CHOLECYSTOKININ IN THE CENTRAL NERVOUS SYSTEM: PHARMACOLOGICAL CHARACTERIZATION, SOLUBILIZATION, AND AUTORADIOGRAPHIC LOCALIZATION OF THE RECEPTORS AND INTERACTIONS WITH CENTRAL DOPAMINERGIC FUNCTIONS

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ABSTRACT

In 1980, cholecystokinin (CCK) was demonstrated to co-exist with dopamine (DA) in certain neurons of the CNS. Together with evidence implicating abnormal DA function in schizophrenia, the CCK/DA co-existence provided a theoretical basis for investigating the potential therapeutic value of CCK as an antipsychotic. Several animal studies provided physiological, pharmacological, biochemical, and behavioural evidence that CCK down-regulated DA function. On this basis, numerous clinical trials were conducted during the early 1980s. Although initial, uncontrolled clinical trials were very positive, subsequent controlled trials failed to support these preliminary results.

Since the animal studies were carried out on rodents, little was known about CCK in the brain of higher mammalian species. In the present study, CCK receptors were characterized in the brains of higher mammalian species. The pharmacological characteristics of CCK receptors appear to have been well preserved in the mammalian brain. However, marked differences in the distribution of CCK receptors were observed in several brain areas. There were basically two main types of species-specific differences. The absence or presence of CCK receptors in a given structure and differences in the distribution within a given structure. Although the reasons for such species-specific differences in receptor distribution are not clear at the present time, this evidence cautions against simple extrapolation of data obtained in animal models directly to clinical applications.

Moreover, although some animals studies provided evidence of an inhibitory effect of CCK on DA function, which would be compatible with a

potential antischizophrenic action, others reported a lack of modulation or an enhancement of DA function. Circling behaviour is commonly used to assess DA function. In the present study, unilateral intracranial microinjections of CCK_8 induced a dose-dependent contraversive circling bias in rats. This CCK_8-induced contraversive circling bias was attenuated by the DA receptor antagonist haloperidol. This suggests that, under the acute conditions employed in this study, CCK_8 may induce contraversive circling by exerting a unilateral facilitatory influence on DA neurotransmission.

To further elucidate the mechanism(s) underlying this CCK-induced circling behaviour, the effects of CCK peptides on ligand binding at DA receptors and on DA-stimulated adenylate cyclase were investigated in the rat striatum. Under the assay conditions employed, CCK_8 has no significant acute effects on binding at the DA receptor or on DA-stimulated adenylate cyclase. It is possible that CCK induced its acute facilitatory influence on DA function in the circling behaviour paradigm by altering DA turnover or release.

Another problem with the animal studies which were used as the basis for the clinical application was that the vast majority were of an acute nature. In the present study, long-term administration of CCK_8 did not significantly alter DA D_2 receptor densities nor the expression of DA D_2 receptor mRNA. In contrast, long-term haloperidol administrations significantly increased CCK binding in the nucleus accumbens, olfactory tubercle, and frontal cortex.

The results of the present study indicate that care must be taken when extrapolating evidence on CCK function obtained in animals to the clinical application. Moreover, further studies on CCK function in the CNS and CCK/DA interactions are required before this peptide can be considered for further clinical trials in schizophrenic patients.

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Earlier versions of this thesis were printed on reused paper. The final version was backprinted on 100% recycled, acid-free, 20 lb bond paper containing at least 15% post-consumer waste. Wherever possible, several figures and tables were printed on the same page. This was done to minimize the use of our precious and definitely exhaustible Canadian forests.

Cette thèse est dédiée à ma mère, Fleurette Morency. The academic successes that I have enjoyed to date are directly attributable to the motivation, drive, and never-ending strive for perfection that I have inherited from my mother. Although she may not be able to read and understand the contents of this thesis, my mother has always been, and still is, able to read me like an open book. It is with great pride that I dedicate this thesis to her.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
[^{125}I]BH	[^{125}I]3-(4-hydroxyphenyl)proprionic acid N-hydroxysuccinimide ester
Boc	t-Butyloxycarbonyl
B_{max}	Maximal receptor density
BSA	Bovine serum albumin
Bt_2cGMP	Dibutyryl cyclic GMP
cAMP	Adenosine 3',5'-cyclic-monophosphate
CCK	Cholecystokinin
CCK_4	C-terminal CCK tetrapeptide, CCK_{30-33}
CCK_7	Sulfated C-terminal CCK heptapeptide, CCK_{27-33}
CCK_8	Sulfated C-terminal CCK octapeptide, CCK_{26-33}
CCK_{8U}	Unsulfated C-terminal CCK octapeptide
CCK_{33}	CCK tricontatriapeptide, CCK_{1-33}
CCK-A	Alimentary-type CCK receptor
CCK-B	Brain-type CCK receptor
CCK-LI	CCK-like immunoreactivity
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propan-Sulfonate
CI-988	4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo [3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]-1-phenyl] amino}-4-oxo-[R-(R*,R*)]-butanoate N-methyl-D-glucamine, also referred to as PD 134308

CNS	Central nervous system
CR 1409	D,L,-4-(3,4-dichloro-benzoyl-amino)-5-(di-n-pentyl-amino)-5-oxo-pentoic acid
CSF	Cerebrospinal fluid
DA	Dopamine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethyleneglycol-bis-(βamino ethyl ether)N,N'-tetra-acetic acid
Gpp(NH)p	5'-guanylyl imidophosphate
GTP	Guanosine 5'-triphosphate
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid
HPLC	High pressure liquid chromatography
$[^3H]pCCK_8$	[propionyl-^{3}H]propionylatedCCK_8
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Concentration inhibiting 50% of binding
IC	Intracranial
ICV	Intracerebroventricular
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
K_d	Dissociation constant
K_i	Inhibition constant

L-364,718	3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-2-carboxamide, also referred to as MK-329 or devazepine
L-365,260	3R(+)-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-N'-(3-methylphenyl)-urea
MCID	Microcomputer imaging device
MPFCx	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
N-CBZ-CCK_{27-32}	N-carbobenzoxy-CCK_{27-32}
Nle	N-methylnorleucine residues
NPA	N-propylnorapomorphine
N-terminal	Amino terminal
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
Pentagastrin	Boc-β-Ala-CCK_4
PLG	L-Prolyl-L-Leucyl-Glycinamide
PMSF	Phenylmethyl-sulfonyl fluoride
PNS	Peripheral nervous system
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNF 8702	[N-methyl-$Nle^{28,31}$]CCK_{8U}
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
Tween 80	Polyoxyethelene sorbitan mono-oleate

CHAPTER I

INTRODUCTION

Ivy and Oldberg (1928) first described cholecystokinin (CCK) as a substance released from the upper intestine to produce gallbladder contraction. Several years later, Harper and Raper (1943) discovered that the duodenal mucosa contained a hormone which stimulated the secretion of enzymes from the pancreas; the hormone was termed pancreozymin for that reason. Although CCK and pancreozymin were originally thought to be completely separate hormones, Jorpes and Mutt (1966; Mutt & Jorpes, 1968) found that methanol-insoluble extracts of the duodeno-jejunal mucosa stimulated both gallbladder contraction and pancreatic enzyme secretion. Once purified and characterized, CCK and pancreozymin proved to be identical. Since it was originally recognized for its action on the gallbladder, this peptide is now commonly referred to as CCK. It has numerous effects on the gastrointestinal tract and biliary systems, as outlined in Table I. Detailed descriptions of these effects can be obtained from a number of reviews (e.g., Allescher & Ahmad, 1991; Dockray, 1983; Goldfine & Williams, 1983; Marx et al., 1987; Mutt, 1980; Thompson, 1987) and will not be further discussed.

As with many other peptides initially identified in the intestine, CCK was subsequently demonstrated to exist in the CNS. In 1975, Vanderhaeghen et al. reported the existence of a gastrin-like immunoreactivity in the rat brain. The

Table I

Effects of CCK on the Gastrointestinal, Pancreatic and Biliary Systems.

GASTROINTESTINAL TRACT:

Inhibition of gastric emptying.

Inhibition of lower oesophageal sphincter pressure.

Inhibition of water and electrolyte absorption in the jejunum.

Stimulation of intestinal motility.

Stimulation of gastric acid secretion.

Competitive inhibition of gastrin-stimulated gastric acid secretion.

Increase of mucosal blood flow.

PANCREAS:

Stimulation of pancreatic enzyme secretion.

Stimulation of insulin and glucagon release.

Enhancement of secretin-induced bicarbonate secretion.

Trophic effect on the pancreas.

GALLBLADDER and LIVER:

Stimulation of gallbladder contraction

Relaxation of the sphincter of Oddi.

Stimulation of hepatic bile secretion.

following year, Dockray (1976) demonstrated that the bulk of immunoreactivity was due to CCK, a gastrin-like material which cross-reacted with gastrin antibodies due to the common C-terminal of gastrin and CCK (see Figure 1). In addition, CCK has also been located in the PNS (Dalsgaard et al., 1983; Dockray et al., 1981b; Hutchinson et al., 1981; Larsson & Rehfeld, 1979).

CCK was originally isolated and sequenced by Mutt and Jorpes (1966) as a tricontatriapeptide (CCK_{33}). However, several molecular forms of CCK (ranging in length from 4 to 58 amino acids) have been described since (Brownstein & Rehfeld, 1985; Dockray, 1983; Turkelson & Solomon, 1990). In the brain, at least five molecular forms have been identified: CCK_{39}, CCK_{33}, CCK_{12}, CCK_8, and CCK_4. Of these, CCK_8 is the most abundant form (Dockray, 1983; Larsson & Rehfeld, 1979; Marley et al., 1984; Miller et al., 1984; Rehfeld, 1978; 1985). Apart from size heterogeneity, CCK peptides also display a substantial number of species variations (Jansen & Lamers, 1985; Rehfeld, 1985; Williams et al., 1986). Therefore, unless otherwise specified, the use of the term CCK in this thesis implies a variety of CCK-related peptides.

I-1 DISTRIBUTION OF CCK IN THE CNS

I-1.1 Immunohistochemistry and Radioimmunoassay

Immunoreactive CCK has been localized in several mammalian species in both the CNS and PNS by immunohistochemistry and radioimmunoassay. The distribution of CCK in the rat brain has been studied more extensively than any species (see reviews Beinfeld, 1983; Dockray, 1983; Emson & Marley, 1983; Fallon & Seroogy, 1985; Morley, 1982; Seroogy & Fallon, 1989), but CCK has

Figure 1 Amino acid sequence of some CCK-related peptides.

Regions of homologies with CCK_8 are underlined. Note that gastrin-related peptides have the sulfated tyrosine residue on the sixth amino acid from the C-terminal whereas CCK-related peptides have it on the seventh amino acid from the C-terminal. $Gastrin_{17}$ also exists in the unsulfated form (i.e., $Gastrin_{17}$ I). Adapted from Morency & Mishra (1987).

Peptide	Sequence
CCK_{33}	NH_2-Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-$CONH_2$ 
CCK_8	NH_2-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-$CONH_2$ 
CCK_{8U}	NH_2-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-$CONH_2$
Caerulein	NH_2-Glp-Gln-Asp-Tyr-Thr-Gly-Trp-Met-Asp-Phe-$CONH_2$ 
$Gastrin_{17}$ II	NH_2-Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe $CONH_2$ 

also been mapped in the hamster (Miceli et al., 1987), guinea pig (Larsson & Rehfeld, 1979), porcine (Rehfeld, 1978), bovine (Barden et al., 1981), monkey (Beinfeld et al., 1983), and human brain (Emson et al., 1982). Although a few species-specific differences have been noted, CCK mapping studies in these mammalian species have yielded fairly similar results.

In the forebrain, CCK cell bodies are densely packed in several sublaminae of the neocortex (Gall et al., 1987; Innis et al., 1979), with especially high numbers in the piriform and endopiriform cortex, claustrum, and periamygdaloid area (Beinfeld et al., 1981; Miceli et al., 1987). CCK-immunoreactive cell bodies are also found within the external plexiform layer of the olfactory bulb (Larsson & Rehfeld, 1979; Seroogy et al., 1985).

CCK-immunoreactive neurons are found in the hilus of the dentate nucleus and in all layers of the hippocampus proper (Gall, 1984; Handelmann et al., 1981; Harris et al., 1985; Miceli et al., 1987; Sloviter & Nilaver, 1987) as well as in the amygdala, thalamus, and striatum (Beinfeld et al., 1981; Gilles et al., 1983; Larsson & Rehfeld, 1979; Meyer et al., 1982; Miceli et al., 1987; Schiffman et al., 1989). In the hypothalamus, CCK-LI is found within the paraventricular nucleus (Kiss et al., 1984; Micevych et al., 1987) and preoptic nucleus (Miceli et al., 1987; Micevych et al., 1987). In the brainstem, CCK distribution closely follows that of DA. CCK neurons are densely packed in the ventral mesencephalon, many of which are colocalized with DA (Hökfelt et al., 1980a; Seroogy et al., 1989a).

In addition, CCK-LI is found in projection neurons. High CCK-LI levels have been detected in fibres of the lateral olfactory tract (Gall et al., 1987; Miceli

et al., 1987), anterior commissure (Cho et al., 1984), retrohippocampal pathways (Greenwood et al., 1981; Köhler & Chan-Palay, 1982), medial perforant path (Fredens et al., 1984; 1987), and stria terminalis (Miceli et al., 1987).

Although the immunocytochemical distribution of CCK-LI has been well documented, the interpretation of results with antibody techniques is limited by the potential for cross-reactivity between structurally related antigens. Generally, antisera directed against the C-terminal of CCK are used for immunohistochemistry and these do not discriminate between the different molecular forms of CCK. Moreover, C-terminal antisera is known to cross-react with calcitonin gene-related peptide and gastrin (Ju et al., 1986; Rehfeld & Hansen, 1984).

I-1.2 In Situ Hybridization

More recently, CCK mRNA has been localized in the brain by in situ hybridization histochemical techniques, thereby offering a solution to these specificity problems. In addition, in situ hybridization has the added advantage of providing a specific analysis of the distribution of CCK mRNA at both the tissue and the single cell levels. The distribution of cell bodies containing CCK mRNA correlated well with the distribution pattern of CCK-LI. Briefly, numerous cells containing CCK mRNA were detected in the cerebral cortex, the hippocampus, the olfactory bulb, the claustrum, and the amygdala (de Belleroche et al., 1990; Ingram et al., 1989; Lanaud et al., 1989; Savastava et al., 1988; 1990). In addition, the absence of CCK mRNA in the basal ganglia (de Belleroche et al., 1990; Ingram et al., 1989; Savastava et al., 1988; 1990) is in agreement with previous immunohistochemical studies where CCK cell bodies were not detected even after direct colchicine injections in this nucleus (Gilles et

al., 1983; Vanderhaeghen, 1985). The cell bodies of origin that give rise to dense CCK innervation to this area are in the claustrum/piriform cortex (Meyer et al., 1982).

Substantial levels of CCK mRNA were detected in the ventral tegmental area and in the substantia nigra pars compacta of rats, cats, and monkeys (Burgunder & Young, 1990; Ingram et al., 1989; Savastava et al., 1988; Schalling et al., 1990) but not in the hamster, guinea pig, or human ventral mesencephalon (Palacios et al., 1989; Schalling et al., 1990). Further evidence for the colocalization of CCK and DA in the mesencephalon was obtained with in situ hybridization. Coexpression of CCK mRNA and tyrosine hydroxylase mRNA was demonstrated in cell bodies distributed throughout the substantia nigra pars compacta and the ventral tegmental area (Kiyama et al., 1991; Savastava et al., 1989; Seroogy et al., 1989b). The degree of colocalization (i.e., percentage of double-labelled cells) observed in these studies was much higher than reported in early immunohistochemical studies. This difference between the two techniques used was attributed to the lack of sensitivity of immunohistochemical studies and/or to elution of antibodies required for double immunohistochemical staining (Kiyama et al., 1991; Savastava et al., 1989; Seroogy et al., 1989b).

Two brain regions were identified where CCK-immunoreactive neurons had been previously reported but where CCK mRNA was not detected; the bed nucleus of stria terminalis and the nucleus of the solitary tract (Ingram et al., 1989). Since the identity of the CCK peptide in these areas was never confirmed with HPLC or gel chromatography, the identity of the specific peptide detected by immunohistochemical studies is questionable given the lack of specificity discussed above.

Conversely, CCK mRNA was localized in some neurons that were not known to express the CCK peptide. These areas include many of the thalamic nuclei, the Edinger-Westphal nucleus, the motor trigeminal nucleus, the nucleus of the facial nerve, and the lateral vestibular nucleus (Burgunder & Young, 1988; Ingram et al., 1989; Lanaud et al., 1989; Savastava et al., 1988; Seroogy et al., 1989b; Sutin & Jacobowitz, 1990; Voigt & Uhl, 1988). Although the presence of CCK mRNA does not necessarily indicate the phenotypic expression of the peptide, several explanations have been offered for the lack of immunocytochemical detection of CCK in these areas (Ingram et al., 1989; Seroogy et al., 1989b): 1) the peptide may be present within the cell bodies but in amounts below the sensitivity limits of present immunocytochemical techniques, 2) the peptide may be synthesized but then rapidly exported from the soma to the axon, 3) the CCK mRNA may be translated but processed differently than in other neurons resulting in unrecognized CCK fragments - Reinfeld (1987) has reported differential post-translational modification of CCK in endocrine cells of the pituitary, 4) the CCK mRNA could remain untranslated, and 5) the hybridization probes could be cross-hybridizing with mRNAs unrelated to CCK. It should be noted that similar discrepancies between the distributions of labelled perikarya obtained with in situ hybridization versus immunocytochemical techniques have been reported for other peptides (Harlan et al., 1987; Segerson et al., 1987; Siegel & Young, 1985).

I-1.3 Phylogeny

CCK-LI has been found in a wide variety of nervous systems, including those of coelenterates, the most primitive multicellular animals

(Grimmelikhuijzen et al., 1980), advanced invertebrates such as the snail (Osborne et al., 1982), and insects (Dockray et al., 1981a). The occurrence of CCK in both nerves and endocrine cells was established in primitive vertebrates such as the cyclostomes and has since been well conserved (Dockray, 1979). Taken together, this phylogenetic evidence would suggest that the neuronal function of CCK was established early in the emergence of the nervous systems and that its hormonal role is a relatively recent addition.

I-2 CCK AS A NEUROTRANSMITTER

To be considered as a neurotransmitter, an endogenous substance must satisfy certain criteria (see Carpenter & Reese, 1981; Renaud, 1978): The substance should be present in the presynaptic terminal; it should be synthesized in the neuron and released upon nerve stimulation; when applied exogenously in physiological concentrations, it should interact with specific receptors and mimic exactly the actions of the endogenously released substance; and a specific mechanism should exist for removing or inactivating the substance once it has been released. Since the initial identification of neuronal CCK, several lines of evidence have supported a role for this peptide as a neurotransmitter.

I.2.1 Subcellular Distribution

High concentrations of CCK occur in the synaptosomal fraction of brain homogenates (Dodd et al., 1980; Emson et al., 1980a; Pinget et al., 1978; 1979) and, more specifically, CCK has been located in synaptic vesicles by electron microscopy (Hendry et al., 1983). Since the capacity for peptide synthesis in neurons is limited to the cell soma, CCK must be transported to nerve endings

intra-axonally. The existence of axonal transport mechanisms has been demonstrated in the vagus nerve and the medial forebrain bundle by showing that ligation and lesion of nerve fibres, respectively, lead to CCK accumulation on the side of the cell body (Dockray et al., 1981b; Williams et al., 1981). Moreover, medial forebrain bundle lesions lead to a CCK depletion in areas innervated by the A10 cells, such as the nucleus accumbens, the bed nucleus of the stria terminalis, and the central nucleus of the amygdala (Hökfelt et al., 1980b; Williams et al., 1981). Gel chromatography of the CCK-related peptides isolated from different subcellular fractions indicated that the majority of the CCK-LI in the soluble fraction was of high molecular weight (equivalent to CCK_{33} or larger), while the synaptosomal fraction and vesicles contained primarily lower molecular forms of CCK-LI (CCK_4-CCK_8) (Emson et al., 1980a; Golterman et al., 1981). This suggests that there is processing of CCK as it migrates from the cell body of the neuron (soluble fraction) to the nerve terminal.

I-2.2 Biosynthesis

The biosynthesis of CCK has been demonstrated in the CNS. Using [^{35}S]methionine to label CCK and immunoadsorption to isolate this newly formed [^{35}S]CCK, Golterman et al. (1980ab; 1981; Golterman 1982ab; 1985) observed a rapid (within 15 min) and substantial incorporation of [^{35}S]methionine into high molecular weight forms of CCK equivalent in size to CCK_{33} or larger. Two CCK-converting enzymes have been isolated from aqueous extracts of porcine and bovine brains which convert CCK_{33} to CCK_{12} and CCK_8, respectively (Malesci et al., 1980; Ryder et al., 1980; Straus et al., 1978).

I-2.3 Release

The release of CCK from CNS tissue has been demonstrated both in vitro and in vivo. CCK is released in vitro from rat synaptosomal fractions (Dodd et al., 1980; Pinget et al., 1979) and from slices of rat cortex and hypothalamus (Emson et al., 1980a). In these studies, the release was evoked by a depolarizing stimulus in the form of elevated external K^+ concentrations and was demonstrated to be Ca^{2+}-dependent. In addition, the release of CCK-LI has been demonstrated in vitro in the neostriatum (Meyer & Krauss, 1983), in the hippocampus (Verhage et al., 1991), and in the pituitary neurointermediate lobe (Marley et al., 1983). In vivo, a Ca^{2+}-dependent release of CCK-LI was induced in cat spinal cords by both K^+ depolarization and by increasing the intensity of sciatic nerve stimulation (Yaksh et al., 1982). Similarly, in vivo release of CCK-LI has been demonstrated from the cat cerebral cortex in response to either K^+ or veratridine stimulation (Wang et al., 1983). In both cases, the released CCK-LI co-migrated with CCK_8 on a Sephadex G50 column, although the cortical extracts contained some material which co-chromatographed with CCK_4, CCK_{33}, and CCK_{39}.

I-2.4 Neuronal Effects

Electrophysiological studies indicate that iontophoretic application of CCK produces either excitation or inhibition among the CNS neurons examined (e.g., Denavit-Saubie et al., 1985; Dodd & Kelly, 1981; Jeftinija et al., 1981; MacVicar et al., 1987; Morin et al., 1983; Oomura et al., 1978; Phillis & Kirkpatrick, 1980). For example, Oomura et al. (1978) and Phillis & Kirkpatrick (1980) have applied CCK peptides to cortical pyramidal neurons in rats and found that CCK produced a slow onset excitatory response. A similar excitatory response was observed

after the iontophoretic application of CCK_8 to cat dorsal horn interneurons in vivo and in rat dorsal horn neurons using an in vitro slice technique (Jeftinija et al., 1981). In contrast, inhibition was observed in the hippocampus (Mac Vicar et al., 1987) and in the nucleus tractus solitarius (Denavit-Saubie et al., 1985; Morin et al., 1983). The CCK_8-induced excitation of midbrain DAergic neurons and cortical DA-sensitive neurons was blocked by both iontophoretic or systemic administrations of proglumide, a CCK receptor antagonist (Bunney et al., 1985; Chiodo & Bunney, 1983).

I-2.5 Inactivation

Incubation of synaptosomes with radiolabelled CCK failed to show any significant accumulation of labelled CCK into the synaptosomes, suggesting the lack of a reuptake mechanism. Instead, there is a rapid degradation of exogenous CCK by synaptosome fractions (Dodd et al., 1980; Deschodt-Lanckman et al., 1981). Rapid degradation and inactivation of endogenous CCK is achieved by a serine peptidase. By studying the fate of endogenous CCK_8 released by depolarization of rat cortical slices, Rose et al. (1988; 1989) observed that serine-alkylating reagents (e.g., PMSF) markedly protected CCK_8-LI, suggesting the involvement of a serine peptidase. Active site-directed inactivators and transition-state inhibitors of serine peptidases were also effective in protecting CCK_8-LI. In contrast, peptidase inhibitors belonging to various other classes (i.e., metallo-peptidase, thiol-endopeptidase, and carboxypeptidase) had little or no effect on the recovery of CCK_8-LI (Rose et al., 1988; 1989). CCK_{29-33} (Gly-Trp-Met-Asp-Phe-NH_2), CCK_{29-31} (Gly-Trp-Met), and CCK_{30-31} (Trp-Met) were found to be the major metabolites of CCK_8 degradation (Deschodt-Lanckman, 1985; Rose et al.,

1988; 1989).

I-3 CCK RECEPTORS

High-affinity and saturable binding sites for CCK have been identified in the pancreas, gallbladder, fundic gland, ileum, stomach, PNS, and CNS of various species. These binding sites are specific for CCK peptides in that unrelated peptides such as insulin, substance P, vasoactive intestinal polypeptide, bombesin, secretin, and enkephalin do not compete with radiolabelled CCK for binding (see Morency & Mishra, 1987).

