitable due to high binding of the sense control in both cases.

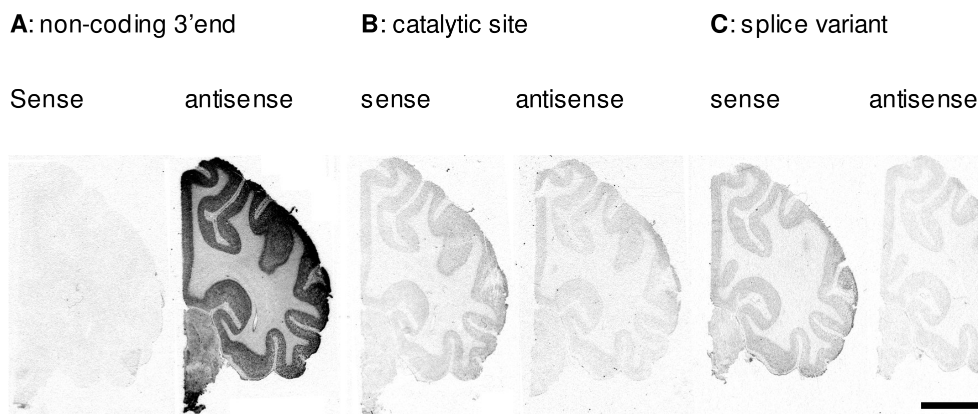

Fig.6: ISH of sense and antisense mRNA probes to different sequences of TPP II; bar 1 cm. Slides derive from the same set of hybridizations.

Distribution of TPPII mRNA in monkey brain

Our studies on primate brain shows a broad distribution of TPP II mRNA to cortical and sub cortical structures. It is expressed all over cerebral cortex, with higher levels in frontal regions, in caudate, putamen, claustrum, thalamus, hippocampus, amygdala, substantia nigra/VTA and cerebellum (Paper II). In the cerebral cortex, TPP II mRNA is relatively evenly expressed over the cortical layers.

The distribution pattern of TPP II mRNA in monkey brain corresponds to the distribution of TPP II peptide in rat brain as studied using immunohistochemistry [46]. Comparative distribution of TPP II mRNA in a monkey brain section as compared to markers for CCK-ergic transmission (mRNAs encoding CCK receptors and CCK peptide) is shown in Fig. 7. TPP II is thus found in regions rich in CCK, a distribution pattern compatible with it's presumed involvement in the degradation of CCK. In vitro studies of TPP II show a preference for degrading CCK8 and CCK5, but also possible involvement in degrading other brain peptides [151]. Recent studies have also demonstrated that TPP II is involved in antigen processing [86].
The probe used in this study recognizes both the membrane-bound and cytosolic form of the peptide, the latter one probably involved in general intracellular turnover of proteins [172]. Taken together, these findings imply a broad distribution of TPP II.

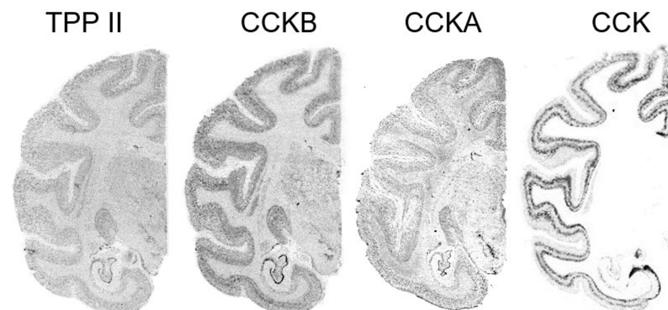

Fig. 7: Comparative distribution of mRNAs for TPP II, CCK_B and $_A$ receptors and CCK peptide in coronal sections of *Cynomolgus* monkey brain corresponding to a level situated 9 mm caudal to the anterior commisure [106].

Distribution of CCK_A receptor mRNA in monkey brain (Paper III)

Using a human probe against CCK_A receptor mRNA on monkey brain slides, we found distribution over the cortical areas with higher levels in discrete brain regions such as frontal cortex in the proximity of the frontal pole, para-amigdaloid area, substantia nigra, hippocampus, DG, hypothalamic areas, lateral geniculate body, cerebellum (Fig. 8), in concordance with earlier descriptions of distribution of CCK_A mRNA in rat. This distribution is also in line with immunolocalization studies of the CCK_A receptor in rat [114], but more wide spread than suggested by receptor autoradiography studies.

Existence of CCK_A receptors in the different brain areas is suggested by *in vivo* animal studies. Microinjection of L-364718 (selective CCK_A receptor antagonist) in the posterior accumbens region of rat antagonizes CCK-induced potentiation of dopamine-induced hyperlocomotion [29]. The existence of CCK_A receptors in the striatum is also suggested by the inhibitory effect of CCK_A antagonists on CCK-induced excitation of striatal neurons [35]. Selective CCK_A receptor antagonists influence midbrain dopaminergic neurons [154], in line with existence of CCK_A receptors in this area.

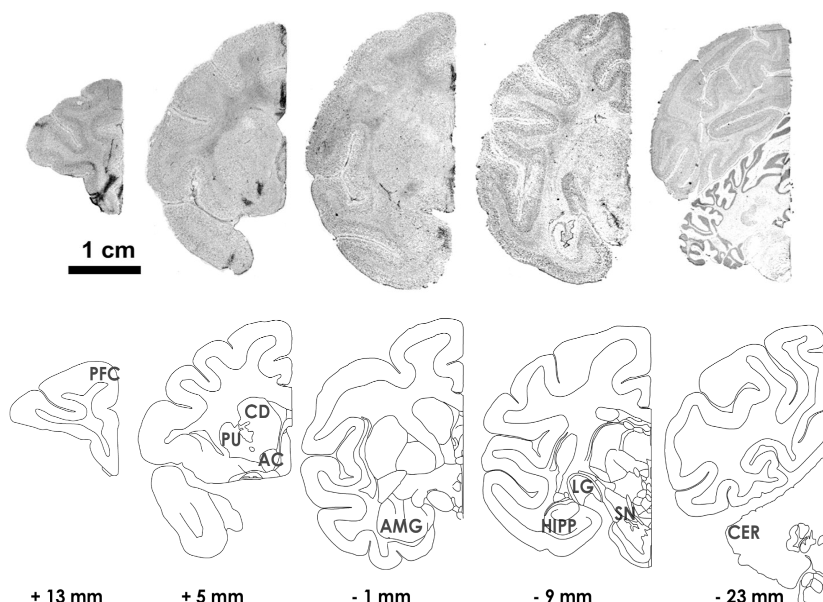

Fig. 8. Distribution of CCK_A receptor mRNA in monkey coronal brain sections (upper panel). Lower panel represents corresponding maps of coronal monkey brain sections, numbers in mm represent position relative to the anterior commisure, [106]. PFC = prefrontal cortex, AC = nucleus accumbens, CD = caudate, PU = putamen, AMG = amygdala, HIPP = hippocampus, LG = lateral geniculate body, SN = substantia nigra, CER = cerebellum.
The shapes of coronal slices used for this study differ from the ones in the primate brain atlas [106]. This is a post-mortem tissue handling artifact, as the hemispheres were separated and allowed to freeze placed on the medial surface, resulting in a lateral to medial compression of the structures and a pronounced medial-rostral shift/dislocation of temporal structures as compared to their expected anatomical position.

Ligand binding studies on the CCK_A receptor

We were able to displace the binding of tritiated devazepide from monkey and human brain slides using CCK8S as a binding competitor. However, the non-specific binding in several sets of experiments was 70-80% of the total binding, making evaluation of distribution studies difficult. The ligand used in this study was the racemate (±) L364718. Earlier studies have demonstrated the different features of the two optical isomers of this substance, (-) L364718 being 100 times more specific for the CCK_A receptor and (+) L364718 mostly accounting for the background signal [9, 41]. At present, the availability of radiolabeled specific CCK_A ligands is low. However, new agonist and antagonists have been synthesized. SR146131 is a selective CCK_A agonist [13, 14] of potential future use in distribution studies.

4.2.2 Studies of post-mortem human BA 10

In the region of main interest in this thesis, the PFC, there are previous *post mortem* human findings of decreases in CCK mRNA levels [183], CCK_B receptor mRNA [187] and CCK_B receptor binding [48] following schizophrenia.
The levels of TPP II mRNA (Fig. 9) in this study did not show a statistically significant decrease (Table 6). We have found decreased levels of CCK_A receptor mRNA (Fig. 9) in schizophrenia (Table 6), however, ligand binding studies for this receptor (Fig. 9) were difficult to evaluate due to high unspecific binding of the ligand. We could not find significant decreases of CCK mRNA (Fig. 9) levels in BA 10 (Table 6).