As summarized in Table II, pharmacological studies have provided evidence for three receptor subtypes of the CCK/gastrin family. The first type, CCK-A, was originally characterized in peripheral tissues such as pancreas and gallbladder. It has recently been identified in isolated areas of the CNS (see below). CCK receptors of a different type, CCK-B, are more widely distributed in the CNS. The final receptor type, the stomach gastrin receptor, appears to be closely related to the CCK-B receptor.

It should be noted that other terminologies are used in the literature for CCK-A and CCK-B receptors, i.e., pancreatic-type and cerebral-cortical type receptors (e.g., Jensen et al., 1990) or peripheral-type and central-type receptors (e.g., Chang & Lotti, 1988). However, these terminologies are potentially confusing; as will be described below, 1) both CCK-A and gastrin receptors are peripheral, 2) gastrin receptors have been identified in the pancreas, and 3) CCK-A receptors have been identified in the CNS.

Table II

Classification of CCK/Gastrin Receptor Subtypes.[1]

Receptor Subtypes	Characteristic Tissue	Agonist Selectivity	Antagonists	Location
CCK-A	Pancreas	$CCK_8 >>> CCK_{8U} >$ gastrin $\geq CCK_4$	L-364,718, CR-1409, and A-64,718	Pancreatic acini, gallbladder muscle, pyloric sphincter, lower esophageal sphincter, anterior pituitary cells, spinal cord, interpeduncular nuclei, area postrema, posterior hypothalamic nuclei, nucleus tractus solitarius.
CCK-B	Cerebral cortex	$CCK_8 > CCK_{8U} \geq$ gastrin $> CCK_4$	L-365,260, PD 135158 and CI-988	Several CNS regions with highest densities in cerebral cortex, nucleus accumbens, caudate nucleus, olfactory bulb, and hippocampus.
Gastrin	Parietal cells	$CCK_8 =$ gastrin $>> CCK_{8U} >> CCK_4$	L-365,260, PD 135158 and CI-988	Parietal cells, pancreatic acinar cells, gastric glands, GI smooth muscle.

[1] Adapted from Jensen et al. (1990).

I-3.1 Pharmacological Characterization

Because of the lack of suitable receptor antagonists, the different receptor subtypes were originally distinguished by their relative affinities for various CCK peptides. Until very recently, only three classes of CCK receptor antagonists had been reported (see Figure 2): 1) derivatives of cyclic nucleotides such as Bt_2cGMP; 2) modified amino acids such as proglumide and benzotript; and 3) C-terminal CCK fragments or partial sequences of the C-terminal such as N-CBZ-CCK_{27-32}. Unfortunately, all of these antagonists were plagued by low affinities for CCK receptors and did not discriminate effectively between the different receptor subtypes (see Morency & Mishra, 1987).

Within the last five years, a series of "second-generation" antagonists have been developed. The first class of antagonists (e.g., CR-1409 and CR-1505) represents a major improvement over the parent amino acid derivative proglumide (Rovati & Makovec, 1988). The second class (e.g., L-364,718 and L-365,260) represents a new chemical class of CCK antagonists, i.e., non-peptide compounds that possess a benzodiazepine ring (see Chang & Lotti, 1988; Evans et al., 1988). The third class of antagonists (e.g., A-64,718) are hybrid structures of CR-1409 and L-364,718 (see Nadzan et al., 1988). In addition, several CCK-derived peptides and pseudopeptides have been synthesized and reported to be potent CCK receptor antagonists (Horwell et al., 1991; Hughes et al., 1990; Martinez et al., 1988). These "second-generation" antagonists not only possess higher affinities but can also discriminate between the different receptor subtypes. Briefly, L-364,718, CR-1409, and A-64,718 have high selectivity for CCK-A receptors whereas L-365,260 and CI-988 have high selectivity for

CCK-B/gastrin receptors (Chang & Lotti, 1988; Freidinger, 1989; Horwell et al., 1991; Hughes et al., 1990; Nadzan et al., 1988; Rovati & Makovec, 1988; Silverman et al., 1987; Woodruff & Hughes, 1991). At present, the different types of CCK antagonists have similar affinities for CCK-B and gastrin receptors and these receptors can only be distinguished by the relative affinities for agonists.

I-3.1.1 CCK-A Receptors

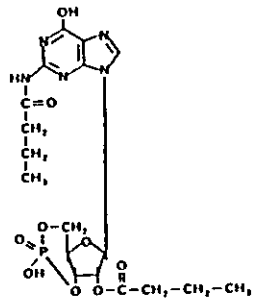
Results from several studies suggest that the C-terminal tetrapeptide sequence (CCK_4) is sufficient for binding to pancreatic CCK-A receptors, but the C-terminal heptapeptide sequence (CCK_7) with the sulfated tyrosine is necessary for full biological activity and potency. Thus, CCK fragments of seven or more amino acids appear to act as full agonists. Briefly, when compared to CCK_{33}, CCK_{10} and CCK_8 were about three times more potent, CCK_7 equipotent, CCK_6 300-1000 times less potent and CCK_5 and CCK_4 1000-3000 times less potent in their binding affinity and in their ability to stimulate amylase secretion (Jensen et al., 1980; 1982; Miller et al., 1981; Robbercht et al., 1978; Sankaran et al., 1980; 1982; Steigerwalt & Williams, 1981).

Caerulein, a decapeptide originally isolated from the skin of an Australian frog (Anastasi et al., 1968) possesses the same C-terminal octapeptide sequence as CCK except for a threonine instead of a methionine residue as the sixth amino acid from the C-terminal (see Figure 1). It is approximately twice as potent as CCK_8 and 6-8 times more potent than CCK_{33} in its ability to bind pancreatic CCK receptors and stimulate amylase release (Jensen et al., 1980; Sankaran et al., 1980). In contrast, sulfated and unsulfated $gastrin_{17}$ are about 100 and 1000

Figure 2 Structure of common CCK antagonists.

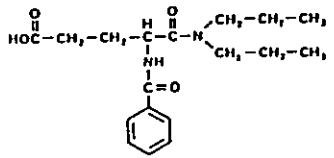
The structures of the most common CCK antagonists from each of the different classes are depicted in this figure. Apart from L-365,260, the remaining CCK antagonists illustrated have little or no affinity for CCK-B/gastrin receptors. Adapted from Jensen et al. (1990) and Morency & Mishra (1987).

DERIVATIVES OF CYCLIC NUCLEOTIDES

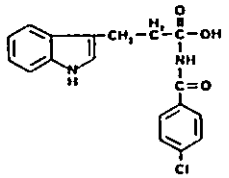


Bt_2-cGMP

DERIVATIVES OF AMINO ACIDS

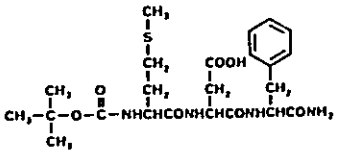


Proglumide

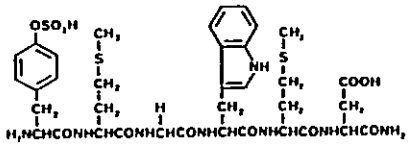


Benzotript

CCK FRAGMENTS OF C-TERMINAL

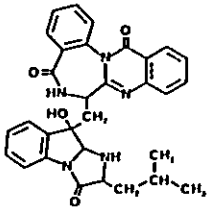


BOC-CCK_{31-33}

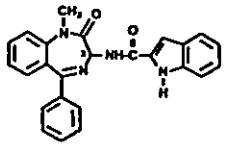


CCK_{27-32}-NH_2

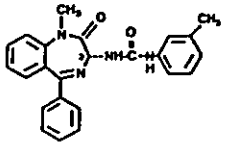
NONPEPTIDAL ANTAGONISTS



Asperlicin



L-364,718



L-365,260

times less potent than CCK_{33}, respectively (Jensen et al., 1980; Sankaran et al., 1980; Steigerwalt & Williams, 1981). As illustrated in Figure 1, peptides of the gastrin family have the sulfated tyrosyl residue at the sixth amino acid from the C-terminal instead of the seventh amino acid like peptides of the CCK and caerulein families. Therefore, both the presence and position of the sulfated tyrosine appear to be critical. Chemical modifications and substitutions of amino acids in C-terminal fragments of CCK generally results in a decrease of binding affinity and biological potency (Desbuquois, 1985). Functionally, the most important component is the sulfated tyrosyl residue. Desulfation of CCK_8 and CCK_7, as well as caerulein, decreases the ability of these peptides to bind to pancreatic CCK-A receptors and to stimulate amylase secretion by 300 to 5000 fold (Innis et al., 1979; Jensen et al., 1980; 1982; Miller et al., 1981; Robbercht et al., 1978; Sankaran et al., 1980; 1982; Steigerwalt & Williams, 1981). Replacing the sulfated tyrosyl residue by a phosphate or shifting it to the left or right in the amino acid sequence decreases biological potency to the same extent as desulfation and leaves gastrin-like activity (Bodanszky et al., 1977; Emson & Marley, 1983; Kaminski et al., 1977; Mutt, 1978; Robbercht et al., 1978).

As with the CCK agonists, there was a close correlation between the ability of these CCK antagonists to inhibit binding of radiolabelled CCK and their ability to inhibit CCK-stimulated amylase release. In terms of ability to inhibit binding of [^{3}H]L-364,718 or [^{125}I]BH-CCK to pancreatic CCK-A receptors, L-364,718 has the greatest affinity reported (0.08 to 2.0 nM). L-364,718 has approximately a 50-fold higher affinity than A-63,718, 150-fold higher than CR-1409, 250-fold higher than L-365,260, 2700-fold higher than N-CBZ-CCK_{27-32},

over 100,000-fold greater than Bt_2cGMP, benzotript, and proglumide (for reviews see Gardner & Jensen, 1984; Jensen et al., 1990; Zucker et al., 1988). The antagonism was characterized by a parallel shift of the CCK concentration-response curve to the right without significant reduction in the maximal biological response. In addition, inhibition of biological responses was reversible and specific for CCK-related peptides; these antagonists did not inhibit the actions of unrelated peptides or compounds (see Gardner & Jensen, 1984; Jensen et al., 1990; Zucker et al., 1988).

Gastrin and CCK share their C-terminal pentapeptide amide, the region of gastrin which represents that hormone's receptor binding domain. In the mammalian stomach, gastrin receptors on parietal cells have equal affinity for gastrins and CCK (Sankaran et al., 1980) and a much higher affinity for L-365,260 than for the CCK-A antagonist, L-364,718 (Jensen et al., 1990). Interestingly, gastrin receptors were recently identified in the pancreas. Yu et al. (1990) used both [^{125}I]BH-CCK_8 and [^{125}I]gastrin to characterize CCK binding sites in guinea pig pancreatic acini. As expected, CCK-A receptors had a 445 to 133,333 fold greater affinity for CCK_8 than $gastrin_{17}$I, CCK_{8U}, or CCK_4 and interacted with high affinity with the two selective CCK-A antagonists, CR-1409 and L-364,718. In addition, these investigators detected a small proportion of CCK binding sites which had a high affinity for both gastrin and CCK_8 and relatively high affinities for CCK_{8U}, CCK_4, and pentagastrin. This class of receptors also had a 100 fold lower affinity for CR-1409 and L-364,718 compared to the pancreatic CCK-A receptors (Yu et al., 1990).

CCK-A receptors have also been identified in other tissues. Structure-

activity studies of ligand binding and/or biological activity have demonstrated that CCK-A receptors on pancreatic acinar cells resemble those in the gallbladder, in the ileum (Chang & Lotti, 1986; Gaudreau et al., 1987; Schjoldager et al., 1988; Steigerwalt et al., 1984; Von Schrenck et al., 1988), in the anterior pituitary cells (Reisine & Jensen, 1986), and in the lower oesophageal sphincter (Ratton & Goyal, 1986).

CCK-A receptors have also been identified in several areas of the CNS including the posterior hypothalamic nuclei, interpeduncular nuclei, area postrema, nucleus tractus solitarius (Hill et al., 1987ac; Moran et al., 1986), and spinal cord (Hill et al., 1988). It should be noted that CCK-A receptors were only identified in the monkey and human spinal cord; CCK binding sites in the rodent spinal cord were of the CCK-B subclass (Hill et al., 1988). Species-specific differences were also noted in the interpeduncular nucleus. In the rat, CCK-A binding sites were confined to two discrete regions of the interpeduncular nucleus closely adjacent to the midline. The localization differed somewhat in the mouse in that CCK-A binding was associated with the peripheral rather than the central regions of the interpeduncular nucleus. In contrast, CCK-A binding sites were absent from any regions of the interpeduncular nucleus in the guinea pig (Hill et al., 1987c).

I-3.1.2 CCK-B Receptors

CCK-B receptors were initially identified and characterized in the rat brain over a decade ago (Hays et al., 1980; Saito et al., 1980). Specific binding for [^{125}I]BH-CCK$_{33}$ was found to be the highest in the cerebral cortex, olfactory bulb, and caudate nucleus with appreciable binding also present in the hypothalamus

and hippocampus. Much lower binding or no binding at all was observed in the midbrain, hindbrain and cerebellum (for review, see Morency & Mishra, 1987). In this respect, the regional distribution of CCK binding sites was said to correlate well with the distribution of CCK immunoreactivity (Hays et al., 1980; Saito et al., 1980).

As with the CCK-A receptors, the C-terminal tetrapeptide is the minimal structural requirement for binding to CCK-B receptors (Knight et al., 1984; Steigerwalt & Williams, 1984). In contrast to CCK-A receptors, CCK-B binding sites in mammalian brain show much less sensitivity in their ability to discriminate between CCK-related peptides. Although the relative order of potency of these is very similar for CCK-A and CCK-B receptors (see Table II), CCK fragments of seven amino acids or less, unsulfated CCK fragments and gastrins have a much greater affinity for CCK-B receptors. There is less than a 50-fold difference in potency of the various CCK analogues for CCK-B receptors whereas 1000-5000 fold differences were observed in the pancreatic tissues (Innis & Snyder, 1980ab; Takeda et al., 1989; Van Dijk et al., 1984). Additionally, as mentioned above, another difference between CCK-A and CCK-B receptors is in their interaction with antagonists. In terms of ability to inhibit binding of [^{3}H]L-365,260 or [^{125}I]BH-CCK to cortical CCK-B receptors, L-365,260 has the greatest affinity reported (1.4-1.8 nM). L-365,260 has approximately a 100-fold higher affinity than L-364,718, 1000-fold higher than CR-1409, and over 50,000-fold greater affinity than asperlicin (Chang et al., 1989). Proglumide and benzotript are virtually inactive at the CCK-B receptors (e.g., Lin & Miller, 1985; Wennogle et al., 1985).

A more detailed review of the literature on the pharmacological characterization and localization of CCK-B receptors in the brain is presented in the next chapter.

I-3.2 Biochemical Characterization

The technique of irreversible affinity labelling has been applied for identification and molecular characterization of many different receptors. Affinity labelling of receptor sites consists of two parts: first, the binding of ligand to receptor (affinity component) and second, the formation of a covalent bond (labelling component). This technique has been used for the characterization of the CCK receptors (for review see Miller, 1990). To determine the size and subunit composition of CCK receptors, cross-linked radiolabelled membranes are commonly analyzed by SDS-PAGE.

I-3.2.1 CCK-A Receptors

During early studies, [^{125}I]BH-CCK$_{33}$ was cross-linked to pancreatic membranes with photoactive homobifunctional cross-linking agents or with heterobifunctional cross-linking agents. Briefly, when [^{125}I]BH-CCK is cross-linked to its receptors on pancreatic membranes in the presence of a reducing agent (β-mercaptoethanol or DTT), the major labelled band was at M_r~85000. When membranes were not reduced, a major band was labelled at M_r~130000; this band could be converted to M_r~85000 upon reduction. As depicted in Figure 3, these data suggested that the pancreatic receptor is a M_r~130000 protein consisting of two subunits: A M_r~85000 binding domain connected by a disulfide bond to a M_r~45000 subunit of unknown function (for review, see Morency & Mishra, 1987).

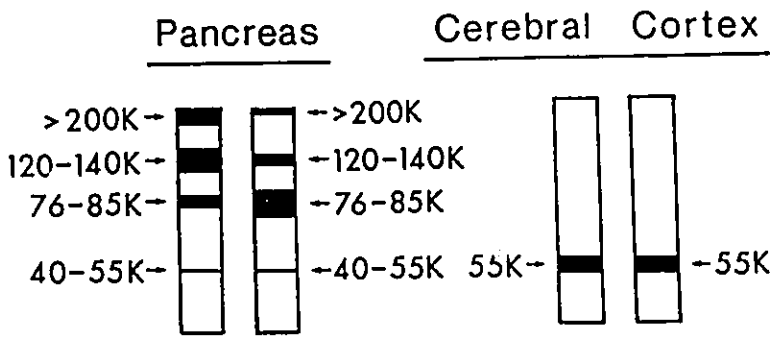
Several lines of evidence suggest that these proteins are constituents of the pancreatic CCK-A receptors. First, when [^{125}I]BH-CCK_{33} was added in the presence of excess unlabelled CCK_8, bands were completely absent. Second, the concentrations of unlabelled CCK_8 required to inhibit binding were similar to those required to inhibit cross-linking. Finally, Bt_2cGMP, a weak CCK antagonist, inhibited both binding and cross-linking of [^{125}I]BH-CCK_{33} under similar conditions (see Morency & Mishra, 1987). Later attempts to biochemically characterize the pancreatic CCK-A receptors by affinity labelling involved short CCK peptides with an intrinsic photoactivable chemical group (e.g., Fourmy et al., 1989; Klueppelberg et al., 1989ab; Pearson et al., 1987; Powers et al., 1988). These probes offer two significant advantages, their small sizes and monovalency, thereby increasing the likelihood of labelling the active binding site. Indeed, photoaffinity labelling of pancreatic membranes with these monofunctional probes identified an M_r80000-95000 protein with a M_r42000 core protein.

Similarly, structural characterization of the gallbladder CCK-A receptor using monofunctional photoactivable probes identified a protein with an M_r85000-95000 in the human gallbladder (Schjoldager et al., 1989) and with an M_r70000-85000 in the bovine gallbladder (Shaw et al., 1987; Schjoldager et al., 1988). Further biochemical characterization revealed that CCK receptors in human and bovine gallbladders are both N-linked complex glycoproteins with different carbohydrate domains and similar M_r43000 protein cores (Schjoldager et al., 1990).

Finally, the purification of the pancreatic CCK-A receptor was recently accomplished by Duong et al. (1989) and Szecowka et al. (1989). Although

Figure 3 Diagrammatic representation of the subunit structure of CCK-A and CCK-B receptors as determined by affinity cross-linking.

Electrophoretic mobilities on SDS-PAGE performed under reducing conditions and without reduction are illustrated. Cross-linking of [^{125}I]BH-CCK$_{33}$ to pancreatic CCK-A and cortical CCK-B receptors was inhibited by addition of excess unlabelled CCK$_8$. Adapted from Morency & Mishra (1987).



(1) Reducing agent	−	+	−	+
(2) Inhibition by CCK_8	Yes		Yes	

different purification schemes were used, both groups obtained a predominant polypeptide of M_r80000-90000 and a minor polypeptide of M_r55000-57000. Duong et al. (1989) reported an additional minor polypeptide of M_r26000. Binding to the purified receptor preparation was comparable to that observed in the crude solubilized pancreatic membrane preparation (Duong et al., 1989; Szecowka et al., 1989).

I-3.2.2 CCK-B Receptors

As mentioned previously, the CCK-A and CCK-B receptors differ markedly in selectivity for various analogues, suggesting they may have different structural components. Indeed, when [^{125}I]BH-CCK_{33} was cross-linked to brain membrane receptors, SDS-PAGE revealed only one broad labelled band of about M_r~55,000 (Sakamoto et al., 1984). Interestingly, treatment of the solubilized receptors with a reducing agent had no major effect on this band, indicating that it was not connected to a non-binding component by a disulfide link as the pancreatic binding proteins (see Figure 3). The appearance of this band could be blocked by incubation with CCK_8 (100 nM) or CCK_4 (1 μM). It should be noted that the same concentration of CCK_4 did not alter the cross-linking of [^{125}I]BH-CCK_{33} to pancreatic membranes (Sakamoto et al., 1983), illustrating the differential selectivity of CCK-A and CCK-B receptors previously observed in binding studies. Thus, CCK-B receptors appear to be both structurally and functionally distinct from the pancreatic CCK-A receptors.

To date, no confirmation or additional characterization of CCK-B receptors is available. However, McVittie et al. (1990) recently presented evidence that they have cloned a CCK/gastrin-responsive G protein-coupled

receptor. Using the polymerase chain reaction method, a 2.2 kb message was amplified from rat striatal mRNA. This clone was found to encode for a 326 amino acid protein which contains seven transmembrane domains as predicted by hydropathy analysis. In situ hybridization analysis indicated a high abundance of mRNA in the cortex, hippocampus, and thalamus with lower levels in the striatum, olfactory bulb, mesencephalon, and pituitary. To establish the identity of this receptor, RNA was transcribed from the cDNA clone and expressed in Xenopus oocytes. Of the numerous agonists tested for their ability to induce $^{45}Ca^{2+}$ efflux activity, both CCK and gastrin exhibited the greatest stimulation of efflux in RNA- but not H_2O-injected oocytes (McVittie et al., 1990). The cloning and sequencing of the CCK-B receptor will facilitate comparisons between the CCK receptors classes.

I-4 CCK FUNCTIONS IN THE CNS

The functional role of CCK in the CNS has been an area of intensive investigation over the past 15 years. CCK has been implicated in a wide variety of physiological functions, including analgesia, anxiety, respiration, satiety, sedation, and thermoregulation (for review, see Albus, 1988; Baber et al., 1989; Baile et al., 1986; Morley & Levine, 1985; Sheehan & de Belleroche, 1984; Silver & Morley, 1991). However, the role of CCK in satiety, nociception, and the effects of CCK in the mesocorticolimbic and nigrostriatal pathways (relevant to CCK/DA interactions) have been the topics attracting the most interest.

I-4.1 CCK and Feeding

There is considerable literature on the inhibitory effects of CCK on

feeding behaviour (for review, see Baile et al., 1986; Blundell, 1991; Silver & Morley, 1991; Stacher, 1986). Systemic administration of CCK reduces food intake in numerous animal species and produces a sensation of satiation in humans. These effects are readily blocked by various CCK-A antagonists (for review, see Dourish et al., 1988; Schneider et al., 1988). However, because of the large supraphysiological doses of CCK required to induce these effects, controversy existed over whether the effects of CCK represented a true satiating effect or are merely secondary to toxicity or aversion.

The development of specific antagonists has provided renewed support for the role of CCK in satiety mechanisms. It was reasoned that if endogenous CCK plays a significant role in producing satiety, then CCK antagonists should increase food intake by antagonizing the effects of endogenously released CCK. However, it has been more difficult to confirm a role for endogenous CCK by demonstrating increased food intake in otherwise normal animals treated with CCK-A antagonists, though some success has been reported in rodents (see Dourish et al., 1988; Schneider et al., 1988) and in humans (Wolkowitz et al., 1990).

Recently studies by Dourish and colleagues may have provided an explanation for the weak antisatiety effects of CCK-A antagonists. When comparing the CCK-A antagonist L-364,718 and the CCK-B/gastrin antagonist L-365,260, both increased the frequency of feeding and decreased the onset of resting after access to food in rats that had been starved overnight. L-365,260, however, was 100 times more potent than L-364,718 (Dourish et al., 1989b). This rank order of potency agrees with the ability of these drugs to displace

radiolabelled CCK from brain and stomach membranes (Section I-3.1). In contrast, the hypophagic effects of exogenous CCK_8 were blocked by L-364,718 but not L-365,260 (Dourish et al., 1989a). When considered together, these data suggest that exogenous CCK inhibits feeding by an action on CCK-A receptors whereas CCK antagonists increase food intake by blocking the action of endogenous CCK/gastrin at CCK-B/gastrin receptors.

The role of CCK in the central control of feeding has been more controversial compared to its peripheral effect. For example, some investigators (e.g., Sheehan & de Belleroche, 1984) have argued that CCK-induced satiety is clearly a peripheral action because, in the rat, CCK is more effective when given peripherally than centrally and bilateral vagotomy abolishes the response to peripherally administered CCK_8. However, convincing evidence for CCK's satiety effect in the CNS has been accumulating. For example, although CCK is less effective in decreasing food intake when injected centrally as compare to peripheral injections in rodents, the converse has been observed in other species (see Silver & Morley, 1991). Furthermore, in the rat, microinjections of CCK into the ventricles produced a dose-related depression of gastric contraction (Bueno & Ferre, 1982), free feeding (Schick et al., 1986), food-rewarded lever pressing (Maddison, 1977), and running in an alley to get food (Zhang et al., 1986).

Suppression of feeding in food-deprived rats has also been achieved by intracranial infusion of small amounts of CCK_8 in various brain areas: medial pontine area (Schick et al., 1990); lateral medulla (Schick et al., 1990); lateral hypothalamus (Willis et al., 1984); and paraventricular nucleus (Schwartz et al., 1988), suprachiasmatic nucleus (Mori et al., 1986), and ventromedial nucleus

(Stern et al., 1976) of the hypothalamus. Injections of equal doses of CCK in various thalamic nuclei, in the medial aspects of the amygdala, in the substantia nigra, in mesencephalic central grey, or the medial medulla were without effect (Schick et al., 1990). Interestingly, it has recently been demonstrated that CCK is released from the vicinity of the lateral hypothalamus in cats and primates after loading of the stomach (Schick et al., 1987; 1989).

Although their respective contributions are still not fully understood, it is now generally accepted that both central and peripheral CCK affect food intake (see Silver & Morley, 1991).

I-4.2 CCK and Nociception

The presence of CCK in several areas known to be involved in nociception (e.g., periaqueductal grey, dorsal horn of the spinal cord) provided the initial basis for studies on the possible analgesic actions of this neuropeptide (for review, see Baber et al., 1989; Dourish et al., 1988; Stacher, 1986). Peripheral or central administration of high doses of CCK produced an analgesic effect (Hill et al., 1987b; Jurna & Zetler, 1981; Zetler, 1980). In contrast, small doses of CCK, that are probably in the physiological range, blocked opiate-mediated analgesia (Faris et al., 1983; Itoh et al., 1982b; O'Neill et al., 1989).

CCK has been shown to be released in response to opiate injection (Tang et al., 1984) and it was proposed that the released peptide could act via a negative feedback mechanism to return the organism to its basal level of pain sensitivity (Baber et al., 1989; Watkins et al., 1985). This proposal received support from studies with the weak non-specific antagonist, proglumide. This agent potentiated morphine analgesia and prevented the development of

tolerance (Paneri et al., 1987; Watkins et al., 1984; 1985ab). However, proglumide had no effect on pain threshold when given alone and did not potentiate various forms of non-opiate analgesia (Watkins et al., 1984; 1985a). Furthermore, proglumide has been shown to enhance the analgesia induced by endogenous opiates (Watkins et al., 1985b) and enhance opiate-induced analgesia in humans (Lavigne et al., 1989; Price et al., 1985).

These observations have now been extended to the current, more potent and selective antagonists. These "second-generation" CCK antagonists also enhanced morphine analgesia and prevented morphine tolerance; L-364,718 had a similar potency in primates and rodents (for review, see Baber et al., 1989). This enhancement of morphine analgesia was achieved selectively without potentiating the morphine-induced respiratory depression, a dangerous and life-threatening side effect of this drug (Dourish et al., 1990b).

The rank order of potency for enhancing morphine analgesia in rats was determined to be L-365,260>L-364,718>L-365,031 (Dourish et al., 1990a). This rank order correlates well with the potency of these antagonists in blocking CCK-B receptors and suggests that CCK/opiate interactions in this species are mediated by CCK-B receptors. As mentioned previously (Section I-3.1.1), profound interspecies differences exist in spinal cord CCK receptors (i.e., CCK-B receptors in rodents vs CCK-A receptors in primates and man). Thus, the similar potency of L-364,718 in potentiating morphine analgesia in rodents and primates, together with the lack of effect of this drug on morphine-induced respiratory depression, a response mediated by opiate receptors in the brain stem and spinal cord (Ling et al., 1985), suggests a supraspinal mechanism for the mediation of

CCK antagonist potentiation of opiate analgesia. Indeed, O'Neill et al. (1990) recently reported that blockade of CCK-B receptors by L-365,260 induced analgesia in the squirrel monkey. Therefore, specific CCK antagonists may have a therapeutic role in enhancing exogenous and/or endogenous opioid analgesia and in preventing tolerance to opioid analgesics.

I-4.3 CCK and Dopamine

As was described in Section I-1, the coexistence of CCK and DA has been demonstrated within neurons of the ventral mesencephalon with immunohistochemical and/or in situ hybridization techniques. In the rat, a high degree of colocalization was observed in the substantia nigra pars compacta, especially the intermediate and dorsal regions, and pars lateralis. In contrast, very little colocalization was observed in the substantia nigra pars lateralis. Other areas of the ventral midbrain that exhibited a moderate proportion of neurons containing both CCK and tyrosine hydroxylase included the ventral tegmental area, interfascicular nucleus, and rostral and caudal linear nuclei (for review, see Fallon, 1988; Hökfelt et al., 1985). Recently, Seroogy et al. (1989a) combined these double-labelling colocalization techniques with fluorescence retrograde tracing in order to determine some of the forebrain projections of these neurons. Ventral midbrain neurons containing both CCK and tyrosine hydroxylase were found to project to the nucleus accumbens, prefrontal cortex, and caudate-putamen (Seroogy et al., 1989a).

The discovery of CCK being co-localized with DA in some areas of the brain has generated considerable interest in potential CCK/DA interactions. The value of this intense research activity is underscored by growing evidence

implicating abnormal DA function in patients with neuropsychiatric disorders including Parkinson's disease and schizophrenia (e.g., Carlton & Manowitz, 1984; Haracz, 1982; Hornykiewicz, 1985; Lee & Seeman, 1980; Matthysse, 1974; Meltzer & Stahl, 1976; Mishra, 1986; Snyder, 1976; 1981; Van Kammen, 1979).

Biochemical, pharmacological, electrophysiological and behavioural investigations have suggested a modulatory role for CCK on central DA function. However, many inconsistencies exist in this literature, especially concerning the facilitatory versus inhibitory effects of CCK upon several DA-mediated functions (for review, see Nair et al., 1986). What follows is not intended to be an exhaustive review of the literature on CCK/DA interactions but rather to exemplify the inconsistencies found in this literature. A more detailed review of the pertinent literature will be presented in subsequent chapters. Thus, CCK has been reported by some investigators to induce decreases in basal DA release (Altar, 1988; 1989; Altar & Boyar, 1989; Blaha et al., 1987; Cosi et al., 1989; Lane et al., 1986; 1987; Markstein & Hökfelt, 1984), K^+-induced DA release (Voigt & Wang, 1984; Voigt et al., 1986), DA turnover (Altar, 1989; Altar et al., 1988; Fekete et al.,1981a; Fuxe et al.,1980; Marshal et al., 1983; Matsumoto et al., 1984), and DA binding (Agnati et al., 1983ab; Fuxe et al., 1981ab; 1983; Marshal et al., 1983; Murphy & Schuster, 1982). In contrast, other investigators have reported that CCK increases basal DA release (Marshall et al., 1990; Voigt et al., 1986), K^+-induced DA release (Hamilton et al., 1984; Hetey et al., 1988; Kovács et al., 1981; Starr, 1982; Vickroy & Biandhi, 1989; Vickroy et al., 1988), DA turnover (Fekete et al., 1981b; Kovács et al., 1981; Laitinen et al., 1990), and DA binding (Agnati & Fuxe, 1983; Agnati et al., 1984; Dumbrille-Ross & Seeman,

1984; Murphy & Schuster, 1982). Still others have reported that CCK did not significantly alter basal DA release (Hamilton et al., 1984; Starr, 1982; Von Voightlander & Losey, 1982), K^+-induced DA release (Hamilton et al., 1984; Vickroy & Biandhi, 1989), DA turnover (Kovács et al., 1981; Matsumoto et al., 1984; Widerlöv et al., 1983ab), and DA binding (Dumbrille-Ross & Seeman; 1984; Fuxe et al., 1983; Murphy, 1985). Finally, while some behavioural studies have demonstrated neuroleptic-like effects of CCK-peptides (Cohen et al., 1982; De Witte et al., 1988; Fekete et al., 1984; Katsuura & Itoh, 1982; Van Ree et al., 1983; Zetler, 1981; 1983), others have reported potentiation of DA-mediated behaviours (Crawley et al., 1985ab; 1986; Daugé et al., 1990; Ellinwood et al., 1983; Pettit & Mueller, 1989; Vaccarino & Rankin, 1989) and still others reported a lack of interactions (Hamilton et al., 1984; Schneider et al., 1983; Widerlöv et al., 1983a). The reason for some of these discrepancies have been related to differences in species, brain regions, dose of CCK, and experimental procedures. However, in several studies, discrepant findings were obtained despite similar methodologies.

Although the majority of CCK/DA interaction studies have focused on the effects of CCK on DA functions, it is worth noting that DAergic agents have been shown to modulate CCK content (Frey, 1983; Yoshikawa et al., 1989ab), CCK release (Artaud et al., 1989; Butcher et al., 1989; Meyer & Krauss, 1983; Meyer et al., 1984), CCK binding (Chang et al., 1983; Debonnel et al., 1990; Fukamauchi et al., 1987; Morency & Mishra, 1987; Suzuki & Moroji, 1989), and CCK-mediated behaviours (Morency et al., 1987a; Pfaus & Phillips, 1987; Worms et al., 1986).

I-5 CCK IN NEUROLOGICAL AND PSYCHIATRIC DISORDERS

The growing evidence implicating abnormal DA function in patients with neurological and psychiatric disorders such as schizophrenia and Parkinson's disease (e.g., Carlton & Manowitz, 1984; Haracz, 1982; Hornykiewicz, 1985; Lee & Seeman, 1980; Matthysse, 1974; Meltzer & Stahl, 1976; Snyder, 1976; 1981; Van Kammen, 1979) in conjunction with the demonstrated CCK/DA co-existence and interactions in animal studies provided a theoretical basis for investigating the involvement and potential therapeutic value of CCK in these disorders. Starting from the premise that CCK might be involved in the pathophysiology of certain neurological and psychiatric disorders, several investigators have sought to measure potential abnormalities of CCK systems in afflicted patients. Since direct measures are not possible in humans, studies have concentrated on two fronts: measures of CCK levels in the CSF and studies on post-mortem brains.

I-5.1 Levels of CCK in the CSF

As summarized in Table III, the levels of CCK have been measured in the CSF of patients with anorexia nervosa, depression, mania, Parkinson's disease, and schizophrenia. Conflicting results are prevalent in this literature. For example, Lostra et al. (1985) observed a depletion of CCK in the CSF of drug-free Parkinson's patients whereas Verbank et al. (1984) did not detect any significant differences.

Although no significant changes in CCK levels have been observed in the CSF of neuroleptic-treated schizophrenic patients (Lostra et al., 1985; Tamminga et al., 1986; Verbanck et al., 1984), conflicting results have been reported for drug-free schizophrenics. Verbank et al. (1984) and Garver et al. (1990)

Table III

CCK in Neurological and Psychiatric Diseases: Levels of CCK in the Cerebrospinal Fluid.

Study	N[1]	Patient Diagnosis	Diagnosis Criteria[2]	Drug status	Results
SCHIZOPHRENIA:					
Gerner & Yamada (1982)	39	Controls	RDC	Drug-free for 7 days.	No significant differences.
	23	Anorexia nervosa			
	10	Manics			
	28	Primary major depression			
	13	Schizophrenia		Neuroleptic-free for 14 days.	
Gjerris et al. (1984)	17	Controls	ICD-9	Drug-free for 7 days.	No significant differences.
	17	Endogenous depression	NCRS		
	11	Non-endogenous depression	BRMES		
	7	Manics			
	6	Schizophrenia	BPRS		
Verbanck et al. (1984)	51	Controls			
	13	Parkinson's disease	CC/HVA	PD drug-free for 7+ days.	Decreased levels in patients with bipolar depression and in drug-free schizophrenics.
	9	Bipolar manic-depression	FC	MD drug-free for 14+ days.	
	9	Untreated schizophrenia	FC	US drug-free for 6+ weeks.	
	6	Treated schizophrenia	FC	TS treated with haloperidol.	
Gerner et al. (1985)	31	Controls	RDC	Drug-free for 14-77 days.	Elevated levels in schizophrenics.
	72	Chronic schizophrenia			

Lotstra et al. (1985)	68	Controls			
	20	Parkinson's disease	CC	PD drug-free for 7+ days.	Decreased levels in patients with Parkinson's and in drug-free schizophrenics.
	30	Primary major depression	RDC	PMD drug-free for 14+ days.	
	16	Untreated schizophrenia	RDC	US drug-free for 6+ weeks.	
	10	Treated schizophrenia	RDC	TS treated with haloperidol.	
Rafaelsen & Gjerris (1985)	15	Controls	ICD-9	Not available.	No significant differences.
	26	Endogenous depression	NCRS		
	14	Non-endogenous depression	BRMES		
	7	Schizophrenia	BPRS		
Tamminga et al. (1986)	8	Controls	DSM-III/RDC		
	9	Untreated schizophrenia		US drug-free for 4+ weeks.	Elevated levels in drug-free schizophrenics.
	3	Treated schizophrenia		TS treated with neuroleptics.	
Garver et al. (1990)	6	Controls	DSM-III	Drug-free for 14+ days.	Decreased levels in schizophrenics.
	11	Schizophrenia			

1 N = number of patients in the study.

2 BPRS (Brief Psychiatric Rating Scale); BRMES (Bech-Rafaelsen MElancholia Scale); CC (Clinical Criteria); DSM-III (Diagnostic and Statistical Manual of Mental Disorders III); HVA (Low levels of homovanillic acid in CSF); FC (Feighner's criteria); ICD-9 (International Classification of Disease - Ninth Edition); NCRS (New-Castle Rating Scale); RDC (Research Diagnostic Criteria).

observed a depletion of CCK in lumbar CSF in drug-free schizophrenics compared to controls, suggesting that a loss of CCK in the brain was a part of the psychopathology of the disease. Gerner & Yamada (1982) also found reduced CCK levels in CSF of untreated schizophrenics; however, the difference was not statistically significant. Similarly, no significant differences in the levels of CCK in CSF of drug-free schizophrenics were reported by Gjerris et al. (1984) and Rafaelsen & Gjerris (1985). In contrast, Gerner et al. (1985) and Tamminga et al. (1986) reported increased levels of CCK in lumbar CSF, not decreased. Whether the conflicting results can be explained or not, the relevance of lumbar CSF measures remains a controversial issue. It is by no means certain whether the CCK in lumbar CSF is derived from spinal cord tissue or the cerebrum (Tamminga et al., 1985).

I-5.2 Post-mortem Studies

In addition to CCK measurements in CSF, investigators have also examined post-mortem brain tissues (see Table IV). Decreased CCK-LI levels were reported in the substantia nigra of Parkinson's patients (Studler et al., 1982) and in the globus pallidus and substantia nigra of Huntington's patients (Emson et al., 1980b). In addition, CCK binding was decreased in the basal ganglia and cerebral cortex of Huntington's patients (Hays et al., 1981; Hays & Paul, 1982). These alterations, however, may simply result from the degeneration occurring in these neurodegenerative disorders.

Sanders et al. (1982) reported increased CCK levels in the white matter but not the grey matter of the temporal and occipital cortex of Alzheimer-type patients. Rossor et al. (1981) had previously reported that there was no

significant differences in CCK-LI concentrations between controls and patients with senile dementia of Alzheimer type. However, these investigators did not separate grey from white matter in the cortex and, therefore, may have missed an increased CCK level.

When comparing CCK levels in schizophrenics and normal controls, no significant differences in CCK levels were detected in the entorhinal cortex by Perry et al. (1981), and in the amygdala, nucleus accumbens, caudate nucleus, substantia nigra, hippocampus, frontal and temporal cortex by Kleinman et al. (1983; 1985). In contrast, significant decreases in CCK-LI levels were reported in the temporal cortex of schizophrenics by Ferrier and colleagues (Ferrier et al., 1983; 1985; Roberts et al., 1983). Another interesting finding from their studies was that CCK was also significantly reduced in the hippocampus and amygdala in those patients who had been rated as having negative symptoms. These authors noted that CCK may be worth studying as a potential therapeutic agent in this generally treatment-resistant group.

Significant reductions in CCK binding have been detected in the hippocampus and frontal cortex of schizophrenics (Farmery et al., 1985; Ferrier et al., 1983; 1985). Although the schizophrenics were receiving neuroleptics, these authors suggest that this would not account for the findings since a drug effect would be expected to occur in all brain regions. Moreover, animal studies have shown that chronic neuroleptic treatment increases CCK binding (e.g., Chang et al., 1983; Fukamauchi et al., 1987; Morency & Mishra, 1987).

More recent studies have focused on the localization of CCK mRNA in post-mortem brains by in situ hybridization. Interestingly, Schalling et al. (1989;

Table IV

CCK in Neurological and Psychiatric Diseases: Post-mortem Studies.

Study	N[1]	Patient Diagnosis	Results
SCHIZOPHRENIA:			
Perry et al. (1981)	12 5	Controls Schizophrenia	No significant differences in CCK concentration in the entorhinal cortex (Brodman Area 28).
Roberts et al. (1983)	12 14	Controls Schizophrenia	Decreased CCK-LI in the temporal cortex; decreased CCK-LI in the amygdala and in the hippocampus of patients with morbid negative symptoms.
Ferrier et al. (1983; 1985)	12 14	Controls Schizophrenia	Decreased CCK-LI in the temporal cortex (Brodman Area 21 & 22); decreased CCK-LI in the amygdala and hippocampus of patients with negative symptoms. No significant changes in the frontal, parietal, and cingulate cortex, dorso-medial and lateral thalamus, globus pallidus, and putamen. Decreased CCK binding in the hippocampus and in the frontal cortex of schizophrenics.
Farmery et al. (1985)	16 16	Controls Schizophrenia	Decreased CCK binding in the hippocampus and in the frontal cortex of schizophrenics.
Kleinman et al. (1983; 1985)	30 11 6 11	Controls Paranoid schizophrenia Undifferentiated schizo. Other psych. disorders	No significant differences in CCK-LI in the amygdala, nucleus accumbens, caudate nucleus, frontal cortex, substantia nigra, hippocampus, and temporal cortex.
Schalling et al. (1989)	1	Schizophrenia	Presence of CCK mRNA in dopamine cells in the ventral mesencephalon. These authors cite a study by Palacios et al. (1989) in which the presence of CCK mRNA was not detected in substantia nigra dopaminergic cells of 11 control adults.
Schalling et al. (1990)	5 5	Controls Schizophrenia	Extensive codistribution of CCK mRNA and tyrosine hydroxylase mRNA was observed in the ventral mesencephalon of 5/5 schizophrenics. A weaker signal was observed in 2/5 controls.

PARKINSON'S DISEASE:		
Studler et al. (1982)	13 Controls 10 Parkinson's disease	Decreased CCK-LI in substantia nigra of Parkinson's disease; normal levels in the frontal cortex, striatum, and nucleus accumbens.
HUNTINGTON'S DISEASE:		
Emson et al. (1980b)	10 Controls 10 Huntington's disease	Decreased CCK-LI in the globus pallidus and in the substantia nigra of Huntington's disease; no significant difference in the striatum.
Hays et al. (1981)	7/5 Controls 7/5 Huntington's disease	Decreased CCK binding ($\downarrow B_{max}$) in the basal ganglia (n=7) and in the cerebral cortex (n=5) of Huntington's disease with no change in apparent binding affinity (K_d).
Hays and Paul (1982)	9/11 Controls 9/11 Huntington's disease	Decreased CCK binding in the putamen (n=9) and in the cerebral cortex (n=11) of Huntington's disease.
ALZHEIMER'S DISEASE:		
Rossor et al. (1981)	10 Controls 10 Alzheimer's dementia	No significant differences in CCK-LI.
Sanders et al. (1982)	12/7 Controls 13/6 Alzheimer's dementia	Increased CCK levels in white matter from occipital (n=13) and temporal cortex (n=6) of Alzheimer's dementia but not in grey matter.
Hays and Paul (1982)	15 Controls 15 Alzheimer's dementia	No significant changes in CCK binding in the temporal and cingulate cortex.

[1] N = number of patients in the study.

1990) have reported extensive codistribution of CCK and tyrosine hydroxylase mRNAs in the ventral mesencephalon of all six schizophrenic brains studied to date. In contrast, a weaker signal was observed in two of the five control brains. Furthermore, the presence of CCK mRNA was not detected in the substantia nigra DAergic cells from 11 control adults in a recent study by Palacios et al. (1989). This raises the exciting possibility that the presence of CCK mRNA in DA cells is a result of the disease or the neuroleptic treatment or both.

I-5.3 Clinical Trials

The clinical effects of CCK have been most widely studied in schizophrenia and will be reviewed here; a few clinical trials with other disorders have been conducted and are summarized in Tables V and VI.

According to the DA hypothesis of schizophrenia, a hyperactive DA system is responsible for at least some of the symptoms associated with this disease (Carlton & Manowitz, 1984; Haracz, 1982; Meltzer & Stahl, 1976). This is further supported by the almost perfect correlation between the clinical potency of neuroleptic drugs and their affinity for the DA D_2 receptor (for review, see Seeman, 1980). Most evidence indicates that the therapeutic actions of neuroleptic drugs is related to a reduction of DA activity in the mesolimbic and mesocortical regions rather than in the nigrostriatal area.

The distribution of CCK in high concentrations in cortical and limbic regions and the documented co-existence of CCK and DA within neurons in the ventral mesencephalon projecting mainly to these regions revealed a unique potential for CCK as a potential antipsychotic. This evidence, combined with the biochemical, electrophysiological, and behavioural evidence demonstrating down-

regulating effects of CCK on DA functions in animals studies (for review, see Section I-4.3; Vanderhaeghen & Crawley, 1985), provided the major impetus for investigations of the possible clinical therapeutic effects of CCK in schizophrenia.

Initial clinial trials of the possible antipsychotic effects of CCK were first carried out in open, uncontrolled studies. In the first preliminary clinical trial, Moroji et al. (1982) administered a single dose of caerulein to 20 chronic schizophrenic patients stabilized on neuroleptics. Sixteen of their patients were noted to have improved mood and three experienced some relief of auditory hallucinations two hours after the injection. There was also a statistically significant improvement in some items of the BPRS. The improvement peaked at one week after the injection and was sustained for a further two weeks. These investigators drew the conclusion that caerulein had a long-acting antipsychotic effect in chronic schizophrenia. Similar positive findings have been reported in seven other open studies with caerulein (Albus et al., 1984; Boza & Retondo, 1985; Itoh et al., 1982a; Moroji et al., 1985; Van Ree et al., 1984; Yamagani et al., 1986) and three studies with CCK fragments (Bloom et al., 1983; Nair et al., 1982; 1983). In these studies, most patients were suffering from chronic schizophrenia and all were maintained on neuroleptics (see Table V).

Such open treatment studies, however, are of limited value because of their methodological shortcomings. Although the results from these open studies were apparently consistent, the lack of blindness on the part of the observer makes it all but impossible to overcome the confounding effect of observer bias on the assessments. To establish therapeutic efficacy, it is necessary to conduct double-blind placebo-controlled studies.

Table V

CCK in Neurological and Psychiatric Diseases: Clinical Trials - Preliminary Studies.

Study	N[1]	Patient Diagnosis[2] (Criteria[3])	Neuroleptics	Drug[4]	Study Design (Rating Scales[5])	Results
SCHIZOPHRENIA:						
Moroji et al. (1982a)	12	CS (DSM-III)	+	CER, 0.3 μg/kg IM x 1	Open uncontrolled (BPRS)	Improved effect.
Moroji et al. (1982b)	20	CS Includes 12 patients from 1st study. (DSM-III)	+	CER, 0.6 μg/kg IM x 1	Open uncontrolled (BPRS)	16/20 improved.
Itoh et al. (1982a)	58	CS (unstated)	+	CER, 20-40 μg IM x 1	Open uncontrolled (BPRS; GIR)	23/58 improved effect.
Nair et al. (1982)	6	CPS Neuroleptic-resistant. (RDC; DSM-III)	+	CCK_{33}, 75 IDU IV x 1	Open uncontrolled (BPRS; SS-PSE; CGI)	6/6 improved.
Nair et al. (1983)	21	CS Neuroleptic-resistant. (RDC)	+	CCK_{33}, 75 or 150 IDU IV x 1	Open uncontrolled (BPRS; SS-PSE; NOSIE)	11/21 improved effect.
Bloom et al. (1983)	8	CS Neuroleptic-resistant. (FC)	+	CCK_8, 0.04 μg/kg IV x 1	Open uncontrolled (BPRS; SS-PSE; NOSIE; SARS)	3/8 good BPRS score. 2/8 fair BPRS score.
Albus et al. (1984)	6	CS (unstated)	+	CER, 0.3 μg/kg IM x 2	Open uncontrolled (BPRS)	6/6 improved effect.
Moroji et al. (1985)	20	CS Neuroleptic-resistant. (DSM-III)	+	CER, 40 μg/kg IM x 1	Open uncontrolled (BPRS)	Improved effect.
	12	Responders to CER from above study. (DSM-III)	+	CER, 40 μg/kg IM x 1/wk for 1 year	Open uncontrolled (BPRS)	11/12 sustained improvement for 1 year.
Boza & Retondo (1985)	9	CS (DSM-III)	+	CER, 0.5-0.8 μg/kg IM x 1	Open uncontrolled (BPRS)	Improved effect. BPRS not seen clinically.