Table 6. Actin corrected values for CCK, TPP II and GAPDH mRNA in human *post mortem* BA 10; measurements are made on calibrated optical density levels, arbitrary units. Levels between probes are not comparable as measurements derive from different hybridizations. C = control group; S = schizophrenia group. Shown p-values are two-sided. **Bold** style indicates significant results at $p < 0.05$ level.

	Median		Min		Max		P values
	C	S	C	S	C	S	C vs S
TPP II/actin	2.88	1.42	1.70	0.64	10.68	3.78	0.093
CCKA/actin	4.04	2.69	3.35	2.06	9.56	6.12	**0.026**
CCK/actin	4.56	3.66	3.31	2.40	5.40	5.66	0.365
GAPDH/actin	1.15	1.10	0.74	0.87	1.35	1.37	0.937

Due to the small sample size, further studies may be needed to further pursue the hypothesis of decreases in CCK-ergic function in the PFC. Some earlier studies have found correlations of low CCK-levels in the CSF to negative symptomatology[11] and lack of response to antipsychotic treatment in schizophrenia [56]. CCK appears to be required for neuroleptic-induced depolarization-inactivation of mesolimbic dopamine neurons [56]. Taken together, the findings described above might imply that patients low in CCK function may be resistant to the antipsychotic effects of neuroleptics [56].

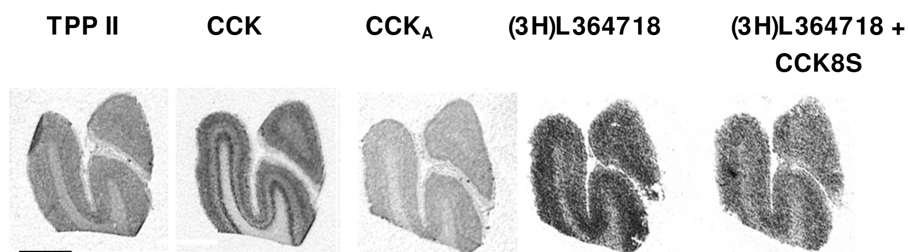

Fig. 9: From left to right, TPP II, CCK, CCK_A mRNA ISH and CCK_A ligand binding with and without blocker, respectively, in human BA 10. Bar 1 cm.

Possible functional connection between CCK and TPP II

We found localization of TPP II mRNA to regions rich in CCK.

To address the possibility of a functional connection between TPP II and its presumed substrate CCK, we examined possible correlations between expression levels for mRNAs encoding TPP II and CCK in BA10 from patients having a schizophrenia diagnosis as compared to a control group. We found a positive correlation of the levels of mRNAs studied, more pronounced in the patient group (Fig. 10).

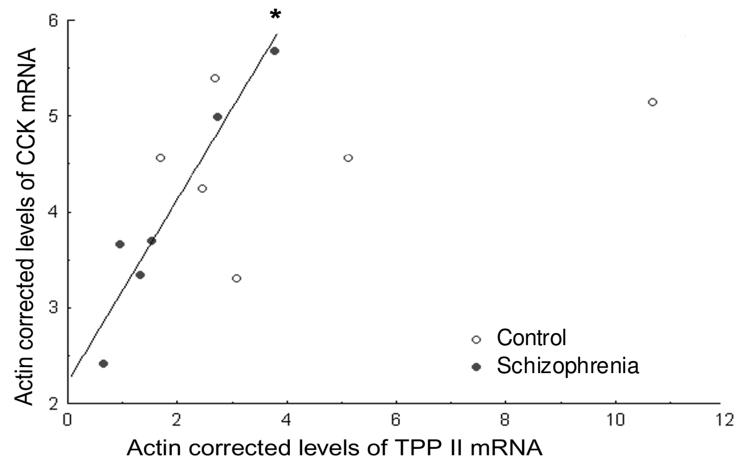

Fig. 10. Correlations between actin-corrected TPP II and CCK mRNAs in human BA 10. The levels of TPP II and CCK mRNA showed a positive correlation in the whole group (Kendalls tau 0.51, $p < 0.05$) The correlation was more pronounced in the schizophrenia group (Kendalls tau 0.86, $p < 0.05$). *Regression line shown for the schizophrenia group.