Yamagani et al. (1986)	21	CS (DSM-III)	+	CER, 0.6-2.0 μg/kg IM x 1/wk x 8 wks	Open uncontrolled (BPRS)	Improved effect mainly at doses 1.0-1.2 μg/kg.
Van Ree et al. (1984)	6	CS (PSE; RDC; DSM-III)	+	CER, 40 μg IM x 1/wk x 2 wk	Single-blind. Placebo followed by CER. (BPRS)	Improved effect.
DYSKINESIA:						
Nishikawa et al. (1985; 1986)	7	CS with oral TD (EMG; MV)	+	CER, 0.8 μg/kg IM x 1	Open uncontrolled	2/7 Significant decrease in choreic movements 2 hrs after the injection lasting up to 6 weeks.
Hashimoto & Yanagisawa (1990)	11	HD (n=3); DID (n=2); IOD (n=4); SC (n=1); SLE (n=1)	9+ 2–	CER, 0.8 μg/kg IM every 5-8 days x 5 (n=4); x 4 (n=2); x 2 (n=5)	Open uncontrolled (EMG; AIMS)	Significant decrease in choreic movements minutes after the injection and for several days.
PANIC/ANXIETY DISORDERS:						
de Montigny (1989)	10	Healthy subjects with no history of anxiety, panic-like disorders, phobia, obsessive-compulsive symptoms, or epilepsy.	–	CCK_4, 20-100 μg IV	Open uncontrolled (PAS; CRAS)	7/10 CCK_4 induced panic-like attacks and 10/10 experienced GI discomfort.
				Pretreatments with lorazepam (n=4), meprobamate (n=2), naloxone (n=3), or dimenhydrinate (n=1)		Lorazepam prevented CCK_4-induced panic-like attacks. The antiemetic dimenhydrinate did not did not reduce GI discomfort.
				CCK_8, 35 & 40 μg IV (n=2)		CCK_8 induced severe GI symptoms at low doses.

1 N = number of patients in the study.

2 CS (Chronic schizophrenia); CPS (Chronic paranoid schizophrenia); DID (Drug-induced dyskinesia); HD (Huntington's disease); IOD (Idiopathic orofacial dyskinesia); SC (Senile chorea); SLE (Systemic lupus erythematodes); TD (Tardive dyskinesia).

3 CC (Clinical criteria); DSM-III (Diagnostic and Statistical Manual of Mental Disorders III); FC (Feighner's criteria); RDC (Research Diagnostic Criteria).

4 IM x (number of intramuscular injections); IV x (number of intravenous injections).

5 AIMS (Abnormal Involuntary Movement Scale); BPRS (Brief Psychiatric Rating Scale); CGI (Clinical Global Impression); CRAS (Clinician Rated Anxiety Scale); EMG (Electromyogram); GIR (Global Improvement Rating); MV (Microvibration); NOSIE (Nurse Observation Scale for In-patient Examination); PAS (Panic Assessment Scale); PSE (Present State Examination); SARS (Simpson-Angus extrapyramidal Rating Scale); SS-PSE (Schizophrenia subscale on the Present State Examination).

Table VI

CCK in Neurological and Psychiatric Diseases: Clinical Trials - Double-blind Studies.

Study	N[1]	Patient Diagnosis[2] (Criteria[3])	Conventional Treatments[4]	Drug[5]	Rating Scales[6]	Results
SCHIZOPHRENIA:						
Hommer et al. (1984; 1985)	8	CS (DSM-III; RDC)	+	CER, 0.3 → 0.6 μg IM x 2/day x 4 days	ATRS; BPRS; B-HS	No significant antipsychotic effect.
	4	CS (DSM-III; RDC)	2+ 2−	CCK_8, 0.6 μg IV x 1/day x 4 days	ATRS; BPRS; B-HS	No significant antipsychotic effect.
Lotstra et al. (1984)	9	APS (n=3); CPS (n=2); CCS (n=2); UCS (n=2). (RDC)	− −	CER, 30 pM/kg IM & 300 pM/kg IM	CPRS	No significant antipsychotic effect.
Nair et al. (1984)	18	CS neuroleptic-resistant (RDC)	+	CCK_8, 10 μg IV x 1/wk x 8 wks (n=10); placebo (n=8)	BPRS; SS-PSE	Significant antipsychotic effects on some BPRS and SS-PSE items.
Mattes et al. (1985)	17	CS or SCS (DSM-III)	+	CER, 0.6 μg/kg IM x 1/wk x 2 wks (n=9); placebo (n=8)	BPRS; SCL-90; GIR	No significant antipsychotic effect.
Nair et al. (1985)	26	CS neuroleptic-resistant (RDC)	+	CCK_8, 10 μg IV x 1/wk x 8 wks (n=14); placebo (n=12)	BPRS; SS-PSE	Significant antipsychotic effects on some BPRS and SS-PSE items.
Albus et al. (1986)	20	CS (RDC)	+	CER, 0.3 μg/kg IM x 1/wk x 3 wks (n=10); placebo (n=10)	BPRS; NOSIE	No significant antipsychotic effect.
Itoh et al. (1986)	265	CS (DSM-III)	+	CER, 0.6-0.8 μg/kg IM x 1/wk x 4 wks (n=135); placebo (n=130)	BPRS; general ratings	No significant antipsychotic effect.

Tamminga et al. (1986)	5	CS (RDC; DSM-III)	–	CER, 0.3 μg/kg IM x 1	BPRS	No significant antipsychotic effect.
	5	same patients	–	CER, rising dose over 10 days 0.3 → 1.5 μg/kg IM x 5 interspersed with 5 placebo	BPRS	No significant antipsychotic effect.
	6	CS (RDC; DSM-III)	+	CER, 0.3 μg/kg IM x 5 interspersed with 5 placebo	Parallel groups to above	No significant antipsychotic effect.
Verhoeven et al. (1986)	29	CS (DSM-III)	+	CER, 40 μg IM x 7 over 3 wks (n=15); placebo (n=14)	BPRS; GAS	8/15 improved (significant ↓ of psychotic symptoms).
Peselow et al. (1987)	30	CS Neuroleptic-resistant (DSM-III)	+	CCK_8, 0.02 mcg/kg IV (n=14) or 0.04 mcg/kg IV (n=16)	BPRS; ATSEB; HAS; SPSS-PSE	No significant antipsychotic effect.
Innis et al. (1986)	4	CPS (n=2); UCS (n=2) (DSM-III)	+	Proglumide (CCK antagonist), 1600 & 3200 mg/day interspersed with placebo	SCRS; AIMS	No significant antipsychotic effect.
PARKINSON'S DISEASE:						
Bruno et al. (1985)	11	PD evidencing the "on-off" phenomenon.	+	CER, 0.3 μg/kg IM increasing by 0.3 μg/kg increments daily.	Clinical evaluation	No significant neurologic improvements.

1 N = number of patients in the study.

2 CS (Chronic schizophrenia); APS (Acute paranoid schizophrenia); CCS (Chronic catatonic schizophrenia); CPS (Chronic paranoid schizophrenia); PD (Parkinson's disease); SCS (Sub-chronic schizophrenia); UCS (Undifferetiated chronic schizophrenia).

3 DSM-III (Diagnostic and Statistical Manual of Mental Disorders III); GIR (Global Improvement Rating); RDC (Research Diagnostic Criteria); SCL-90 (Symptom check List).

4 Conventional treatments are neuroleptics for schizophrenia and L-Dopa in combination with carbidopa for Parkinson's disease.

5 IM x (number of intramuscular injections); IV x (number of intravenous injections).

6 AIMS (Abnormal Involuntary Movements Scale); ATRS (Abrams-Taylor Rating Scale for emotional blunting); B-HS (Bunney-Hamburg Scale); BPRS (Brief Psychiatric Rating Scale); CPRS (Comprehensive Psychopathology Rating Scale); HAS (Hamilton Anxiety Scale); NOSIE (Nurse Observation Scale for In-patient Examination); SCRS (Short Clinical Rating Scale); SPSS-PSE (Schneiderian "Positive" Symptom Scale abstracted from the Present State Examination); SS-PSE (Schizophrenia subscale on the Present State Examination).

As summarized in Table VI, there have been 10 groups of investigators who have performed double-blind studies of CCK in schizophrenia. Of the 14 studies, significant antipsychotic effects were reported in only three (Nair et al., 1984; 1985; Verhoeven et al., 1986). Verhoeven et al. (1986) studied 29 patients with chronic schizophrenia, and compared caerulein and placebo. They reported that eight out of 15 patients who received caerulein had a significant decrease in psychotic symptoms. Significant antipsychotic effects were also reported by Nair et al. (1984; 1985) but their studies were plagued by a problem; CCK_8 produced gastrointestinal side effects and this would have unblinded the studies.

Conversely, most of the studies reporting negative results had small numbers of patients (Albus et al., 1986; Hommer et al., 1984; 1985; Lostra et al., 1984; Mattes et al., 1985; Tamminga et al., 1986). Negative results from studies which included less than 20 patients are of little value since the numbers would have been too small to reliably demonstrate significant differences even if these were present.

The methodology used in the studies of CCK in schizophrenia leaves much to be desired. In addition to small patient numbers, another methodological problem was the short length of treatment period. Of the 14 double-blind studies reviewed here, only four studies treated patients for four weeks of longer (Itoh et al., 1986; Lostra et al., 1984; Nair et al., 1984; 1985). Five of the studies treated patients for a week of less, which is quite inadequate. It is unreasonable to expect that a new potential therapeutic agent administered for less than a week, and in several instances consisting of a dose given on a single occasion,

should exert a significant effect on chronic schizophrenics who may have been suffering from the illness for several years.

Another serious flaw arises from the concomitant use of neuroleptics. A significant proportion of patients stabilized on neuroleptics would have responded and thus would have relatively low levels of measurable schizophrenic symptomatology. There might have been insufficient symptoms to be able to adequately test the ability of CCK to ameliorate them. It is quite possible for a new potential drug to be as effective as an antischizophrenic agent but for its efficacy to be masked by the use of concomitant neuroleptics. Only three groups have studied the clinical efficacy of CCK in neuroleptic-free patients in double-blind placebo-controlled studies and no significant antipsychotic properties were detected. However, in these studies the patient numbers were too small to be considered an adequate test: Lostra et al. (1984) reported on six chronic and three acute schizophrenics, Tamminga et al. (1986) reported on five patients, and Hommer et al. (1984) had two neuroleptic-free patients in their study. Therefore, the only conclusion that can be drawn is that CCK does not appear to have striking antischizophrenic features that are outside of the spectrum of activity of neuroleptics.

The basic research that was reviewed in Section I-4.3 would predict that either CCK analogues or antagonists could act as antipsychotics. Yet the possible antipsychotic properties of CCK antagonists have only been tested in one study. Innis et al. (1986) administered proglumide, a weak CCK-A antagonist, to four neuroleptic-treated schizophrenics. Again, this study was plagued by all the above-mentioned methodological problems and, not surprisingly, no significant antipsychotic effect was detected.

In conclusion, the clinical usefulness of CCK in schizophrenia has not been properly tested, in my opinion. Almost all studies have been conducted with small numbers of neuroleptic-treated chronic schizophrenics. Indeed, in a recent review of this literature, Montgomery & Green (1988) commented that "the ethics of exposing over 500 patients to CCK without the possibility of drawing conclusions about efficacy are dubious".

I-6 AIMS OF THIS STUDY

In retrospect, clinical trials evaluating the efficacy of CCK for the treatment of schizophrenia may have been somewhat premature. Although the presence of CCK in the CNS was only recognized in 1975 (Vanderhaeghen et al., 1975) and the colocalization of CCK and DA was only identified in 1980 (Hökfelt et al., 1980ab), results from clinical trials were being published as early as 1982 (see Table V). Indeed, the vast majority of clinical trials have been reported from 1982 to 1986 (see Tables V and VI).

Basic research, however, is a slower process. For example, a review of the literature on CCK receptors completed in 1986 revealed that brain CCK receptors had only been characterized in rodents and non-mammalian species (Morency & Mishra, 1987) and significant species differences in regional distribution and selectivity were already apparent (e.g., Vigna et al., 1984; Williams et al., 1986). Therefore, one objective of the present study was to characterize CCK receptors in the brains of a higher mammalian species, the bovine, and to compare the regional distribution of CCK receptors in various species.

The majority of clinical studies evaluating the putative antipsychotic properties of CCK cited physiological, pharmacological, biochemical, and behavioural evidence of neuroleptic properties of CCK obtained in animals studies as the basis for their investigations. However, even prior to 1986, conflicting evidence was evident in this literature (for review, see Nair et al., 1986). Whereas some reports suggested an inhibitory effect of CCK on DA function, which would be compatible with a potential antischizophrenic action, others reported a lack of modulation or an enhancement of DA function. Thus, a second objective of the present study was to investigate CCK/DA interactions in animals using pharmacological, biochemical, and behavioural measures of central DAergic function.

Moreover, if CCK is to be eventually used as an antischizophrenic drug, it would be useful to investigate the effects of long-term administration of this neuropeptide on central DA functions in animals. Thus, the effects of long-term CCK_8 administration on DA D_2 receptor binding and the expression of DA D_2 receptor mRNA was also investigated in this study.

Finally, as was noted in Section I-4.3, DAergic agents have also been shown to modulate CCK function. Since chronic neuroleptic treatment remains the therapeutic strategy in schizophrenia, it would be worthwhile to examine the effects of long-term blockade of DA D_2 receptors on CCK function. Thus, a final objective of this study will be to examine the effects of long-term administration of haloperidol on CCK binding.

CHAPTER II

CHARACTERIZATION, SOLUBILIZATION, AND LOCALIZATION OF CCK RECEPTORS IN THE CNS

As mentioned in the introduction, CCK binding had only been characterized in the CNS of rodents and lower vertebrate species at the outset of this study. Briefly, a single class of CCK binding sites was identified by Scatchard analysis (i.e., linear Scatchard plot) in membrane preparations and tissues sections of rodent brains. High-affinity, saturable, and specific binding sites for CCK have been characterized in several brain regions (see Table VII).

The various radioligands employed in these studies displayed similar apparent affinity for brain and pancreatic CCK receptors but the estimated receptor density appeared to be significantly greater in the pancreas (see Morency & Mishra, 1987). Although linear Scatchard plots were also observed in pancreatic membranes, curvilinea. Scatchard plots have been described in pancreatic acini (Jensen et al., 1980; Sankaran et al., 1982; Williams et al., 1981). This observation is compatible with the existence of a heterogeneous population of CCK receptors or one class of binding sites undergoing site-site interactions (negative cooperativity) and suggests that perhaps low affinity binding sites are present in the brain but do not survive homogenization. Additional evidence cited in support of a heterogeneous population of CCK receptors are biphasic dissociation curves which have been observed in both

Table VII

Characterization of CCK Binding Sites in Rodent Brains.[a]

Species	Brain area	Ligand	K_d (nM)	B_{max}	Reference
MEMBRANE PREPARATIONS:					
Rat	Cortex	[^{125}I]BH-CCK_{33}	2.56	6.6[b]	Hays et al. (1980)
	Cortex	[^{125}I]BH-CCK_{33}	1.7	27.3[b]	Saito et al. (1980)
Guinea pig	Cortex	[^{125}I]BH-CCK_{33}	0.3	0.7[c]	Innis & Snyder (1980b)
	Cortex	[^{125}I]BH-CCK_8	1	28[b]	Lin & Miller (1985)
Mouse	Cortex	[^{125}I]BH-CCK_{33}	1.27	115[b]	Saito et al. (1981)
	Cortex	[^{125}I]BH-CCK_8	1	204[b]	Wennogle et al. (1985)
	Cortex	[^{125}I]BH-CCK_8	1.9	1.05[c]	Clark et al. (1986)
	Cortex	[^{3}H]pentagastrin	1.4	1.15[c]	Clark et al. (1986)
	Cortex	[^{3}H]Boc-Nle-2,5-CCK_7	0.2	14.8[b]	Durieux et al. (1985)
TISSUE SECTIONS:					
Rat	Cortex	[^{3}H]CCK_8	0.86	14.0[b]	Van Dijk et al. (1984)
	Olfactory bulb	[^{3}H]CCK_8	0.88	34.0[b]	Van Dijk et al. (1984)
	Hippocampus	[^{3}H]CCK_8	1.03	8.5[b]	Van Dijk et al. (1984)
	Midbrain	[^{3}H]CCK_8	0.99	5.6[b]	Van Dijk et al. (1984)
	Striatum	[^{3}H]CCK_8	0.99	19.4[b]	Van Dijk et al. (1984)
	Striatum	[^{3}H]pentagastrin	2.5	36.6[b]	Gaudreau et al. (1983)
	Striatum	[^{125}I]BH-CCK_8	2.5	30.4[b]	Gaudreau et al. (1985)

a Adapted from Morency & Mishra (1987). Values cited were determined by Scatchard analysis.
b fmol/mg protein. c pmol/g original tissue.

pancreatic (Deschodt-Lanckman et al., 1978; Jensen et al., 1980; Miller et al., 1981; Sankaran et al., 1980; 1982) and brain membranes (Innis & Snyder, 1980b; Lin & Miller, 1985; Praissman et al., 1983; Saito et al., 1980; 1981; Wennogle et al., 1985).

In non-mammalian species, Vigna et al. (1984) characterized CCK receptors in bullfrog brain and pancreas. The bullfrog CCK receptors exhibited some interesting differences from their mammalian counterparts. First, Scatchard analysis of competitive inhibition of [^{125}I]BH-CCK_{33} binding by CCK_{33} resulted in curvilinear plots for membranes from bullfrog brain and pancreas whereas linear plots were observed in rodent brain and pancreatic membranes. Second, bullfrog brain membranes had a much higher binding capacity than bullfrog pancreatic membranes; the converse was observed in mammals. Finally, other differences were noted with regard to specificity of CCK analogues and relative potencies of CCK antagonists; unlike the mammalian pattern, the bullfrog pancreas as well as brain contained CCK receptors with nearly identical specificity for CCK-related peptides, gastrins and several analogues (Vigna et al., 1984).

This analysis was later extended to other lower vertebrate species. Vigna and colleagues reported that the bullfrog pattern described above was also found in fish and reptiles (Vigna, 1985; Vigna et al., 1986; Williams et al., 1985). Birds, however, exhibited a pattern of CCK receptor specificity in brain and pancreas very similar to that seen in mammals (Vigna et al., 1986). Thus, it was hypothesized that the receptor found in the brain and pancreas of the ectotherm (i.e., fish, reptile, amphibian) is ancestral to the two distinct CCK receptors seen in the endotherm (i.e., birds, mammals) brain and pancreas (Vigna, 1985; Vigna

et al., 1986; Williams et al., 1985).

Since CCK receptors had only previously been characterized in rodents and lower vertebrate species, the first objectives of the present study was to pharmacologically characterize CCK receptors in the brain of a higher mammalian species. The bovine brain was chosen because it is readily obtained, inexpensive, and provided large amounts of tissue compared to other higher mammalian species.

II-1 OPTIMIZATION OF CCK BINDING ASSAY

An important requirement for investigations of the interaction of any neurotransmitter with its receptor(s) is the availability of a suitable radiolabelled ligand. Radioligands used for initial studies of the interaction of CCK with its receptors were tritiated derivatives of CCK_8 (Milutinovic et al., 1977; Morgat et al., 1977) and caerulein (Christophe et al., 1978; Deschodt-Lanckman et al., 1976; 1978; Robbercht et al., 1978). However, the first generation of tritiated CCK peptides were plagued by low specific activities (<20 Ci/mmol).

Radioiodination provides ligands with higher specific activity (~2000 Ci/mmol). However, direct insertion of ^{125}I into tyrosine causes a loss of biological activity, presumably because of iodination of the essential tyrosyl residue and oxidation of the methionine residue located in the critical C-terminal tetra-peptide. Bolton & Hunter (1973) introduced an alternative method to oxidative iodination whereby a previously radioiodinated acylating agent ([^{125}I]BH) is conjugated via an amide bond to free amine groups on peptides. Although [^{125}I]BH-CCK_{33} was the most frequently used ligand in earlier studies,

[^{125}I]BH-CCK_8 was later preferred because it is less susceptible to proteolytic enzymatic cleavage; since the ^{125}I is in the N-terminal region of the CCK_{33} radioligand and not within the biologically critical C-terminal octapeptide, proteolytic cleavage generates unlabelled, biologically active fragments. In addition, CCK_8 is the predominant species of CCK in the CNS and possesses a higher affinity for the CCK receptors than does CCK_{33} (see Morency & Mishra, 1987). Using a similar process to iodination with the Bolton-Hunter reagent, CCK_8 was labelled with N-succinimidyl[^{3}H]propionate; the specific activity of [^{3}H]pCCK_8 was much higher (60–86 Ci/mmol) than earlier tritiated ligands. Since tritiated ligands offer a higher degree of stability and manageability than radioiodinated ligands, [^{3}H]pCCK_8 was chosen for the characterization of CCK receptors in membrane binding assays.

Several experiments were carried out to establish the optimal conditions for [^{3}H]pCCK_8 binding in bovine cortical membranes. This was done for several reasons. First, this ligand had just become commercially available at the outset of this study; indeed, only one report of [^{3}H]pCCK_8 binding was available (Wennogle et al., 1985). Second, as mentioned above, CCK binding had previously been characterized in the CNS of rodents and lower non-mammalian species but never in the brains of higher mammalian species. Finally, no consistent incubation conditions were found in the literature. For example, whereas some investigators used HEPES as a buffer (e.g., Saito et al., 1980; 1981; Wennogle et al., 1985), others used Tris (e.g., Kritzer et al., 1987; Innis & Snyder, 1980ab). Several other inconsistencies in assay conditions regarding buffer composition, pH, ionic components, incubation time and temperature were

evident in the literature (for review, see Morency & Mishra, 1987). The cortex was chosen as the tissue for optimization experiments because of its relative abundance and prior characterizations of CCK receptors in rodents have been conducted with cortical membranes (see Table VII).

II-1.1 METHODS

II-1.1.1 Materials:

[^{3}H]pCCK$_8$ (60–86 Ci/mmol) was purchased from Amersham (Arlington Hgts., IL, USA). BSA, bacitracin, unlabelled CCK$_8$, HEPES, DTT, PMSF, and soybean trypsin inhibitor were from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of the highest available grades. Fresh bovine brains were purchased from local slaughter-houses.

II-1.1.2 Membrane Preparation:

A crude membrane preparation was used for preliminary experiments. Bovine brains were kept on ice until completion of the dissection (approximately 4 hours after sacrifice). Fronto-cortical grey matter was dissected, immediately frozen on dry ice, and stored at −80°C. The cortical tissue was later thawed on ice, homogenized in 10 vol Tris-EDTA buffer (50 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.4) in a Potter-Elvehjem homogenizer and centrifuged at 3,000 g for 10 min. The supernatant was centrifuged at 16,000 g for 20 min and the resulting pellet was washed once with Tris-EDTA buffer, centrifuged as described above, and resuspended in the assay buffer being tested. Protein concentrations were estimated from a standard curve of BSA (1–25 μg/mL) stained with Coomassie blue G-250, as described by Bradford (1976).

II-1.1.3 Ligand Binding:

Unless otherwise specified, freshly prepared cortical membranes were incubated in polypropylene tubes with 2 nM [^{3}H]pCCK$_8$ for 120 min at 25°C. Non-specific binding was determined in parallel assays in the presence of 1 μM unlabelled CCK$_8$. The reaction was terminated by rapid filtration through Whatman GF/B filters using a Brandel Cell Harvester (M-24RI). Filters were rapidly washed three times with 4 mL ice-cold wash buffer and soaked in a scintillation cocktail (Ready Safe, Beckman). Radioactivity bound to the filters was determined in a Beckman liquid scintillation spectrometer (Model 5800).

II-1.2 RESULTS AND DISCUSSION

II-1.2.1 Buffers:

[^{3}H]pCCK$_8$ binding to bovine cortical membranes was evaluated in HEPES-NaOH (10 mM) and in Tris-HCl (50 mM) buffers at different pH (6.5, 7.0, and 7.4). As illustrated in Figure 4, specific binding was the highest in HEPES buffer at pH 6.5 and was used in subsequent assays.

II-1.2.2 Cations and Chelating Agent:

The effects of divalent cations and monovalent cations on CCK binding have previously been systematically examined by several investigators (for review, see Morency & Mishra, 1987). In the rodent brain, binding of radiolabelled CCK$_{33}$, caerulein, CCK$_8$ and pentagastrin was consistently reported to be enhanced by the presence of divalent cations. In contrast, monovalent cations appeared to have inconsistent effects on binding of radiolabelled CCK peptides. Whereas some investigators have reported that monovalent cations decreased

CCK binding (e.g., Gaudreau, 1983; 1985; Saito et al., 1980), others have reported enhanced or unaltered CCK binding (e.g., Van Dijk et al., 1984; Wennogle et al., 1985).

As expected, the binding of [^{3}H]pCCK$_8$ to CCK receptors in bovine cortical membranes was enhanced by the presence of the divalent cations, Mg^{2+} and Ca^{2+} (see Fig. 5); $MgCl_2$ was found to be more effective than $CaCl_2$. $MgSO_4$ was as effective as $MgCl_2$ (data not shown). In addition, the presence of a chelating agent (1 mM EGTA) was necessary for maximal binding. It is possible that optimal [^{3}H]pCCK$_8$ binding requires Mg^{2+} but is inhibited by trace amounts of some other divalent cation. Indeed, Saito et al. (1980) reported that Cu^{2+}, known to be present in the brain, inhibited CCK binding in the mouse cortex.

As illustrated in Figure 5, [^{3}H]pCCK$_8$ binding was also enhanced by the presence of monovalent cations; maximal binding was obtained in the presence of 130 mM NaCl and 5 mM KCl. The presence of Na^+ and K^+ were also required for optimal [^{3}H]pCCK$_8$ binding in mouse cortical membranes (Wennogle et al., 1985). Therefore, buffers used in subsequent binding assays included 130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA.

II-1.2.3 Bovine Serum Albumin:

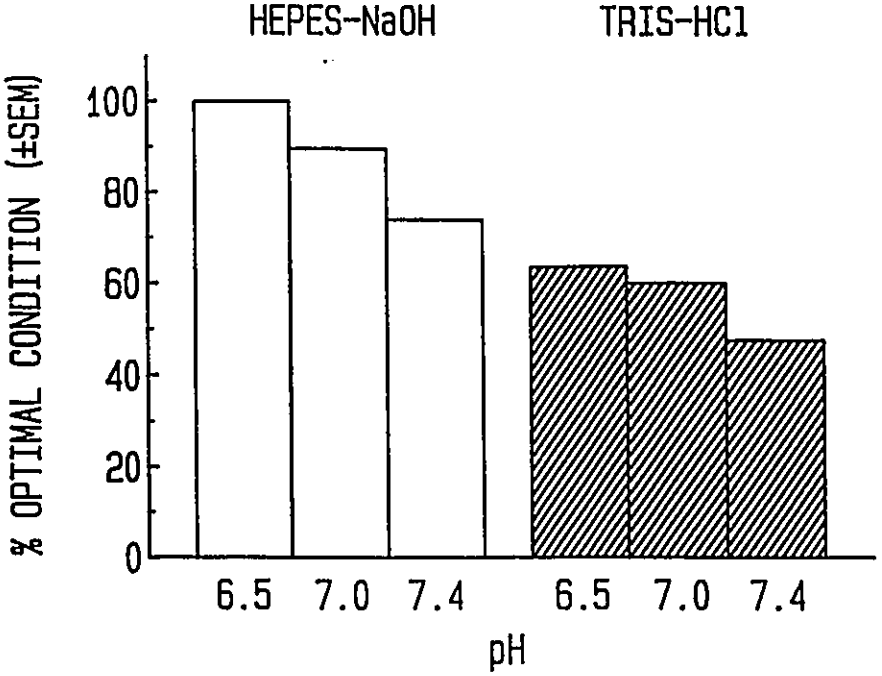
To prevent adsorption of the low amounts of peptides to the incubation tube, 1−10 mg/mL of BSA have been included in buffers by several investigators (see Morency & Mishra, 1987). However, Wennogle et al. (1985) reported that, at these concentrations, BSA slightly stimulated pancreatic binding of [^{125}I]BH-CCK$_8$ but drastically reduced binding of this ligand to mouse cortical membranes. In bovine cortical membranes, [^{3}H]pCCK$_8$ binding was similarly

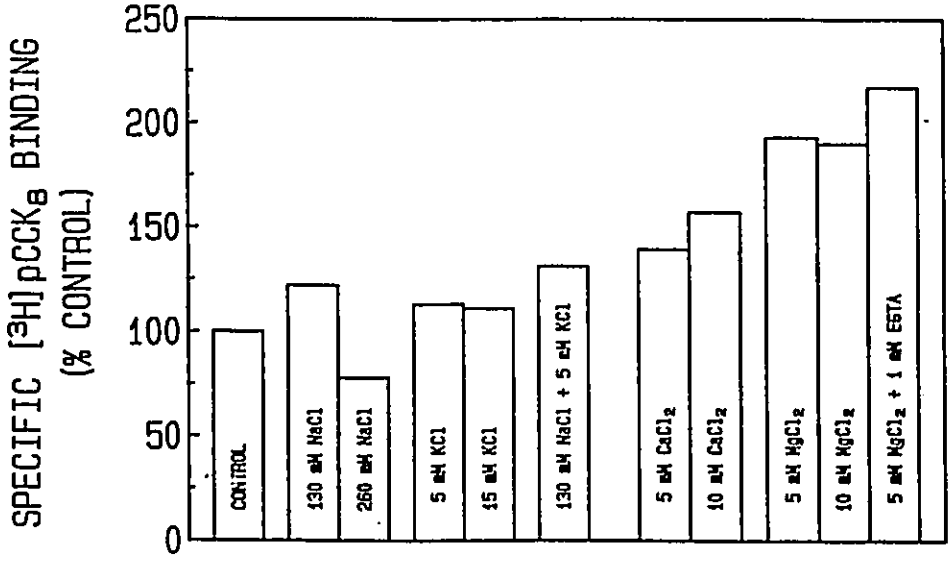
Figure 4 Effects of pH and buffer on [^{3}H]pCCK$_8$ binding in bovine cortical membranes.

[^{3}H]pCCK$_8$ binding to bovine cortical membranes was evaluated in 10 mM HEPES-NaOH and in 50 mM Tris-HCl at pH 6.5, 7.0, and 7.4. Binding was done as described in the methods. Binding data are expressed in percent with the specific binding in HEPES-NaOH at pH 6.5 taken as 100%. Results are the means of three independent determinations with standard deviations less than 10%.

Figure 5 Effects of monovalent and divalent cations and chelating agent on [^{3}H]pCCK$_8$ binding in bovine cortical membranes.

[^{3}H]pCCK$_8$ binding to bovine cortical membranes was evaluated in HEPES-NaOH (10 mM) at pH 6.5 in the presence or absence of monovalent (Na^+, K^+) and divalent cations (Ca^{2+}, Mg^{2+}) and a chelating agent (EGTA) as described in the figure. Results are expressed as percent of control and are the means of three independent determinations with standard deviations less than 10%.


HEPES-NaOH
TRIS-HCl
% OPTIMAL CONDITION (±SEM)
100
80
60
40
20
0
6.5
7.0
7.4
6.5
7.0
7.4
pH


SPECIFIC [3H] pCCK8 BINDING
(% CONTROL)
250
200
150
100
50
0
CONTROL
130 mM NaCl
260 mM NaCl
5 mM KCl
15 mM KCl
130 mM NaCl + 5 mM KCl
5 mM CaCl2
10 mM CaCl2
5 mM MgCl2
10 mM MgCl2
5 mM MgCl2 + 1 mM EGTA

reduced by 1 and 10 mg/mL BSA; lower concentrations (0.01 and 0.1 mg/mL) did not affect binding (Fig. 6). It should be noted that polypropylene tubes are used in our study instead of glass tubes to minimize adsorption of peptides to the incubation tube.

II-1.2.4 Dithiothreitol:

DTT (1−5 mM) has been used in some binding studies of brain CCK receptors (Gaudreau et al., 1983; 1985; Innis & Snyder, 1980ab; Praissman & Walden, 1984; Zarbin et al., 1981; 1983). In our study, although the addition of DTT (1 or 5 mM) enhanced total binding of [^{3}H]pCCK$_8$ to bovine cortical membranes, such an increase was also accompanied by an increase in non-specific binding. Since no significant improvements in specific binding resulted (see Fig. 6) and the ratio of specific binding to total binding was decreased by the presence of DTT, there was no obvious need for its use in future assays.

II-1.2.5 Protease Inhibitors:

Protease inhibitors have also been used in most CCK binding studies to protect the radioligand and receptors from degradation. Bacitracin and soybean trypsin inhibitor have been commonly used for this purpose (see Morency & Mishra, 1987). Although these protease inhibitors are used in our laboratory for work on opioids and PLG-related peptides, they have been used in combination with PMSF (e.g. Kazmi & Mishra, 1986; Mishra et al., 1990; Srivastava et al., 1988). As illustrated in Figure 6, the addition of bacitracin (100 μg/mL) and soybean trypsin inhibitor (5 μg/mL) ameliorated [^{3}H]pCCK$_8$ binding to bovine cortical membranes but optimal binding was observed when these inhibitors were combined with 0.1 mM PMSF. Indeed, Rose et al. (1988; 1989) recently

reported that serine-alkylating reagents such as PMSF markedly protected CCK_8-LI from endogenous degradation by a serine peptidase (see Section I-2.5).

II-1.2.6 Protein Concentration:

$[^3H]pCCK_8$ binding to various concentrations of bovine cortical membranes was found to be linear in the concentration range tested (50−750 µg/mL; data not shown). CCK binding had been previously reported to be linear at concentrations of up to 1.2 mg/mL in the rat cortex (Hays et al., 1981; Praissman et al., 1983); a significant decline was observed at concentrations higher than 1.5 mg/mL (Praissman et al., 1983).

II-1.2.7 Incubation Temperature:

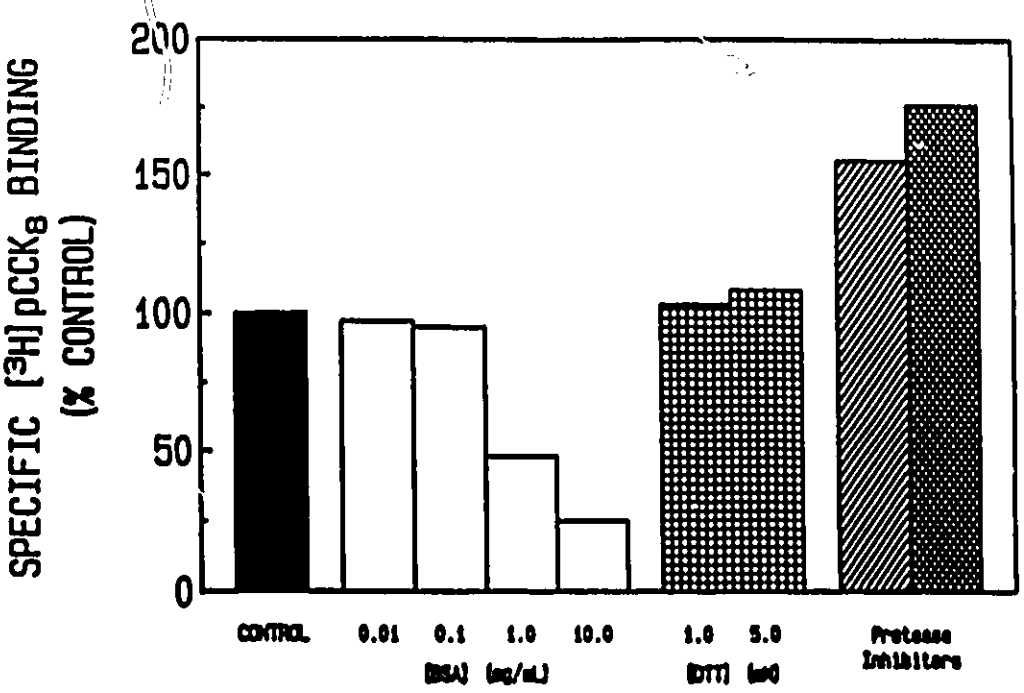
Stepwise reductions in incubation temperature (37→0°C) resulted in a 50−80% decrease in maximal total binding of $[^3H]$caerulein, $[^{125}I]$BH-CCK_8, $[^{125}I]$BH-CCK_{33} and Boc$[^{125}I]$Tyr-$(Thr^{28},Nle^{31})CCK_8$ to pancreatic CCK receptors (Deschodt-Lanckman et al., 1978; Jensen et al., 1980; Miller et al., 1981; Pearson et al., 1986; Williams et al., 1988). In cortical membranes, however, binding of radioiodinated CCK was optimal at 22−25°C, as higher temperatures had deleterious effects on the radioligand and/or membrane receptors (Praissman et al., 1983; Hays et al., 1980). As illustrated in Figure 7, similar results were observed for $[^3H]pCCK_8$ binding to bovine cortical membranes. Following a 120 min incubation, binding was optimal at 25°C whereas incubation at 37°C had a deleterious effect on $[^3H]pCCK_8$ binding. Although the lowest binding was observed at 0°C after a 120 min incubation, overnight incubation at this temperature resulted in $[^3H]pCCK_8$ binding almost equivalent to that observed following 120 min incubation at 25°C.

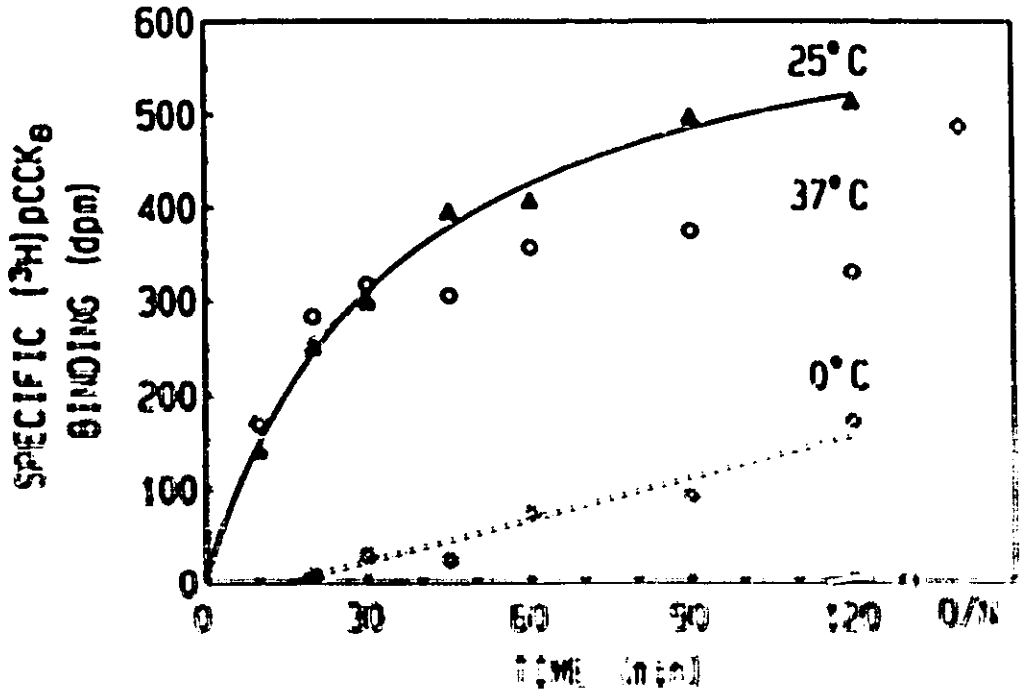
Figure 6 Effects of BSA, DTT, and protease inhibitors on [^{3}H]pCCK$_8$ binding in bovine cortical membranes.

[^{3}H]pCCK$_8$ binding to bovine cortical membranes was evaluated in HEPES-NaOH (10 mM) containing 130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA (pH 6.5) in the absence (control, filled bar) or presence of BSA (open bars), DTT (checkered (+) bars), and protease inhibitors (diagonal (/) lines = 100 μg/mL bacitracin and 5 μg/mL soybean trypsin inhibitor; criss-crossed (X) lines = 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitors, and 0.1 mM PMSF). Binding was done as described in the methods. Results are expressed as percent of control and are the means of three independent determinations performed in triplicates with standard deviations less than 10%.

Figure 7 Effects of incubation temperature on [^{3}H]pCCK$_8$ binding in bovine cortical membranes.

[^{3}H]pCCK$_8$ binding to bovine cortical membranes was evaluated in HEPES-NaOH (10 mM) containing 130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 1 mM EGTA, 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitor, and 0.1 mM PMSF (pH 6.5) at 0, 25, and 37°C. At indicated times, 0.5 mL aliquots were withdrawn and filtered as described in methods. Results are representative of two independent determinations performed in triplicates.


SPECIFIC [3H]pCCK8 BINDING
(% CONTROL)
200
150
100
50
0
CONTROL
0.01
0.1
1.0
10.0
1.0
5.0
Protease
Inhibitors


SPECIFIC [3H]pCCK8
BINDING (dpm)
600
500
400
300
200
100
0
25°C
37°C
0°C
0
30
60
90
120

II-2 PHARMACOLOGICAL CHARACTERIZATION OF CORTICAL CCK RECEPTORS

Having determined the optimal binding conditions, the next objective was to pharmacologically characterize CCK receptors in the bovine cerebral cortex, i.e., Scatchard plot for the estimation of the K_d and B_{max}, Hill plot for resolving potential receptor multiplicity or cooperativity, and association and dissociation kinetics. For comparative purposes, Scatchard and Hill plots of saturation isotherms were also determined in the bovine caudate and in the rat and canine cerebral cortex.

As previously described in the introduction, CCK receptors have been found to exist as two subtypes, CCK-A and CCK-B on the basis of the pattern of distribution and differences in affinity of CCK agonists and antagonists. In rodent brains, the presence of CCK-A receptors was limited to a few isolated nuclei (see Chapter I). However, Barrett et al. (1989) recently reported that high densities of CCK A receptors were present in several areas of the bovine brain, including the cortex. These investigators used [^{3}H]L-364,718 membrane binding assays, but did not demonstrate the characteristic selectivity of CCK-A receptors (e.g., several hundred fold higher affinity for CCK_8 than CCK_{8s}). Thus, competitive inhibition studies were performed in the present study to determine the relative affinities of various CCK agonists and antagonists and identify the receptor subtype present in the bovine cortex.

Regarding the cellular mechanisms responsible for mediating the effects of CCK, previous studies have demonstrated that the pancreatic CCK A receptor regulates phosphatidylinositol turnover and phosphatidyl inositol breakdown via a

guanyl nucleotide binding protein (G-protein) (Merritt et al., 1986; Williams et al., 1987; see review Petersen, 1984). In contrast, central CCK-B receptors have not been convincingly linked to a second messenger system. A characteristic of receptors linked to G-proteins is a decreased affinity for agonists in the presence of guanyl nucleotides. Therefore, the non-hydrolysable GTP analogue Gpp(NH)p was used to investigate the effects of guanyl nucleotides on [^{3}H]pCCK$_8$ binding in the bovine cortex.

II-2.1 METHODS

II-2.1.1 Materials:

The materials were as described in Section II-1.1.1 except that the unlabelled CCK peptides used in the displacement studies were purchased from Peninsula Laboratories (Belmont, CA, USA). Benzotript was purchased from Research Biochemicals Inc. (Natick, MA, USA), PLG and neuropeptide Y from Peninsula Laboratories, neurotensin and substance P from Sigma, and Gpp(NH)p from Boehringer Mannheim Canada (Laval, Qué). L-364,718 and L-365,260 were generously supplied by Dr. Roger M. Freidinger of Merck Sharp & Dohme Research Laboratories (West Point, PA, USA). Male Sprague Dawley rats were purchased from Charles River Canada. Adult mongrel dogs were generously donated by Dr. E.E. Daniel, Dept. of Biomedical Sciences, Division of Pharmacology & Physiology, McMaster University.

II-2.1.2 Membrane Preparation:

Fronto-cortical grey matter and of the caudate were dissected from fresh brains and frozen at -80°C. The tissue was later thawed on ice, homogenized in

10 vol of ice-cold 0.25 M sucrose in a Potter-Elvehjem homogenizer. The crude homogenate was centrifuged at 1,000 g for 10 min at 4°C. The supernatant was saved and the pellet resuspended in 10 vol of 0.25 M sucrose and centrifuged as described above. Both supernatants were pooled and centrifuged at 105,000 g for 1 hour. The resulting pellet was resuspended in Tris-EDTA buffer (50 mM Tris-HCl containing 1 mM EDTA. pH 7.4) and centrifuged at 30,000 g for 20 min. Finally, the pellet was washed once with Tris-EDTA buffer, centrifuged at 30,000 g for 20 min, and resuspended in HEPES assay buffer (10 mM HEPES-NaOH containing 130 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitors, and 0.1 mM PMSF, pH 6.5) to give a protein concentration of 12−14 mg/mL and stored at −80°C for no longer than two weeks.

II-2.1.3 Association and Dissociation Kinetics:

Bovine cortical membranes (−0.4 mg/mL) without (for total binding) or with (for non-specific binding) 1 μM CCK_8 diluted in HEPES assay buffer were incubated at 25°C. The association was started by the addition of [^{3}H]pCCK_8 (1 nM final concentration). At indicated times, 0.5 mL aliquots were taken and rapidly filtered through Whatman GF/B filters using a Brandel Cell Harvester. Filters were washed three times with 4 mL ice-cold wash buffer (10 mM HEPES, 130 mM NaCl and 5 mM KCl, 5 mM $MgCl_2$, pH 6.5) and the radioactivity was determined as previously described in Section II-1.1.3.

After equilibrium was reached (120 min), the dissociation was initiated by the addition of 1 μM unlabelled CCK_8. Again, aliquots were taken at indicated times and filtered as described above.

II-2.1.4 Equilibrium Binding Experiments:

The membranes were incubated at 25°C for 120 min with increasing concentrations of [^{3}H]pCCK$_8$ (0.1−20 nM) and HEPES assay buffer in a final assay volume of 0.5 mL. The reaction was initiated by the addition of 125 µL of the membrane preparation (150−200 µg protein per assay tube). Non-specific binding was determined in parallel assays containing 1 µM CCK$_8$. In some experiments, 100 µM Gpp(NH)p was added to investigate the effects of guanyl nucleotides on [^{3}H]pCCK$_8$ binding in the bovine cortex.

II-2.1.5 Competitive Inhibition Studies:

Bovine cortical membranes were incubated in the absence (for control, i.e., 100% binding) or in the presence of various concentrations of CCK peptides or antagonists. Non-specific binding was only determined in parallel control tubes containing 1 µM CCK$_8$. The specificity of [^{3}H]pCCK$_8$ binding to the bovine cerebral cortex was also assayed with unrelated peptides (i.e., neuropeptide Y, neurotensin, PLG, and substance P).

II-2.1.6 Data Analysis:

Saturation and inhibition binding data were analyzed on an IBM-PC using the weighted nonlinear curve fitting programs, BDATA and CDATA (EMF software, Knoxville, TN, USA), respectively. Statistical analyses were used to compare the "goodness of fit" between a one or two affinity-state model. A two-site model is selected only if a statistically significant improvement (F-test) of the fit of the data is obtained over a one-site model. The IC$_{50}$ values obtained from the competition curves were converted to K$_i$ values (inhibitor constant) using the Cheng & Prusoff (1973) equation. Kinetic rate constants were determined with the Kinetics program of McPherson (1985).

II-2.2 RESULTS

II-2.2.1 Association and Dissociation Kinetics:

The specific binding of [^{3}H]pCCK$_8$ to bovine cortical membranes as a function of time is depicted in Fig. 8. The binding was time-dependent, a plateau being reached at approximately 100 min at 25°C and remaining constant up to 180 min with a half-time of association ($t_{1/2}$) of 21.1 min. At equilibrium, bound [^{3}H]pCCK$_8$ represented less than 1% of the total added in the incubation medium. Thus, at equilibrium, free ligand can be considered equal to the total ligand added. For this reason, the association kinetics can be analyzed as a pseudo-first order reaction according to the equation:

$$\ln (B_e/B_e - B) = (k_{+1}[L] + k_{-1})t,$$

where B_e is the concentration of the bound ligand at equilibrium, B is the concentration of the bound ligand at time t, [L] is the concentration of the ligand added, k_{+1} is the rate constant of association, and k_{-1} is the rate constant of dissociation. The plot of ln ($B_e/B_e - B$) vs time gave a straight line (Fig. 8; bottom left panel). The association rate constant, obtained by the equation $k_{ob} - k_{-1} = k_{+1}[L]$ where k_{ob} is the slope of association, was calculated to be 0.047 $nM^{-1}min^{-1}$.

The addition of excess unlabelled CCK$_8$ (1 μM) induced a rapid dissociation of [^{3}H]pCCK$_8$ binding to bovine cortical membranes. The dissociation proceeded quickly for the initial 20−30 min and slowly thereafter. Such biphasic dissociation profiles might be attributed to multiple independent receptor sites, interconverting affinity states, or negative cooperativity (Limbird, 1986). The plot of ln B/B_o vs time gave two straight line (Fig. 8; bottom right panel).

Only the initial rate of dissociation has been taken into account and the slope of this line, which represents the dissociation rate constant, was 0.042 min^{-1}. The dissociation constant calculated from these values was 1.12 nM ($K_d = k_{-1}/k_{+1}$).

II-2.2.2 Equilibrium Binding Characteristics:

The binding as a function of increasing concentrations of [^{3}H]pCCK$_8$ to bovine cortical membranes is illustrated in Figure 9. A summary of the binding parameters obtained from Scatchard analyses for [^{3}H]pCCK$_8$ binding in the bovine cortical and striatal membranes as well as for rat and canine cortical membranes is given in Table VIII. In all tissues assayed, Scatchard transforms of these binding isotherms were linear, indicating a single population of CCK receptor sites. In no case did a two-site model provide a statistically significant improvement of the fit of the data. This was also supported by the Hill coefficients which did not differ significantly from unity.