The tendency to decrease seen in BA 10 in the group of patients having had a schizophrenia diagnosis should be further studied. Enzyme inhibitors such as butabindide have been proven effective in affecting food intake in rats via CCKA-depending mechanism [151]. The potential use of enzyme blockers in treatment of schizophrenia might be worth while studying.

4.3 PENTAGASTRIN TEST FOR ANXIETY AND FEAR (PAPERS IV, V)

4.3.1 Plasma C-peptide measurement – A possible bioassay for anxiety?

CCK_B receptor stimulation by pentagastrin (or CCK4) is a well documented human model for panic attacks (see 2.8.2). In pancreatic beta-cells, CCK_B receptor agonists stimulate insulin release [149]. During this process, pro-insulin is cleaved resulting in release of equimolar amounts of insulin and C-peptide [174]. C-peptide is stable to hemolysis [136] and has a longer half-life (30 min) as compared to insulin [45], and can thus be regarded as an indirect measure for insulin release.

If CCK_B receptor reactivity is an individual phenotypic characteristic, measurement of C-peptide release following CCK_B receptor stimulation could be a marker of potential use in assessment of inter-individual differences in reactivity to psychotropic drugs acting on this receptor and of susceptibility to develop anxiety disorders (Fig. 11).

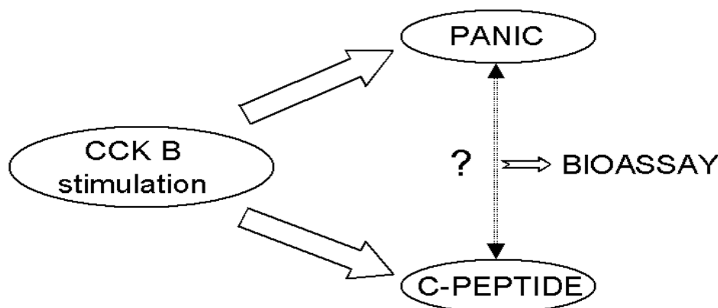

Fig. 11: Hypothesis underlying the possible use of C-peptide in a bioassay for characterization of individual phenotype regarding sensitivity to express anxiety and fear.

4.3.2 Pentagastrin effects on C-peptide and subjective discomfort

In this study (Paper IV), bolus injection of the panicogenic CCK_B agonist pentagastrin gave a dose-dependent increase in C-peptide, significant at 2 and 4 minutes after administration of the highest dose of pentagastrin 0.2 µg/kg ($p < 0.01$ in both cases, Fig. 12). This effect is in concordance with the documented insulin-releasing capacity of CCK_B agonists [149]. Within two minutes from pentagastrin bolus administration, the subjects reported somatic symptoms such as nausea, chest and/or gastrointestinal discomfort, flushing, sweating, paraesthesias, muscular pain, dryness of the mouth, fear, choking, in some subjects accompanied by elation, fear or anxiety. The symptoms disappeared within 4 minutes from the injection time.

Increase in the administered pentagastrin dose was accompanied by higher discomfort ratings on the SADS (Table 7). We observed a dose-dependent significant increase in subjective discomfort as rated on the SADS ($p < 0.001$). Values at dose 0.05 µg/kg were significantly increased as compared to both doses 0.003 and 0.012 µg/kg ($p < 0.001$) and the values after administrating the highest dose of pentagastrin (0.2 µg/kg) were significantly increased as compared to all lower doses ($p < 0.01$ in all cases). The

individual basal levels of C-peptide before administration of pentagastrin dose 0.02 µg/kg correlated positively to the ratings on the SADS following subsequent drug administration (Spearmans $R = 0.47$, $p < 0.05$). However, the relative increase in C-peptide levels 4 minutes after administration of the highest dose of pentagastrin showed a negative correlation to the ratings on the SADS (Spearmans $R = -0.46$, $p < 0.05$).