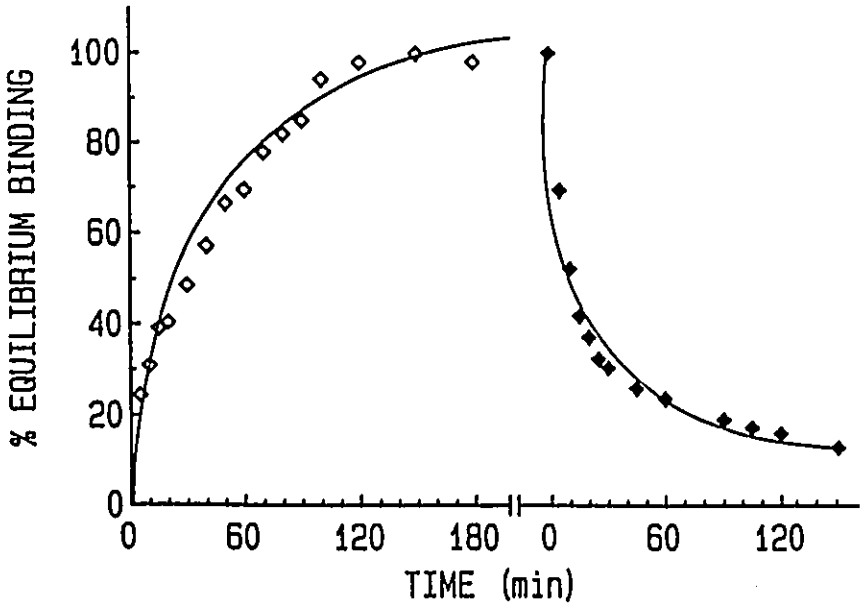
II-2.2.3 Competitive Inhibition Studies:

Competitive inhibition studies with various CCK agonists and antagonists revealed a similar pharmacological profile (see Table IX) as that previously observed in the rodent cortex: 1) the C-terminal tetrapeptide was found to be the minimal structural requirement for binding in the bovine cortex; 2) these receptors showed little sensitivity in their ability to discriminate between CCK-related peptides, indicating that bovine cortical CCK receptors are of the CCK-B subtype; 3) Hill coefficients for displacement by all peptides did not differ significantly from unity, indicating a single population of CCK receptors and the absence of positive or negative cooperative interactions (data not shown); and

Figure 8 Association and dissociation kinetics of [^{3}H]pCCK$_8$ binding to bovine cortical membranes.

UPPER PANEL: [^{3}H]pCCK$_8$ (1 nM) was incubated with bovine cortical membranes. At indicated times, 0.5 mL aliquots were withdrawn and filtered as described in Methods. Dissociation was initiated by the addition of excess (1 μM) unlabelled CCK$_8$ after equilibrium had been achieved (120 min). The data are plotted in terms of % equilibrium binding vs time. The results are representative of three independent experiments carried out in triplicates.

LOWER PANELS: The left panel depicts the association data plotted according to the pseudo first-order reaction ln ($B_e/B_e - B$) vs time, where B_e is the concentration of ligand specifically bound at equilibrium and B is the ligand concentration bound at time t. The right panel shows the dissociation data plotted according to the pseudo first-order reaction dissociation ln B/B_0 vs time, where B and B_0 are the ligand concentration bound at time t and time zero of dissociation, respectively. The K_d of [^{3}H]pCCK$_8$ binding was determined by the equation $K_d = k_{-1}/k_{+1}$ where k_{-1}, the dissociation rate constant, is the slope of the dissociation plot. The association rate constant, k_{+1}, was obtained by the equation $k_{ob} - k_{-1} = k_{+1}[L]$ where k_{ob} is the slope of association and [L] is the concentration of the ligand.


% EQUILIBRIUM BINDING
100
80
60
40
20
0
0
60
120
180
0
60
120
TIME (min)

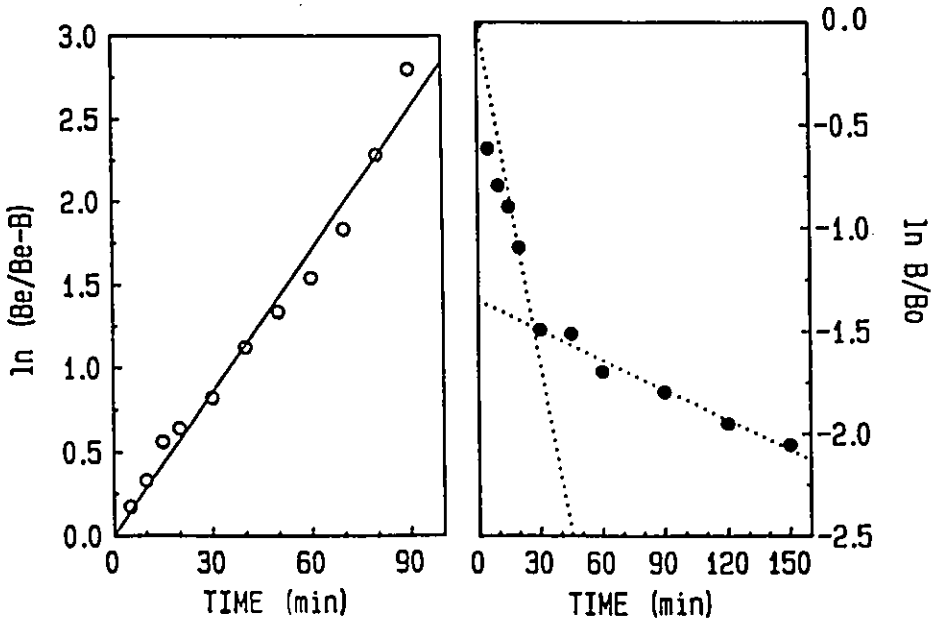

ln (Be/Be-B)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
30
60
90
TIME (min)
ln B/Bo
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
0
30
60
90
120
150
TIME (min)

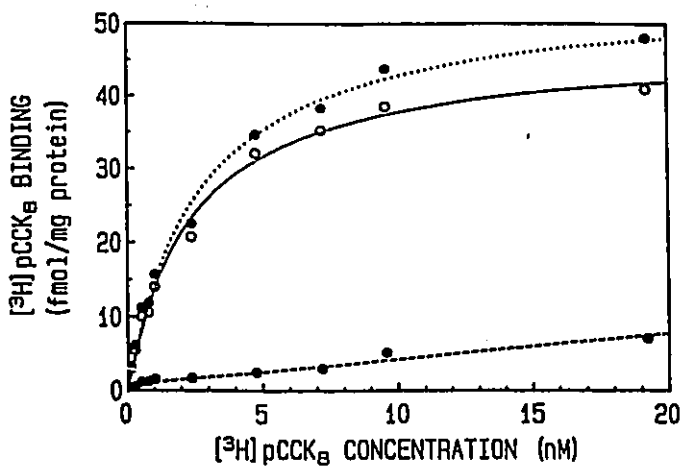
Figure 9 Characterization of [^{3}H]pCCK$_8$ binding to bovine cortical membranes.

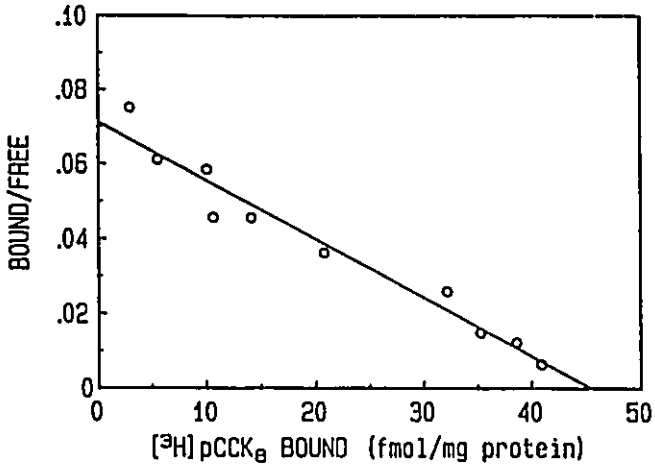
Cortical membranes were incubated with 0.1−20 nM [^{3}H]pCCK$_8$ in HEPES assay buffer at 25°C for 120 min. Non-specific binding was determined in parallel assays in the presence of 1 μM unlabelled CCK$_8$. The reaction was initiated by the addition of membranes. After the 120 min incubation at 25°C, the reaction was stopped and the bound ligand was separated from the free by rapid filtration. The results are representative of three independent experiments carried out in triplicates.

UPPER PANEL - SATURATION ISOTHERM: As illustrated in this figure, [^{3}H]pCCK$_8$ binding was saturable. The total binding (dotted line) and specific binding (solid line) curves were estimated by the rectangular hyperbola equation [Y = A*X/(B+X)] with a standard error of less than 10%. Non-specific binding (dashed line) was estimated by linear regression.

MIDDLE PANEL - SCATCHARD PLOT: Saturation data were analyzed by the linear least-square curve-fitting program BDATA (EMF Software, Knoxville, TX).

LOWER PANEL - HILL PLOT: Hill coefficients were analyzed by the linear least-square curve-fitting program BDATA (EMF Software, Knoxville, TX). Hill coefficients obtained from the three independent determinations did not differ significantly from unity.


[3H]pCCK8 BINDING
(fmol/mg protein)
50
40
30
20
10
0
0
5
10
15
20
[3H]pCCK8 CONCENTRATION (nM)


BOUND/FREE
.10
.08
.06
.04
.02
0
0
10
20
30
40
50
[3H]pCCK8 BOUND (fmol/mg protein)

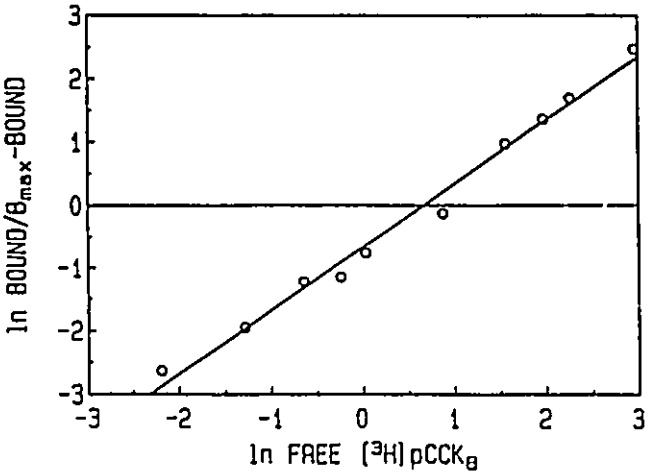

ln BOUND/Bmax-BOUND
3
2
1
0
-1
-2
-3
-3
-2
-1
0
1
2
3
ln FREE [3H]pCCK8

Table VIII

Characterization of CCK Binding Sites: Equilibrium Binding Studies.[1]

Species	Tissue	N	K_d (nM)	B_{max} (fmol/mg protein)	n_H
Bovine	Cortical membranes	6	0.88 ± 0.04	47.9 ± 5.2	1.01 ± 0.03
	Cortical membranes + Gpp(NH)p[2]	3	2.57 ± 0.29*	41.4 ± 8.7	0.95 ± 0.06
	Striatal membranes	3	0.83 ± 0.09	54.5 ± 4.3	1.02 ± 0.04
Canine	Cortical membranes	3	0.76 ± 0.06	43.1 ± 5.8	0.97 ± 0.05
Rat	Cortical membranes	3	0.91 ± 0.09	58.5 ± 3.9	0.98 ± 0.03

1 Cortical or striatal membranes were incubated with 0.1-20 nM [^{3}H]pCCK$_8$ in HEPES assay buffer at 25°C for 120 min. Non-specific binding was determined in parallel assays in the presence of 1 μM unlabelled CCK$_8$. The reaction was initiated by the addition of membranes. After the 120 min incubation at 25°C, the reaction was stopped and the bound ligand was separated from the free by rapid filtration. The results are the means ± SEM of three independent experiments carried out in triplicates. Saturation data were analyzed by the linear least-square curve-fitting program BDATA (EMF Software, Knoxville, TX). A representative Scatchard plot is illustrated in Figure 9.

2 Equilibrium binding studies were done in the absence or presence of 100 μM Gpp(NH)p in parallel assays using the same membrane preparation. The binding parameters estimated by Scatchard analysis were therefore compared by paired Student's t-tests.

* The addition of Gpp(NH)p significantly increased the dissociation constant; $t = 5.12$, $p<0.05$.

4) unrelated peptides (i.e., neuropeptide Y, neurotensin, PLG, substance P) did not displace [^{3}H]pCCK$_8$ binding (Table IX). As was the case with equilibrium binding studies, a two-site model never provided a statistically significant improvement of the fit of the data.

II-2.2.4 Effects of Guanine Nucleotides:

The presence of the non-hydrolysable guanine nucleotide analogue, Gpp(NH)p, decreased the specific binding of [^{3}H]pCCK$_8$ in bovine cortical membranes. Scatchard analysis of binding isotherms done in the presence and absence of 100 μM Gpp(NH)p indicated that the guanine nucleotide produced a statistically significant (paired Student's t = 5.12, $p<0.05$) 3-fold decrease in binding affinity without significantly affecting the maximum number of binding sites (Table VIII).

II-2.3 DISCUSSION

[^{3}H]pCCK$_8$ binding to bovine cortical membranes was found to be of high affinity, specific, reversible, and time- and temperature-dependent. Saturation isotherms in equilibrium binding studies revealed that the binding was saturable at 10-20 nM. Scatchard transforms of these binding isotherms were linear, indicating a single population of CCK receptor sites. Estimates of the dissociation constant revealed the presence of high affinity CCK receptors (≈1 nM) in the bovine cortex. Comparable estimates were observed in the bovine caudate, and in the canine and rat cortex (Table VIII). Similar values have been reported in the rodent cortex using a variety of ligands (Table VII and, more recently, Durieux et al., 1988; Gut et al., 1989; Niehoff, 1989; Wennogle et al., 1988).

Table IX

Inhibition of [^{3}H]pCCK$_8$ binding to bovine cortical CCK receptors by various CCK agonists and antagonists and unrelated peptides.[1]

Displacers	K_i (nM)[2] Cortical Membranes	K_i (nM)[2] Solubilized Preparation[3]
Agonists:		
CCK_8	0.66 ± 0.07	0.91 ± 0.07
Caerulein	0.98 ± 0.11	1.33 ± 0.15
CCK_{8U}	4.2 ± 0.3	5.1 ± 0.4
Pentagastrin	5.5 ± 0.6	8.6 ± 0.9
CCK_4	22.7 ± 2.8	26.3 ± 2.5
CCK_3	>10,000	>10,000
Antagonists:		
L-365,260	2.4 ± 0.3	2.9 ± 0.3
L-364,718	283 ± 44	312 ± 54
Proglumide	>10,000	>10,000
Benzotript	>10,000	>10,000
Unrelated Peptides:		
Neurotensin	>10,000	>10,000
Neuropeptide Y	>10,000	>10,000
PLG	>10,000	>10,000
Substance P	>10,000	>10,000

1 Increasing concentrations of the competing ligands were incubated with 1 nM [^{3}H]pCCK$_8$. The values are means ± SEM of three independent determinations performed in triplicate.

2 The IC_{50} values obtained from the competition curves were converted to K_i values (inhibitor constant) using the Cheng and Prusoff (1973) equation, i.e., $K_i = IC_{50}/1+[L]/K_L$, where [L] is the concentration of free labelled ligand and K_L is the equilibrium dissociation constant of the labelled ligand determined by Scatchard analysis.

3 The solubilization of bovine cortical CCK receptors is described in the next section.

Several lines of evidence suggest the lack of CCK receptor heterogeneity and cooperativity in the bovine cortex: 1) Scatchard transforms of the binding isotherms were linear; 2) Hill analyses of both binding isotherms and competitive inhibition studies yielded values close to unity; and 3) computerised analyses of saturation isotherms as well as displacement binding data established that the one-site model provided the best fit for the data. However, a biphasic dissociation curve was obtained in the kinetic studies. Biphasic dissociation curves have also been observed in rodent brains (Innis & Snyder, 1980; Lin & Miller, 1985; Praissman et al., 1983; Saito et al., 1980; 1981; Wennogle et al., 1985) and have been cited as evidence for the presence of a heterogeneous population of brain CCK receptors. In the present kinetic studies, an accurate estimate of the equilibrium dissociation constant ($K_d = k_{-1}/k_{+1}$) was obtained with the dissociation rate constant of the initial, rapid dissociation. This could suggest the presence of a slower dissociating, higher affinity CCK binding site in the bovine cerebral cortex.

Interestingly, two groups (Durieux et al., 1986; Knapp et al., 1990) have reported CCK receptor heterogeneity using custom-designed CCK radioligands. Durieux et al. (1986) reported the occurrence of two different CCK binding sites in guinea pig cerebral cortex using [^{3}H][Nle^{28,31}]Boc-CCK$_7$. Saturation experiments indicated the presence of two different binding sites (K_d = 0.13 nM, B_{max} = 35 fmol/mg; K_d = 6.4 nM, B_{max} = 92 fmol/mg). More recently, Yamamura and colleagues developed a series of CCK analogues, some of which were reported to be "highly potent and extraordinarily selective for the central vs. peripheral receptor" (Hruby et al., 1990). These investigators subsequently

tritiated one of these analogues, [N-methyl-Nle^{28,31}]CCK$_{8U}$ (SNF 8702), and characterized CCK-B receptors in the guinea pig brain (Knapp et al., 1990). Although saturation experiments revealed that [^{3}H]SNF 8702 bound to a single site in the cortex, cerebellum, hippocampus, and pons-medulla, evidence for CCK-B receptor heterogeneity was observed under other conditions. [^{3}H]SNF 8702 binding to membranes prepared from whole guinea pig brain showed curvilinear Scatchard plots as well as biphasic association kinetics. Curvilinear Scatchard plots were also observed membranes from the midbrain-thalamic region. In addition, CCK-B receptor heterogeneity was suggested by the effect of Gpp(NH)p on [^{3}H]SNF 8702 binding to CCK-B receptors. Although Gpp(NH)p inhibited a portion of the total [^{3}H]SNF 8702 binding in all areas tested (i.e., cortex, hippocampus, and cerebellum), a distinct effect on the binding parameters was seen in these three brain regions. [^{3}H]SNF 8702 binding sites in both the cortex and hippocampus showed a two- to three-fold reduction in binding affinity. Gpp(NH)p did not affect the B_{max} in the cortex, but an apparent 30% loss of binding sites was measured in the hippocampus. Scatchard analysis of [^{3}H]SNF 8702 binding to Gpp(NH)p-treated cortical and hippocampal membranes was best fitted by a one-site model. In contrast, Scatchard analysis of [^{3}H]SNF 8702 binding to Gpp(NH)p-treated cerebellar membranes was best fitted by a two-site model, indicating the presence of a low concentration of high-affinity, Gpp(NH)p-insensitive sites and a high concentration of low-affinity, Gpp(NH)p-sensitive site (Knapp et al., 1990). Such results underscore the complexity of CCKergic systems in the CNS and the need for the development of more potent and selective pharmacological agents.

Similar to the effects on [^{3}H]SNF 8702 in the guinea pig cortex, treatment of bovine cortical membranes with Gpp(NH)p resulted in a three-fold reduction in binding affinity of [^{3}H]pCCK$_8$ with no significant changes in receptor densities. Similar observations have been observed in mouse (Wennogle et al., 1988) and pig (Gut et al., 1989) cortical membranes and suggests a close association of the cortical CCK receptor with a G-protein.

Competitive inhibition studies of [^{3}H]pCCK$_8$ binding to bovine cortical CCK receptors with various CCK agonists and antagonists revealed a pharmacological profile consistent with CCK-B receptors previously characterized in the mammalian brain. There was less than a 50-fold difference in potency of the various CCK analogues tested (Table IX) whereas 1000−5000 fold differences have been observed for pancreatic and brain CCK-A receptors (Section I-3.1.1). Furthermore, the specific CCK-B antagonist L-365,260 had over 100-fold higher affinity for bovine cortical CCK receptors than L-364,718, an antagonist specific for CCK-A receptors (Table IX).

However, these previous observations are not consistent with the report of Barrett et al. (1989). Using [^{3}H]L-364,718 membrane binding assays, they detected high densities (56−480 fmol/mg protein) of CCK-A receptors in all six areas of the bovine and rat brain tested (i.e., nucleus accumbens, caudate, cortex, cerebellum, hippocampus, and brainstem). However, these investigators did concede that "(b)ecause of the low number of specifically bound dpm and the low percentage of specific binding in most areas, ... the results must be viewed with caution" (pg 286, Barrett et al., 1989). Moreover, detailed competitive inhibition studies were not done. Instead, these investigators stated that "we

were able to show that the specific binding of [^{3}H]L-364,718 (0.4 nM) was fully inhibited by 10 μM CR 1409 and 10 μM CCK_8 (data not shown). We, therefore, tentatively conclude that CCK-A sites are found in membranes from these selected areas" (pg 286, Barrett et al., 1989; the emphasis is mine). In my opinion, the absence of competitive inhibition studies seriously weakens their conclusion.

The results observed by Barrett et al. (1989) could not be replicated in our laboratory. Less than 10% specific binding was obtained in bovine cortical membranes (data not shown) as well as in bovine cortical sections (Section II-4) with [^{3}H]L-364,718 (87 Ci/mmol, New England Nuclear, Boston, MA). Since the conditions described by these investigators were duplicated and the radioligand was obtained from the same source, the reason(s) for this discrepancy are not apparent at this time. However, it should be noted that the research group at Merck Sharp and Dohme, the developers of L-364,718, did not observe CCK-A receptors in the above-mentioned areas of the rat brain. Using [^{3}H]L-364,718 which was tritiated in their own facilities, these investigators only reported binding in isolated nuclei such as the area postrema, the nucleus tractus solitarius, and the interpeduncular nucleus but not in areas such as the cortex (Hill et al., 1987a). Moreover, similar conclusions have been drawn from studies displacing [^{125}I]BH-CCK_8 binding with selective antagonists (e.g., Chang et al., 1989; Hill & Woodruff, 1990; Hill et al., 1987a; 1990) or various CCK peptides (e.g., Hruby et al., 1990; Miceli & Steiner, 1989; Moran et al., 1986).

II-3 SOLUBILIZATION OF CORTICAL CCK RECEPTORS

Although it is well established that CCK-A and CCK-B receptors represent two distinct subtypes, the extent of their heterogeneity remains unresolved. One of the fundamental approaches in understanding the biochemistry of membrane-bound receptors is their solubilization in a functional state. Moreover, receptor solubilization is the initial step in the process of purifying a membrane-bound receptor. The natural abundance of CCK-A receptors in pancreatic tissues has led to the solubilization and purification of that receptor (Lambert et al., 1985; Szecowka et al., 1985; 1989; Zahidi et al., 1986). Thus, we sought to establish a simple method for an efficient solubilization of brain CCK receptors.

II-3.1 METHODS

II-3.1.1 Materials:

The materials were as previously described in Section II-2.1.1. In addition, soybean phosphatidyl choline, Sephadex G-50, Tween 80, and bovine γ-globulin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CHAPS and n-Octyl-β-D-glucopyranoside were purchased from Boehringer Mannheim Canada (Laval, Qué), cholic acid (sodium salt) from Calbiochem (La Jolla, CA, USA), PEG 6000 from BDH Chemicals (Toronto, Ont), Coomassie blue G-250 and Triton X-100 from BioRad (Richmond, CA, USA), digitonin from Gallard-Schlesinger Inds., Inc (NY, USA) and WAKO Pure Chemical Inds., Ltd. (Japan).

II-3.1.2 Solubilization:

The solubilization procedure for cortical CCK receptors was adapted from that of Srivastava et al. (1990a). Unless otherwise stated, the cortical membrane preparation (see Section II-2.1.2) was diluted with 2 volumes of HEPES assay buffer and incubated with 10 μM CCK_8 for 120 min at 25°C. The membranes were then centrifuged at 12,000 g for 15 min, washed twice with 5 volumes of assay buffer, and resuspended in the original volume of the same buffer. To solubilize, the preincubated membrane was diluted with an equal volume of a detergent solution containing the following (final concentration in membrane-detergent mixture) 0.5% cholic acid, 0.5 M NaCl, 0.1 mM PMSF, 0.1 mM benzamidine, and 1 mg/ml of crude soybean phosphatidyl choline in HEPES buffer, sonicated to clarity. The mixture was stirred gently in an ice-bath for 30 min. The mixture was subsequently centrifuged at 130,000 g for 60 min. The upper 80% of the clear supernatant, taken as solubilized receptors, was chromatographed through a Sephadex G-50 column (5 g in a 2.5 x 11.5 cm column) equilibrated and eluted with HEPES assay buffer. Protein concentration in solubilized preparations was estimated from a standard curve of BSA (1−25 μg/mL) stained with Coomassie blue G-250, as described by Bradford (1976).

II-3.1.3 Ligand Binding:

The binding of [^{3}H]pCCK_8 to solubilized receptor was performed as described in Section II-2.1 except that, at the end of the incubation period, the tubes were placed on ice and 50 μL of ice-cold bovine γ-globulin (20 mg/mL in HEPES wash buffer, pH 6.5) and 250 μL of PEG (30% w/v in HEPES wash buffer, pH 6.5) were added to precipitate the receptor-ligand complex. The tubes

were vortexed and, after a 10 min incubation on ice, the contents of the tubes were filtered on GF/C filters (Whatman) as previously described. The filters were washed five times each with 4 mL of ice-cold PEG (10% w/v in HEPES wash buffer, pH 6.5). Data analyses were performed as described in Section II-2.

II-3.2 RESULTS

II-3.2.1 Solubilization with Various Detergents:

Seven detergents were initially screened for their ability to solubilize functional brain CCK receptors. As depicted in Figure 10, cholic acid was the most effective detergent. For ease of comparison, this figure only includes the optimal concentration of each detergent, although at least three concentrations were tested. Tween 80, Triton X-100, and digitonin (Gallard-Schlesinger) gave virtually inactive preparations. CHAPS was able to solubilize 55%, digitonin (WAKO) 46%, and n-octyl-β-D-glucoside 32% of the activity obtained with cholic acid under similar conditions. Since cholic acid gave the highest yields of functional receptors, it was used in subsequent experiments.