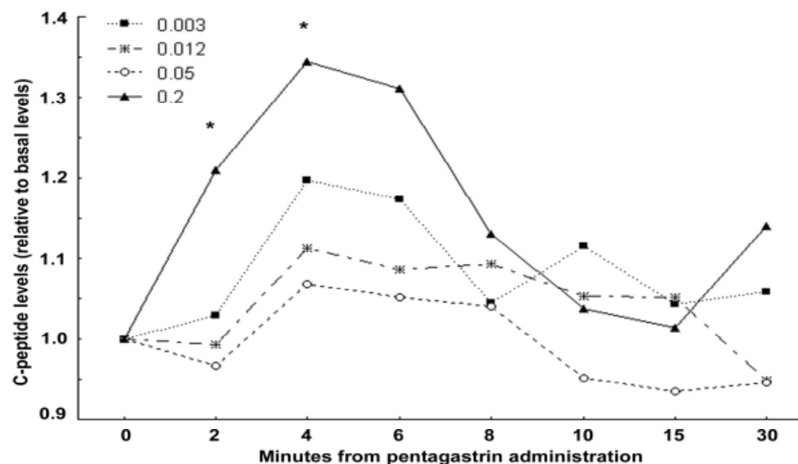

Fig 12: Relative increase in C-peptide plasma levels after administration of 0.003, 0.012, 0.05 or 0.2 µg/kg pentagastrin. * $p< 0.05$

Table 7: Number of individual ratings on every level of the SADS-scale following administration of increasing doses of pentagastrin. The sum in each row should be 20 (the total number of subjects included in the study) except for "test 2", where only rating from 19 subjects are available. **Bold** style indicates doses giving significant increase in discomfort ratings.

Ratings on SADS / Pentagastrin dose	0	1	2	3	4	5
Test 1 0.003 µg/kg	19	1				
Test 2 0.012 µg/kg	15	3	1			
Test 3 0.05 µg/kg	**9**	**6**	**5**			
Test 4 0.2 µg/kg		**3**	**7**	**9**	**1**	

The subjects were previously informed on the doses and expected possible effects of pentagastrin. These individuals with high anticipatory anxiety (fear of fear) might be the group having high pre-test levels of C-peptide.

The highest dose of pentagastrin administrated in this study to an adult of about 70 kg would be 14 µg (0,2 µg/kg). This dose is of similar magnitude as compared to panicogenic doses of CCK4, another well documented CCK_B agonist in human panic research [17]. Doses beginning at 15 µg CCK4 and above, regardless of bodyweight, precipitate panic attacks in up to 75% of the subjects previously diagnosed with panic disorder[17]. However, not all CCK_B agonists administrated peripherally cause panic attacks, e.g. CCK8 has no panicogenic properties [37]. These findings suggest a central

action of peripherally administrated CCK4 and pentagastrin, implying penetration of the brain-blood-barrier by these compounds but not by of CCK8S. Animal studies corroborate this hypothesis [32].

4.3.3 Pentagastrin effects on physiological parameters

Pentagastrin administration resulted also in significant increases in physiological parameters such as heart rate (HR) and peripheral sweating measured as galvanic skin response (GSR) (as described in detail in paper V). Significant increase in GSR ($p < 0.01$) occurred starting 50 seconds after administration of 0.05 µg/kg pentagastrin and after 40 seconds following administration of 0.2 µg/kg pentagastrin ($p < 0.05$ and $p < 0.001$, respectively). Administration of 0.2 µg/kg pentagastrin resulted in a significant increase in heart rate 50-90 seconds after administration as compared to the basal values ($p < 0.001$ at 50, 60, 70 and 90 seconds and $p < 0.05$ at 80 seconds after pentagastrin administration). The HR returned to baseline levels at 120 seconds after pentagastrin administration.

Increases in HR are reported in earlier studies using CCK4 or pentagastrin as a panicogenic agents [2, 17]. The finding of increases in GSR already at a dose as low as 0.05 µg/kg pentagastrin is novel. This increase at 0.2 µg/kg pentagastrin correlated positively to SADS and negatively to C-peptide increase (Paper V).

4.3.4 Psychological characterization

ASI is a measure of the individuals "fear of fear" [111]. ASI scores in the group studied ranged between 0 – 28 (median 7). These ratings are low even when compared to ratings in non-clinical subjects [111]. The ASI scores correlated positively to the ratings on SADS observed following administration of 0.05 and 0.2 µg pentagastrin/kg body weight (Kendall's tau = 0.34, $p < 0.05$ in both cases).

The HAS measures state anxiety, providing in our study the anxiety "baseline" before each pentagastrin trial. The HAS scores in the group had a median value of 1, range 0 - 28. The HAS scores correlated positively to the ratings on SADS following administration of 0.05 and 0.2 µg pentagastrin/kg body weight (Kendall's tau = 0.32 respectively 0.33, $p < 0.05$ in both cases). The HAS scores did not correlate to ASI scores.

Trait anxiety and other personality traits were assessed using the KSP. Due to small sample size, interpretation of the results from KSP ratings is difficult as factor analysis could not be performed. However, among the 15 personality traits measured by the KSP, muscular tension, indirect aggression, verbal aggression and suspicion seemed predictive of the increases in SADS ratings following 0.05 µg/kg pentagastrin. These correlations were not seen following 0.2 µg/kg pentagastrin. These preliminary results might suggest predictive value of personality traits at low pentagastrin doses, possibly identifying individuals more sensitive to experience discomfort (Paper V).

5 CONCLUSIONS

CCK in the PFC is affected by mild stress such as an i.p. saline injection in a region specific fashion. This effect seems to be bi-phasic and is lasting up to at least 8 hours. Moreover, the effect is significantly attenuated by pre-treatment with a single low dose of the psychotomimetic drug ketamine, a glutamate NMDA receptor antagonist.

Use of microdialysis to study *in vivo* release of CCK in the PFC by mild stress might be compromised by the effect of the probe implantation on CCK-ergic mechanisms.

TPP II mRNA is distributed widely in monkey brain, localized to areas rich in CCK. There is a possible functional-anatomical correlation between CCK and TPP II mRNAs, also indicated in studies of BA 10 from individuals having had schizophrenia.

The localization of CCK_A receptor mRNA in monkey brain seems broader than suggested by ligand-binding receptor autoradiography studies in rodents, primates and human. We found CCK_A receptor mRNA in cortical regions, parts of ventral striatum, hippocampus, amygdala, substantia nigra and thalamus in *Cynomolgus* monkey and in human BA 10.

In human *post mortem* PFC (BA 10), the levels of CCK_A mRNA were decreased in a group of individuals having had schizophrenia as compared to material from corresponding control brain tissue.

The use of the CCK_A receptor antagonist devazepide as receptor ligand in post mortem autoradiographic ligand binding studies on human brain sections may not be optimal due to high non-specific binding.

CCK_B receptor stimulation by pentagastrin may reveal phenotype characteristics associated to susceptibility for anxiety reactions or anxiety disorders, measurable through psychotropic reactions and serum levels of C-peptide.

Doses of pentagastrin as low as 0.05 g/kg increase the ratings on SADS, suggesting the possible predictive value of the Pentagastrin test for anxiety and fear.

6 FUTURE PERSPECTIVES

CCK and stress

Stress is regarded as a major factor having negative impact on course and outcome of psychiatric disorders. CCK in the PFC seems to be readily affected by stress. Unfortunately, using *in vivo* microdialysis to study stress effects on CCK in the PFC is a complicated approach as probe implantation seems to interact with CCK-ergic mechanisms. Non-invasive approaches using CCK-receptor ligands in combination with *in vivo* imaging techniques might reveal new cues about CCK-ergic mechanisms in the PFC and other parts of the brain during stress.

Anatomy of CCK-ergic markers

Both TPP II and the CCK_A receptor can be visualized using immunohistochemistry, as a complement to *in situ* mRNA hybridization studies. It would be of interest to study their localization in human brain using different techniques, especially regarding the CCK_A receptor where species differences seem to occur. With respect to pharmacology of schizophrenia drugs such as SR146131, a selective CCK_A agonist, is of interest in further tissue ligand binding and *in vivo* imaging studies.

Enzyme inhibitors such as butabindide have been proven effective in affecting food intake in rats via a CCK_A-depending mechanism. Studies of enzyme inhibitors in the search for potential new antipsychotic agents might be rewarding.

Pentagastrin test for anxiety and fear

The penatagstrin test for anxiety and fear in relation to individual phenotypes may be used in studying individuals with anxiety disorders or other disorders where anxiety is part of the symptomatology. Further diagnostic and prognostic information can be acquired combining this with information from individual genotypes. Finally the Pentagastrin test is suitable to combine with *in vivo* imaging techniques studying the functional anatomy of anxiety and fear.

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