II-3.2.2 Cholic Acid Concentration:

As illustrated in Figure 11, the optimal cholic acid concentration for solubilization of bovine cortical CCK receptors was between 0.5 and 1%; increasing the cholic acid concentration did not result in higher yield of solubilization although the amount of protein solubilized continued to increase up to 1.0%. Therefore, a concentration of 0.5% cholic acid was subsequently employed.

Figure 10 Solubilization of bovine cortical CCK receptors with various detergents.

Bovine cortical membrane preparations (12−14 mg/mL) were solubilized with 0.5% cholic acid, 1% CHAPS, 1% digitonin, 1% n-octyl-β-D-glucoside, 0.1% Triton X-100, or 0.5% Tween 80 in the presence of 0.5 M NaCl, 0.1 mM PMSF, 0.1 mM benzamidine, and 1 mg/mL crude soybean phosphotidylcholine. The mixtures were gently stirred in an ice bath for 30 min and then centrifuged at 130,000 g for 60 min. [^{3}H]pCCK$_8$ binding (2 nM) was assayed as described in the methods. Results are the means ± SEM of three independent experiments.

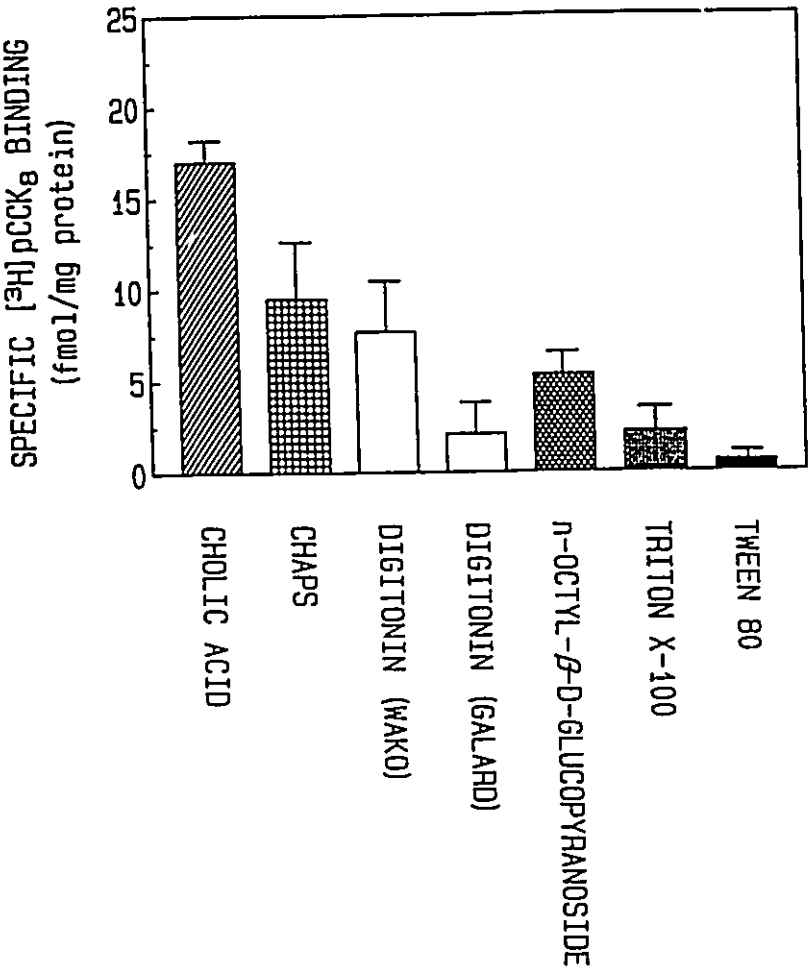
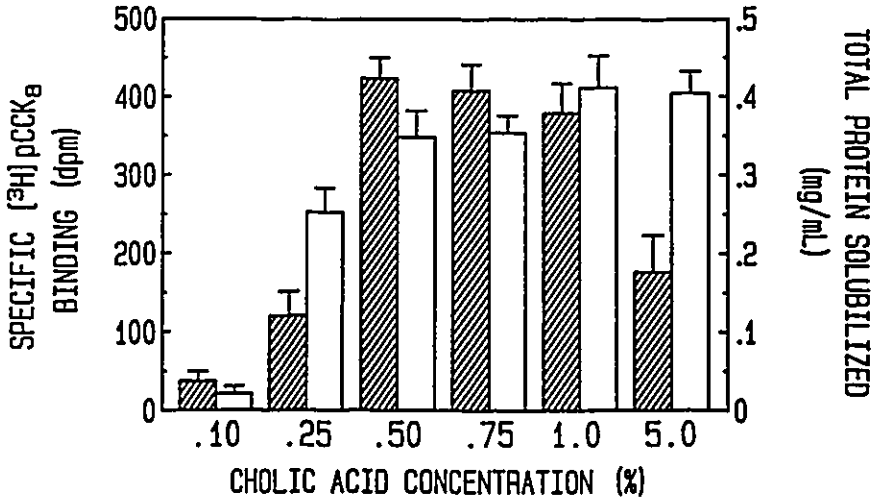

SPECIFIC [3H]pCCK8 BINDING
(fmol/mg protein)
25
20
15
10
5
0
CHOLIC ACID
CHAPS
DIGITONIN (WAKO)
DIGITONIN (GALARD)
n-OCTYL-β-D-GLUCOPYRANOSIDE
TRITON X-100
TWEEN 80

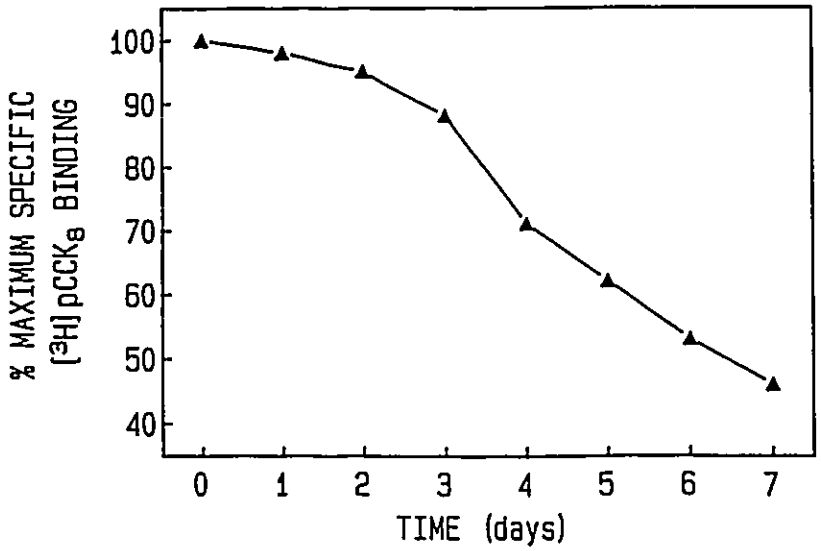
Figure 11 Solubilization of bovine cortical CCK receptors with various concentrations of cholic acid.

As described in the methods, bovine cortical membrane preparations (12–14 mg/mL) were preincubated with 10 μM CCK_8 and solubilized with the indicated concentrations of cholic acid in the presence of 0.5 M NaCl, 0.1 mM PMSF, 0.1 mM benzamidine, and 1 mg/mL crude soybean phosphotidylcholine. The mixture was gently stirred in an ice bath for 30 min and then centrifuged at 130,000 g for 60 min. The supernatants were chromatographed through a Sephadex G-50 column. [^{3}H]pCCK_8 binding (1 nM) was assayed as described in the methods. Results are the mean ± SEM of three independent experiments. Bars with diagonal lines represent specific [^{3}H]pCCK_8 binding (dpm) and opened bars represent the concentrations of protein solubilized (mg/mL).

Figure 12 Stability of solubilized bovine cortical CCK receptors.

The stability of solubilized cortical CCK receptors over time was examined. Bovine cortical membranes (12–14 mg/mL) were preincubated with 10 μM CCK_8 and solubilized with 0.5% cholic acid in the presence of 0.5 M NaCl, 0.1 mM PMSF, 0.1 mM benzamidine, and 1 mg/mL crude soybean phosphotidylcholine. The mixture was gently stirred in an ice bath for 30 min and then centrifuged at 130,000 g for 60 min. The supernatants were chromatographed through a Sephadex G-50 column and stored at 4°C. On a daily basis, the specific [^{3}H]pCCK_8 binding (2 nM) to the solubilized preparation was determined and expressed in terms of percentage of initial binding. [^{3}H]pCCK_8 binding was assayed as described in the methods. Results are representative of three independent experiments performed in triplicates.


SPECIFIC [3H]pCCK8 BINDING (dpm)
500
400
300
200
100
0
.5
.4
.3
.2
.1
0
TOTAL PROTEIN SOLUBILIZED (mg/mL)
.10
.25
.50
.75
1.0
5.0
CHOLIC ACID CONCENTRATION (%)


% MAXIMUM SPECIFIC [3H]pCCK8 BINDING
100
90
80
70
60
50
40
0
1
2
3
4
5
6
7
TIME (days)

The stability of this cholic acid/NaCl-solubilized bovine cortical preparation stored at 4°C was quite good; over 85% of the original [^{3}H]pCCK$_8$ binding activity was still present three days after solubilization (see Fig. 12). Nevertheless, solubilized preparations were never stored longer than 48 hours prior to binding assays.

II-3.2.3 Solubilization Criteria:

The procedure outlined above to solubilize the cortical CCK receptors fulfilled the essential criteria of solubilization (Hjelmeland & Chrambach, 1984ab): (1) filtration of the soluble preparation through 0.22 μM filters (Gelman Sciences, Inc., Rexdale, Ont.) resulted in over 90% recovery of [^{3}H]pCCK$_8$ binding activity; (2) no significant loss of specific [^{3}H]pCCK$_8$ binding upon ultracentrifugation of the solubilized preparation at 130,000 g for three hours; and (3) absence of lamellar membrane fragments under electron microscope (data not shown).

II-3.2.4 Association and Dissociation Kinetics:

The kinetics of [^{3}H]pCCK$_8$ binding to solubilized cortical CCK receptors (see Fig. 13) demonstrated a very similar profile to those in membranes. The binding was time-dependent; association was half-maximal ($t_{1/2}$) after 23.7 min and maximal after approximately 100 min. As observed in the membranes, [^{3}H]pCCK$_8$ bound at equilibrium represented less than 1% of the total added in the incubation medium. Since the free ligand can be considered equal to the total ligand added, the association kinetics can be analyzed as a pseudo-first order reaction. Again, the plot of ln (B_e/B_e-B) vs time gave a straight line (Fig. 13; bottom left panel). The association rate constant was calculated to be 0.034 $nM^{-1}min^{-1}$.

The specific binding at equilibrium was rapidly reversed by the addition of excess unlabelled CCK_8 (1 μM). As in the bovine cortical membranes, dissociation was biphasic in the solubilized cortical preparations. The dissociation proceeded quickly for the initial 20–30 min and slowly thereafter. Again, the plot of ln B/B_o vs time gave two straight line (Fig. 13; bottom right panel). Only the initial rate of dissociation has been taken into account and the slope of this line which represents the dissociation rate constant was 0.040 min^{-1}. The dissociation constant calculated from these values was 1.17 nM ($K_d = k_{-1}/k_{+1}$).

II-3.2.5 Equilibrium Binding Characteristics:

The binding as a function of increasing concentrations of [^{3}H]$pCCK_8$ to bovine cortical membranes is illustrated in Figure 14. Saturation isotherms revealed that [^{3}H]$pCCK_8$ binding to solubilized cortical preparations was saturable. Scatchard transforms of equilibrium binding data gave an apparent dissociation constant (K_d) of 1.09 ± 0.06 nM and a B_{max} of 41.9 ± 3.4 (n=3), comparable to the values obtained in membranes (see Table VII). Scatchard plots of these binding isotherms were linear, indicating a single population of CCK receptor sites. In no case did a two-site model provide a statistically significant improvement of the fit of the data. This was also supported by the Hill coefficients which did not differ significantly from unity (n_H = 1.01 ± 0.05).

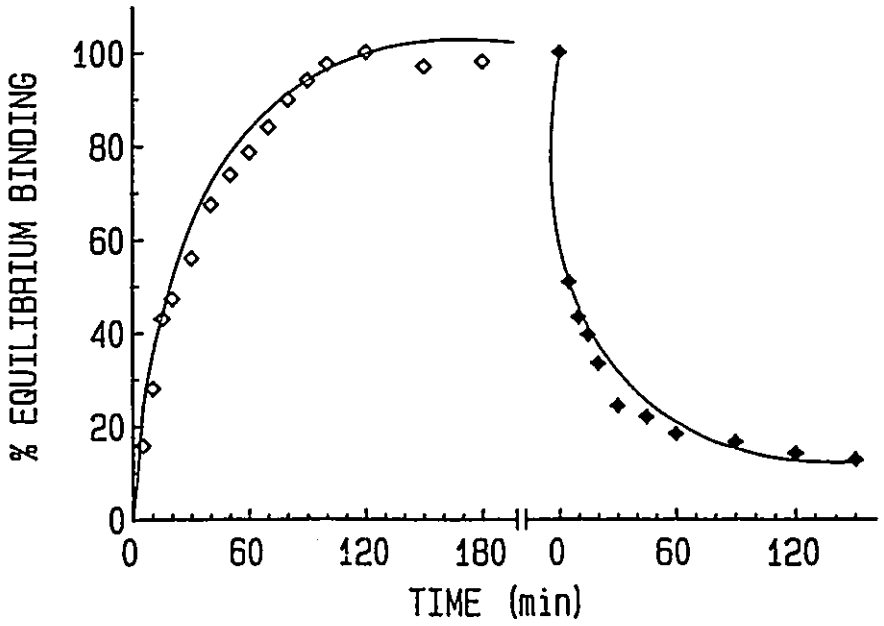
II-3.2.6 Competition Inhibition Studies:

To investigate the pharmacological specificity of the solubilized bovine cortical CCK receptor, a variety of CCK-related peptides and antagonist were tested for their ability to compete with [^{3}H]$pCCK_8$ binding. The solubilized

Figure 13 Association and dissociation kinetics of [^{3}H]pCCK$_8$ binding to solubilized bovine cortical CCK receptors.

UPPER PANEL: [^{3}H]pCCK$_8$ (1 nM) was incubated with solubilized bovine cortical CCK receptors. At indicated times, 0.5 mL aliquots were withdrawn and filtered as described in Methods. Dissociation was initiated by the addition of excess (1 μM) unlabelled CCK$_8$ after equilibrium had been achieved (120 min). The data are plotted in terms of % equilibrium binding vs time. Curves were estimated by the rectangular hyperbola equation [Y = A*X/(B+X)] with a standard error <10%. The results are representative of three independent experiments carried out in triplicates.

LOWER PANELS: The left panel depicts the association data plotted according to the pseudo first-order reaction ln (B_e/B_e-B) vs time, where B_e is the concentration of ligand specifically bound at equilibrium and B is the ligand concentration bound at time t. The right panel shows the dissociation data plotted according to the pseudo first-order reaction dissociation ln B/B_o vs time, where B and B_o are the ligand concentration bound at time t and time zero of dissociation, respectively. The K_d of [^{3}H]pCCK$_8$ binding was determined by the equation $K_d = k_{-1}/k_{+1}$ where k_{-1}, the dissociation rate constant, is the slope of the dissociation plot. The association rate constant, k_{+1}, was obtained by the equation $k_{ob}-k_{-1} = k_{+1}[L]$ where k_{ob} is the slope of association and [L] is the concentration of the ligand.


% EQUILIBRIUM BINDING
100
80
60
40
20
0
0
60
120
180
0
60
120
TIME (min)

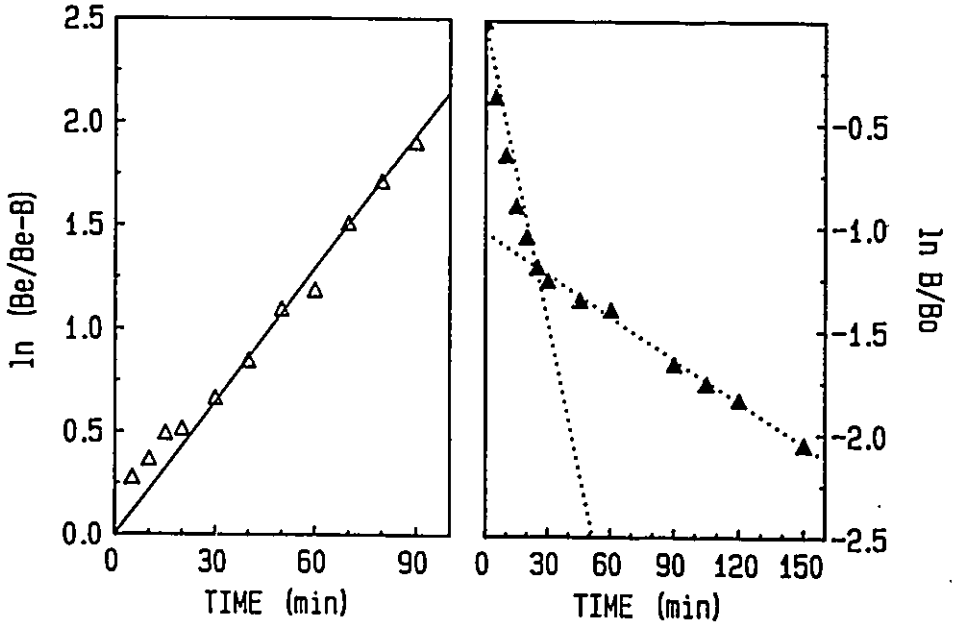
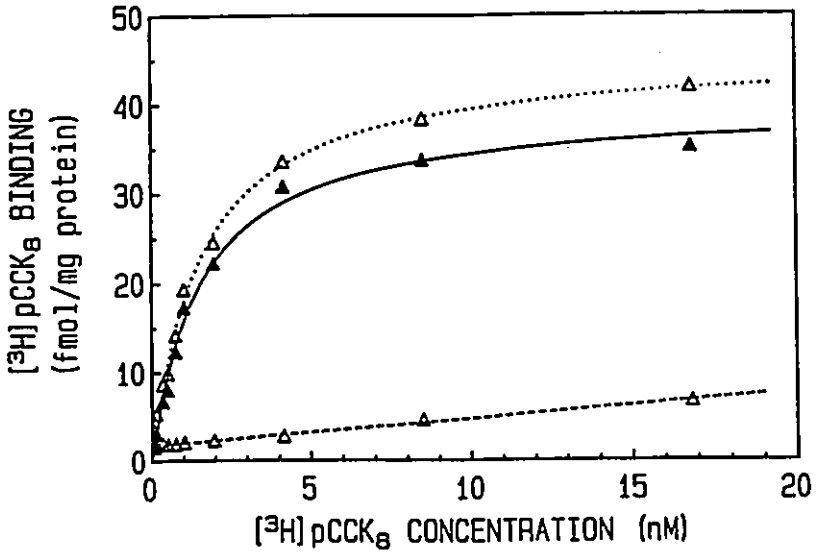

ln (Be/Be-B)
2.5
2.0
1.5
1.0
0.5
0.0
0
30
60
90
TIME (min)
ln B/Bo
-0.5
-1.0
-1.5
-2.0
-2.5
0
30
60
90
120
150
TIME (min)

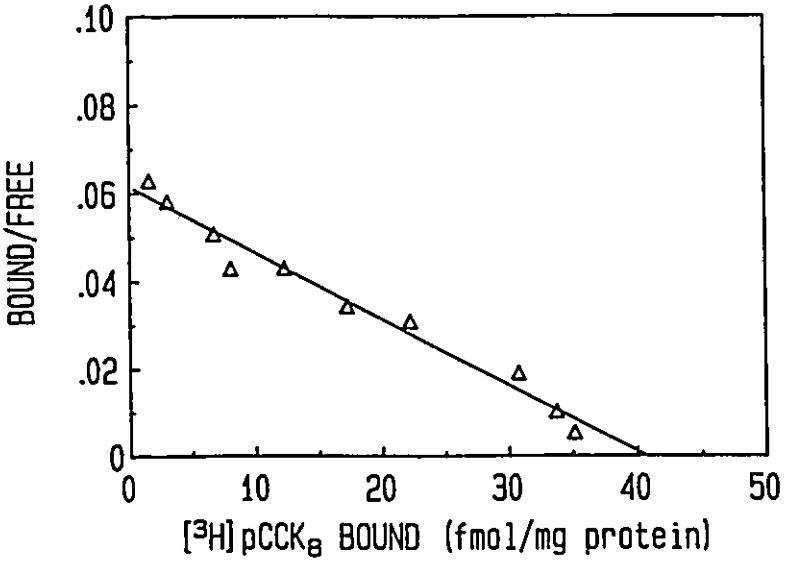
Figure 14 Characterization of [^{3}H]pCCK$_8$ binding to solubilized bovine cortical CCK receptors.

Solubilized bovine cortical CCK receptors were incubated with 0.1−20 nM [^{3}H]pCCK$_8$ in HEPES assay buffer at 25°C for 120 min. Non-specific binding was determined in parallel assays in the presence of 1 μM unlabelled CCK$_8$. The reaction was initiated by the addition of the solubilized preparation. After the 120 min incubation at 25°C, the reaction was stopped and the bound ligand was separated from the free by rapid filtration. The results are representative of three independent experiments carried out in triplicates.

UPPER PANEL - SATURATION ISOTHERM: As in bovine cortical membranes, [^{3}H]pCCK$_8$ binding to solubilized CCK receptors was found to be saturable. The total binding (dotted line) and specific binding (solid line) curves were estimated by the rectangular hyperbola equation [Y = A*X/(B+X)] with a standard error of less than 10%. Non-specific binding (dashed line) was estimated by linear regression.

LOWER PANEL - SCATCHARD PLOT: Saturation data were analyzed by the linear least-square curve-fitting program BDATA (EMF Software, Knoxville, TX).


50
40
30
20
10
0
0
5
10
15
20
[3H]pCCK8 BINDING
(fmol/mg protein)
[3H]pCCK8 CONCENTRATION (nM)


.10
.08
.06
.04
.02
0
0
10
20
30
40
50
BOUND/FREE
[3H]pCCK8 BOUND (fmol/mg protein)

receptors preserved their relative specificity (Table IX) for CCK agonist (CCK_8>caerulein>CCK_{8U}>pentagastrin>CCK_4>>>CCK_3) and CCK antagonists (L-365,260>>L-364,718>>>benzotript). As was observed in competitive inhibition studies with cortical membranes, the pharmacological profile and potencies observed in the solubilized preparation were similar to that reported for CCK-B receptors in rodents; a two-site model never provided a statistically significant improvement of the fit of the data and Hill coefficients for displacement by all peptides did not differ significantly from unity (data not shown). This would suggest the presence of a single population of CCK-B receptors and the absence of positive or negative cooperative interactions.

II-3.2.7 Effects of Guanine Nucleotides:

In the bovine cortical solubilized preparations, as in the membranes, addition of Gpp(NH)p (100 μM) in equilibrium binding assays resulted in a significant decrease in affinity (paired Student's $t = 5.03$, $p<0.05$) without affecting the receptor density: $K_d = 2.76 \pm 0.31$ and $B_{max} = 39.89 \pm 7.9$, and $n_H = 0.92 \pm 0.07$ (n=3). This would suggest a close association of the solubilized cortical CCK receptor with a G-protein.

II-3.3 DISCUSSION

In the present study, a simple method was described for an efficient solubilization of brain CCK binding sites in good yield from bovine cerebral cortex using cholic acid and sodium chloride. This represented the first solubilization of functional brain CCK receptors using a non-denaturing detergent (Morency et al., 1988a). The solubilized cortical CCK receptors fulfilled the

essential criteria of solubilization and exhibit a similar binding characteristics than the membrane-bound receptors. Equilibrium binding studies indicated a single population of high affinity CCK receptors. [^{3}H]pCCK$_8$ binding was found to be specific, time-dependent, and reversible. Competitive inhibition studies suggested that the solubilized receptor sites were of the B-type (i.e., CCK-B), with the same pharmacological profile as that observed in membranes. Finally, Gpp(NH)p significantly reduced the affinity of [^{3}H]pCCK$_8$ to the solubilized preparation, suggesting that the solubilized CCK receptors remained functionally coupled with the G-protein.

CCK-B receptors were also recently solubilized from the pig cerebral cortex with digitonin (Gut et al., 1989). Although these investigators tested the detergents which had been used to solubilized pancreatic CCK-A receptors (i.e., CHAPS, digitonin, Zwittergent 3-14 and 3-08; Lambert et al., 1985; Szecowka et al., 1985; Zahidi et al., 1986), they did not test cholic acid for its ability to solubilize the pig cortical CCK receptors. In the present study, digitonin was not as effective as cholic acid in its ability to solubilize functional bovine cortical CCK receptors. Nevertheless, results observed by Gut et al. (1989) in digitonin-solubilized pig cerebral cortex were very similar to the above-described results observed in cholate-solubilized bovine cerebral cortex: 1) Scatchard plots were linear and gave an apparent dissociation constant comparable to membranes; 2) competitive inhibition studies indicated that the solubilized receptors were of the B-type with the same pharmacological profile as that observed in membranes; 3) binding to the solubilized receptors was time-dependent; and 4) both the membrane-bound and solubilized receptor affinity were attenuated by Gpp(NH)p.

II-4 AUTORADIOGRAPHIC LOCALIZATION OF CCK RECEPTORS

The pharmacological characteristics of CCK receptors appears to have been preserved in the mammalian brain. Analysis by Scatchard plots of dissociation constants and by displacement studies of the rank order of affinity for several CCK-related peptides and antagonists, as well as the linkage of CCK receptors to a G-protein showed comparable results in the brain of several species (see Sections II-2 and II-3). In contrast, species differences have been noted in the distribution of CCK receptors. Most noteworthy is the marked contrast between the high density of CCK receptors in the guinea pig cerebellum and the absence of binding in the rat cerebellum (Sekiguchi & Moroji, 1986; Williams et al., 1986).

Therefore, one of the important questions which immediately follows the characterization of receptors by binding assays is the localization of receptors at the anatomical and cellular levels. This is particularly important in the study of the brain, a highly organized anatomical structure with a very complicated cellular composition. Although microdissection together with biochemical procedures can provide some information on the regional distribution of receptors, these procedures are limited in their anatomical resolution and sensitivity. Receptor autoradiography can circumvent these limitations since, with this method, the receptors are labelled in tissues retaining their anatomical and cellular structures as intact as possible. Labelling of receptors for autoradiography can be achieved in two ways, either in vivo in the intact animal or in vitro by using microtome tissue sections. Autoradiograms, generated by

apposition of the labelled tissue to photographic emulsions, can be analyzed and quantified by using computerized image analysis systems. During the past two decade, the development of in vitro labelling procedures as well as advances in computer-assisted analysis of autoradiograms have resulted in a widespread use of in vitro quantitative receptor autoradiography in the neurosciences (for reviews, see Kuhar, 1985; 1987; Kuhar & Unnerstall, 1991; Kuhar et al., 1986; Niehoff, 1986; Palacios, 1984; 1988; Wamsley & Palacios, 1983).

At the outset of this study, autoradiographic localization of CCK receptors had only been reported in the rodent brain (Gaudreau et al., 1983ab; Mantyh & Mantyh, 1985; Sekiguchi & Moroji, 1986; Van Dijk et al., 1984; Zarbin et al., 1981; 1983; for review, see Morency & Mishra, 1987). Therefore, in the present study, a detailed localization of CCK receptors was determined by in vitro receptor autoradiography in the brains of two higher mammalian species, the bovine and canine. For comparative purposes, CCK receptors were also localized in the rat and chicken brain. Although only rodent brains were used for early characterizations of CCK receptor distributions, detailed autoradiographic localizations have more recently been done in numerous species. Thus, the distributions of CCK receptors in the chicken, rat, canine, and bovine brains characterize in the present study was be compared to recently-reported distributions in the human (Cross et al., 1988; Dietl et al., 1987), monkey (Dietl & Palacios, 1989; Kritzer et al., 1987; 1988; 1990), feline (Dietl & Palacios, 1989; Artaud et al., 1989), guinea pig, rat, mouse, (Dietl & Palacios, 1989; Niehoff, 1989; Sekiguchi & Moroji, 1986), hamster (Miceli & Steiner, 1989), and pigeon brains (Dietl & Palacios, 1989).

Pharmacological characterization of CCK receptors in bovine cortical membranes indicated that the CCK receptors were of the CCK-B subtype. As previously described, this is not consistent with the report of Barrett et al. (1989); they identified CCK-A receptors in membrane preparations from all six areas of the bovine and rat brain tested (i.e., nucleus accumbens, caudate, cortex, cerebellum, hippocampus, and brainstem). Therefore, both the CCK-A and CCK-B antagonists, L-364,718 and L-365,260, were used to characterize CCK receptors in these areas of the bovine and rat brain by receptor autoradiography.

It should be noted that, for these receptor autoradiography studies, [^{125}I]BH-CCK$_8$ was used instead of [^{3}H]pCCK$_8$ because radioiodinated ligands offer two significant advantages over tritiated ligands. First, [^{125}I]-labelled ligands are available in very high specific activities (2000 vs 80 Ci/mmol) and, therefore, require much shorter exposure times compared to tritiated ligands. Second, the weak β rays emitted by tritiated isotopes are differentially absorbed by grey and white brain matter. Thus, given identical radioligand concentrations, the grain density in the emulsion is lower for binding in the white matter than in the grey matter because of its relatively greater density. This grey/white quenching problem can be avoided with radioiodinated ligands (see Kuhar et al., 1986). However, the distributions of [^{3}H]pCCK$_8$ and [^{125}I]BH-CCK$_8$ binding sites were compared in a preliminary experiment in order to ensure that they both labelled the same receptors.

II-4.1 METHODS

II-4.1.1 Materials:

[^{125}I]BH-CCK$_8$ (~2000 Ci/mmol), Hyperfilm-^{3}H, [^{3}H]micro-scales standards, and [^{125}I]micro-scales standards were from Amersham (Arlington Hgts., IL, USA). Frozen chicken brains were purchased from Pel Freeze, Transtar photographic paper from Kodak (Toronto, Ont), carboxymethyl cellulose (sodium salt, medium viscosity) from Sigma, chromic potassium sulfate from Aldrich Chemical Co, Inc (Milwaukee, WI, USA), O.C.T. compound and gelatin (275 Bloom) from Fisher Scientific (Ottawa, Ont).

II-4.1.2 Preparation of Brain Sections:

Bovine, canine, and rat brains were prepared essentially as previously described (Morency et al., 1991). Fresh brains were blocked over ice, rapidly frozen by immersion in hexane cooled in dry ice, and stored at −80°C until sectioning. Such rapid freezing is said to minimize the formation of ice-crystal artifacts (Quirion et al., 1987). Bovine and canine brains were embedded in 3% carboxymethyl cellulose matrix and cut with an LKB Instruments Large Stage Cryomicrotome. Rat and chicken brains were embedded in O.C.T. compound and cut with a Leitz 1720 digital cryostat. Sagittal and coronal sections (20 μm) were thaw-mounted onto glass slides which had been previously subbed using a 0.5% gelatin solution containing 0.05% chromic potassium sulfate. The sections were then dried overnight at 4°C in a desiccator under vacuum and stored at −80°C in sealed plastic bags until use. Appropriate sections were subsequently processed for autoradiography as described below.

II-4.1.3 Quantitative Autoradiography:

On the day of the experiment, frozen tissue sections were thawed and preincubated 15 min at room temperature in HEPES preincubation buffer (10 mM HEPES-NaOH containing 130 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$ at pH 6.5). Brain sections were then incubated for two hours at room temperature in HEPES assay buffer (preincubation buffer containing 1 mM EGTA, 100 µg/mL bacitracin, 5 µg/mL soybean trypsin inhibitors, and 0.1 mM PMSF) in the presence of 7 nM [^{3}H]pCCK$_8$ or 100 pM [^{125}I]BH-CCK$_8$. Non-specific binding was determined on adjacent sections in the presence of 1 µM CCK$_8$. In one study, [^{125}I]BH-CCK$_8$ binding was determined in rat and bovine sections in the presence of the CCK antagonists, L-364,718 (30 nM) and L-365,260 (30 nM), in order to identify CCK-A and CCK-B receptors.

Following the incubation, the sections were then transferred through three 10 min washes of ice-cold preincubation buffer and dipped 10 sec in ice-cold distilled water to remove excess salts. Sections were then rapidly dried under a stream of cold air and stored for 24 hours in a desiccator under vacuum prior to apposition to Hyperfilm-^{3}H together with radioactive micro-scales standards. Exposures were approximately 3–5 days for [^{125}I]BH-CCK$_8$ or 10–14 days for [^{3}H]pCCK$_8$. Autoradiograms were analyzed using an MCID image analyzer (Image Research Inc., St. Catharines, Ont). The autoradiographic images presented below were obtained by using the autoradiograms as negatives and producing positive prints on Transtar photographic paper. For histology, tissue sections which had been apposed to films were subsequently stained for Nissl substance using a Cresyl Violet (1%) stain (Vacca, 1985).

II-4.2 RESULTS

Since [^{125}I]BH-CCK$_8$ was preferred over [^{3}H]pCCK$_8$ for autoradiographical studies, the labelling by these two ligands was compared. As depicted in Figure 15, [^{125}I]BH-CCK$_8$ and [^{3}H]pCCK$_8$ binding were identical in bovine brain sections. Similar results were observed in the rat (data not shown).

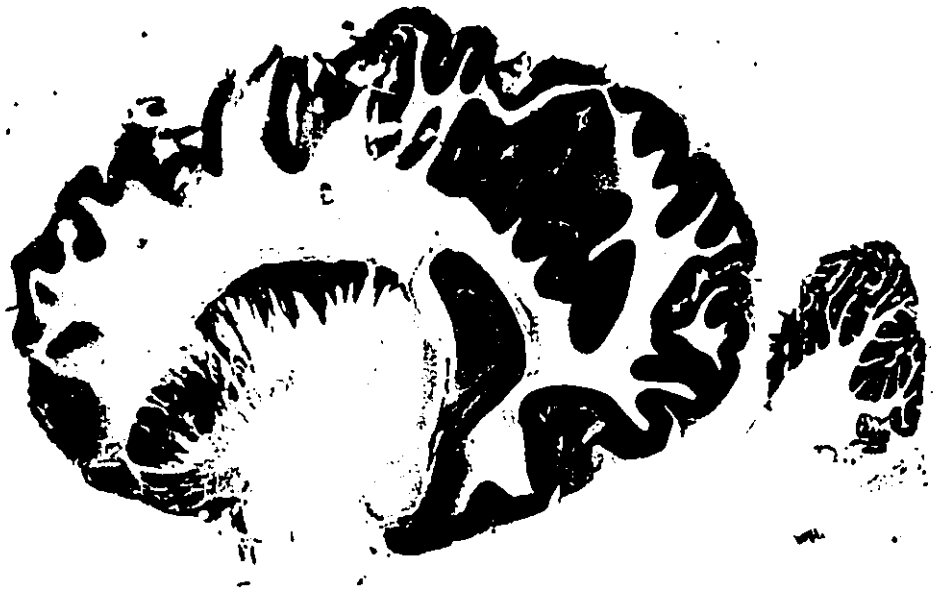
CCK binding sites were found to be heterogeneously distributed in the four species tested. Detailed localizations of CCK receptors in the bovine and canine brains are presented in Figures 16 and 17, respectively. For comparative purposes, the general distribution of [^{125}I]BH-CCK$_8$ binding sites in the bovine, canine, rat, and chicken brains are presented in Figure 18. Microdensitometric determinations of receptor densities for various areas of the bovine, canine, rat, and chicken brains are listed in Tables X–XIII, respectively.

In general, the density of CCK binding sites in the chicken brain was lower than that observed in the mammalian brains (see Fig. 18). This difference could be attributed to differences in the preparation of the chicken brain, i.e., purchased frozen from Pel Freeze versus fresh brains frozen in an hexane/dry ice bath for the other species. However, a similar observation was recently reported by Dietl & Palacios (1989); much lower densities of brain CCK receptors were observed in the brains of lower vertebrates (i.e., pigeon, fish, frog, snake) compared to mammals (i.e., rat, mouse, guinea pig, cat, monkey, human). As will be discussed below, marked species differences were evident in many brain areas. The distributions of CCK receptors in the chicken, rat, canine, and bovine brains characterized in the present study will be compared to recently-reported distributions in several other species.

Figure 15 Comparison of [^{3}H]pCCK$_8$ and [^{125}I]BH-CCK$_8$ binding in bovine brain sections.

Sections were incubated with 7 nM [^{3}H]pCCK$_8$ or 100 pM [^{125}I]BH-CCK$_8$ for 120 min at room temperature. Binding was done as described in the methods. Following the 120 min incubation, the sections were washed, dried, and apposed to Hyperfilm-^{3}H with radioactive micro-scale standards. Exposure was 10-14 days for [^{3}H]pCCK$_8$ binding and 3−5 days for [^{125}I]BH-CCK$_8$ binding. Autoradiographic images were obtained by using the autoradiograms as negatives and producing positive prints on Transtar photographic paper. Thus, dark areas have high densities and light areas have low densities of CCK receptors. Bar = 1cm.

[^{3}H]pCCK$_{8}$



[^{125}I]BH·CCK$_{8}$

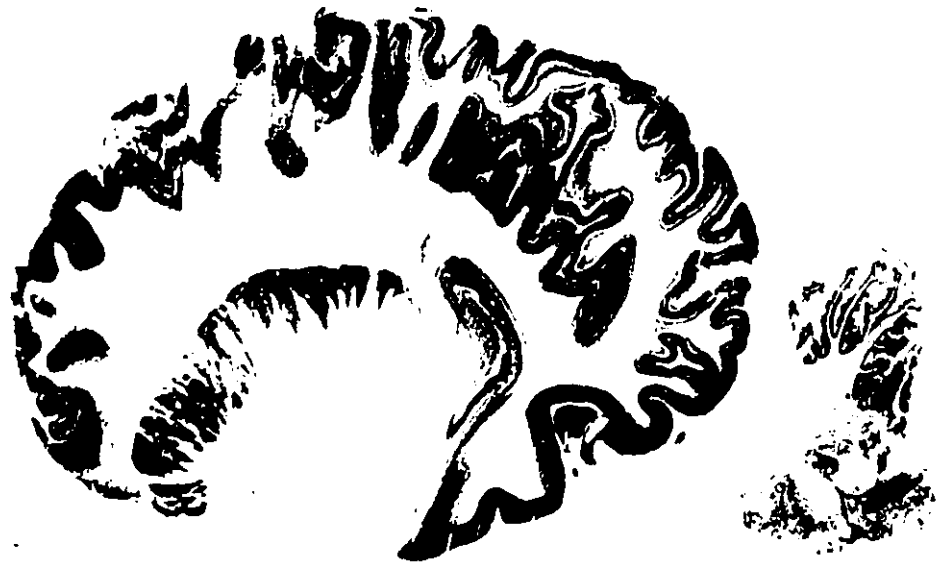


TABLE X

Microdensitometric Determination of [^{125}I]BH-CCK-8 Receptor Densities in Various Areas of the Bovine Brain[1]

BRAIN AREA	Specific Binding
Cortex	
Frontal	10.4 ± 0.4
Temporal	14.8 ± 1.0
Parietal	13.6 ± 0.7
Occipital	18.7 ± 1.4
Basal Ganglia	
Caudate	15.6 ± 0.7
Putamen	7.2 ± 1.0
Nucleus accumbens	13.1 ± 1.2
Globus pallidus	0.6 ± 0.2
Hippocampus	
Presubiculum	13.4 ± 1.2
Subiculum	10.1 ± 0.9
CA1	11.6 ± 0.8
CA2-3	5.9 ± 0.9
Dentate gyrus	14.6 ± 1.1
Amygdala	12.8 ± 1.8
Olfactory tract	9.4 ± 0.9
Thalamus	1.0 ± 0.3
Reticular nucleus	1.2 ± 0.3
Hypothalamus	1.2 ± 0.4
Cerebellum	
Molecular layer	5.1 ± 0.4
Purkinje cell layer	4.6 ± 0.4
Granular layer	4.4 ± 0.4
White matter	0.1 ± 0.0
Brainstem	
Substantia nigra	2.2 ± 0.2
Superior colliculus	2.3 ± 0.2
Inferior colliculus	9.9 ± 1.4
Pons	0.3 ± 0.0
Spinal cord	
Substantia gelatinosa	3.9 ± 0.4
White matter tracts	0.1 ± 0.0

[1] All data are the mean (±SEM) specific binding densities obtained with 100 pM [^{125}I]BH-CCK$_8$ in four animals. Receptor density estimations are expressed in fmol/mg. Brain regions were identified by comparing the Nissl-stained sections to those in the atlas of Yoshikawa (1968).

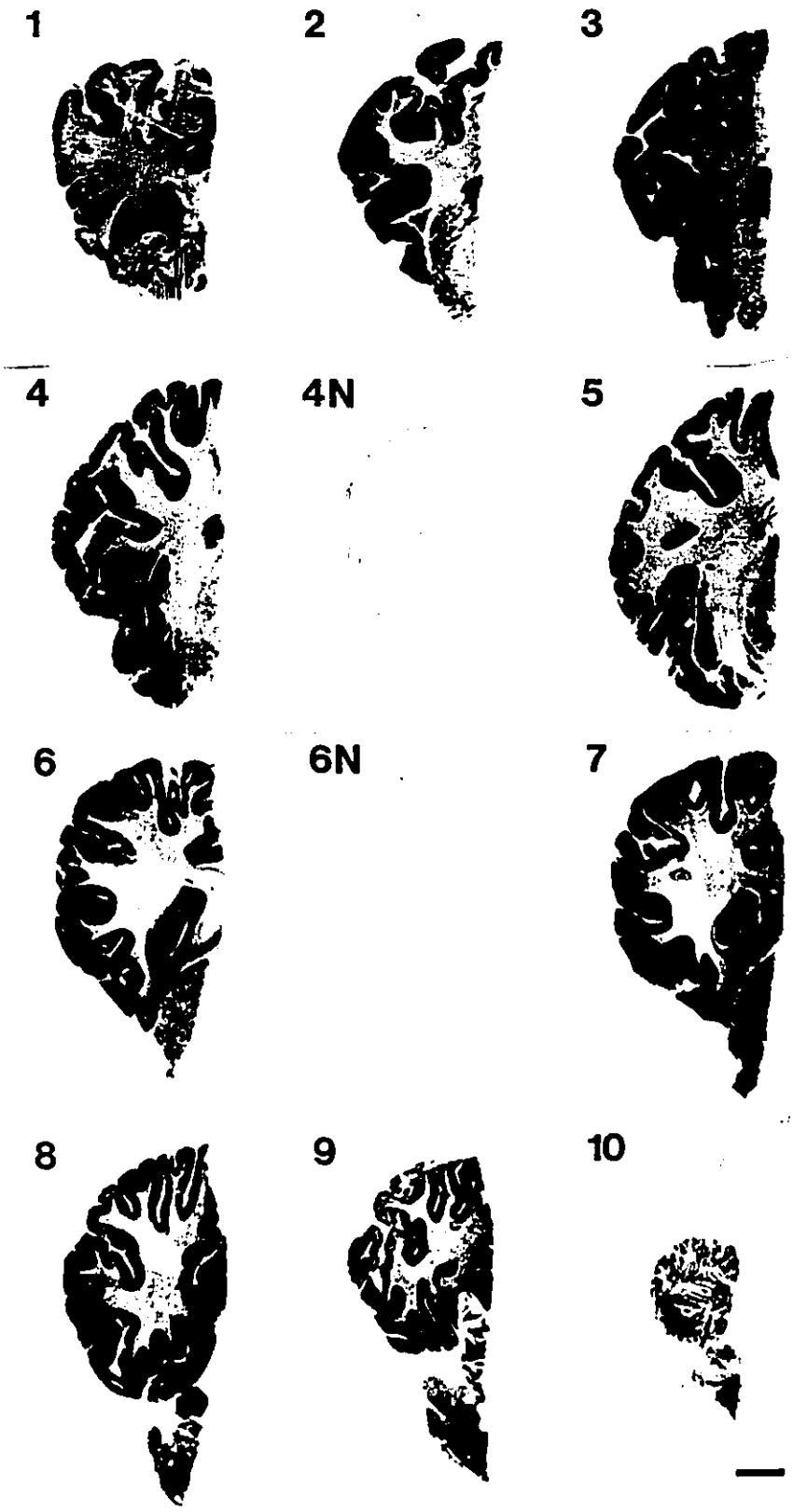
Figure 16 Autoradiographic localization of [^{125}I]BH-CCK$_8$ receptors in sagittal and coronal bovine brain sections.

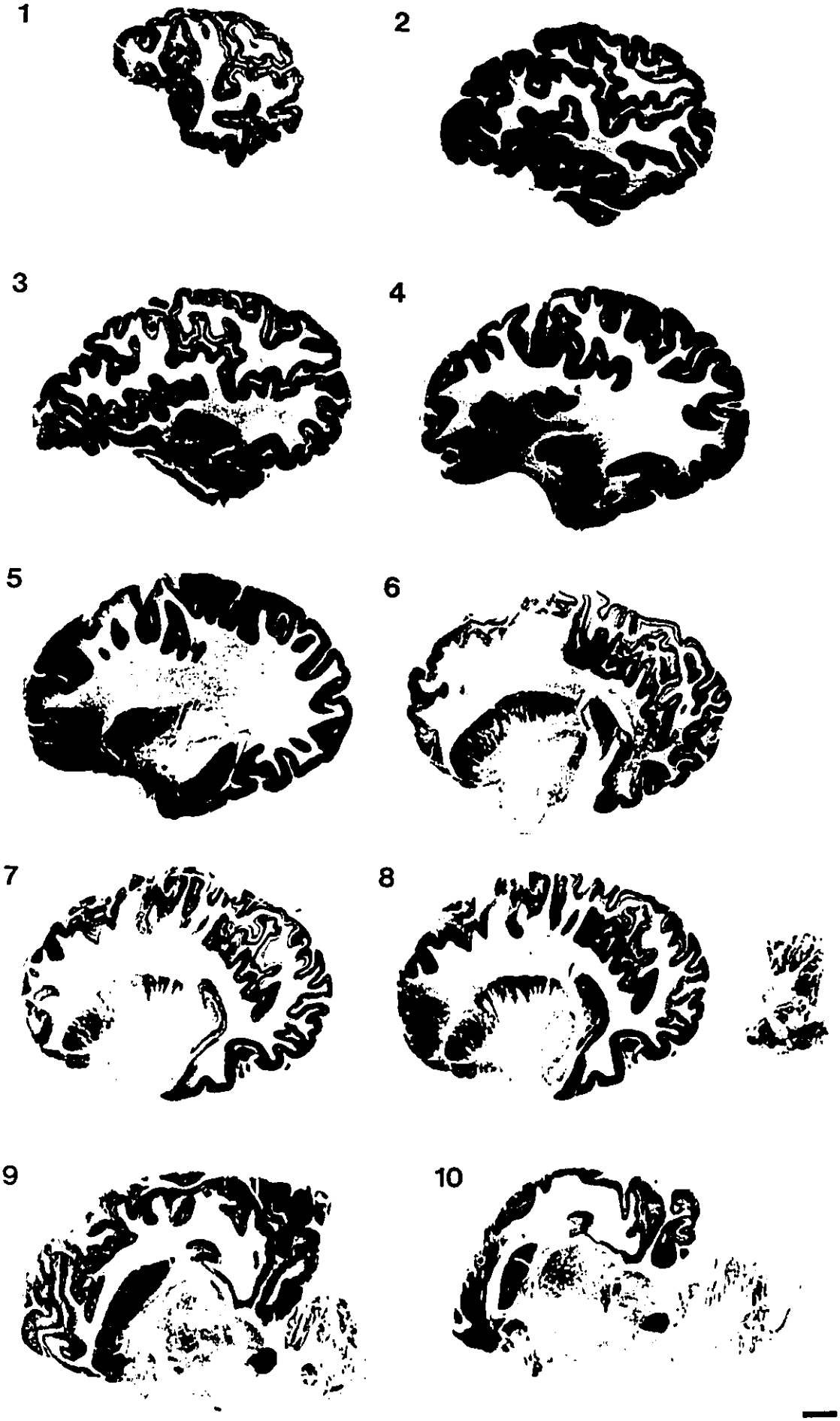
Quantitative autoradiography was done as described in the methods. Briefly, sections were incubated with 100 pM [^{125}I]BH-CCK$_8$ for 120 min at room temperature. Non-specific labelling was determined in adjacent sections with the addition of 1 µM CCK$_8$. Following the incubation, the sections were washed, dried, and apposed to Hyperfilm-^{3}H for 3–5 days with [^{125}I]micro-scales standards. Microdensitometric determinations of [^{125}I]BH-CCK$_8$ receptor densities are listed in Table X. Bar = 1 cm.

FIRST PANEL: Autoradiographic localization of [^{125}I]BH-CCK$_8$ receptors in coronal sections from bovine brain. Sections are presented from the most anterior (1) to the most posterior (10). Sections 4N and 6N represent non-specific labelling.

SECOND PANEL: Autoradiographic localization of [^{125}I]BH-CCK$_8$ receptors in sagittal sections from bovine brain. Sections are presented from the most lateral (1) to the most medial (10).

THIRD PANEL: Brain regions were identified by comparing Nissl-stained sections to those in the atlas of Yoshikawa (1968) and some regions are indicated in this third panel. Abbreviations: A = amygdala; C = caudate nucleus; Cb = cerebellum; Cx = cortex; DG = dendate gyrus; GP = globus pallidus; Hp = hippocampus; IC = inferior colliculus; NA = nucleus accumbens; OB = olfactory bulb; OT = olfactory tract; P = putamen; S = subiculum; SC = superior colliculus; SG = substantia gelatinosa of the spinal cord.


1
2
3
4
4N
5
6
6N
7
8
9
10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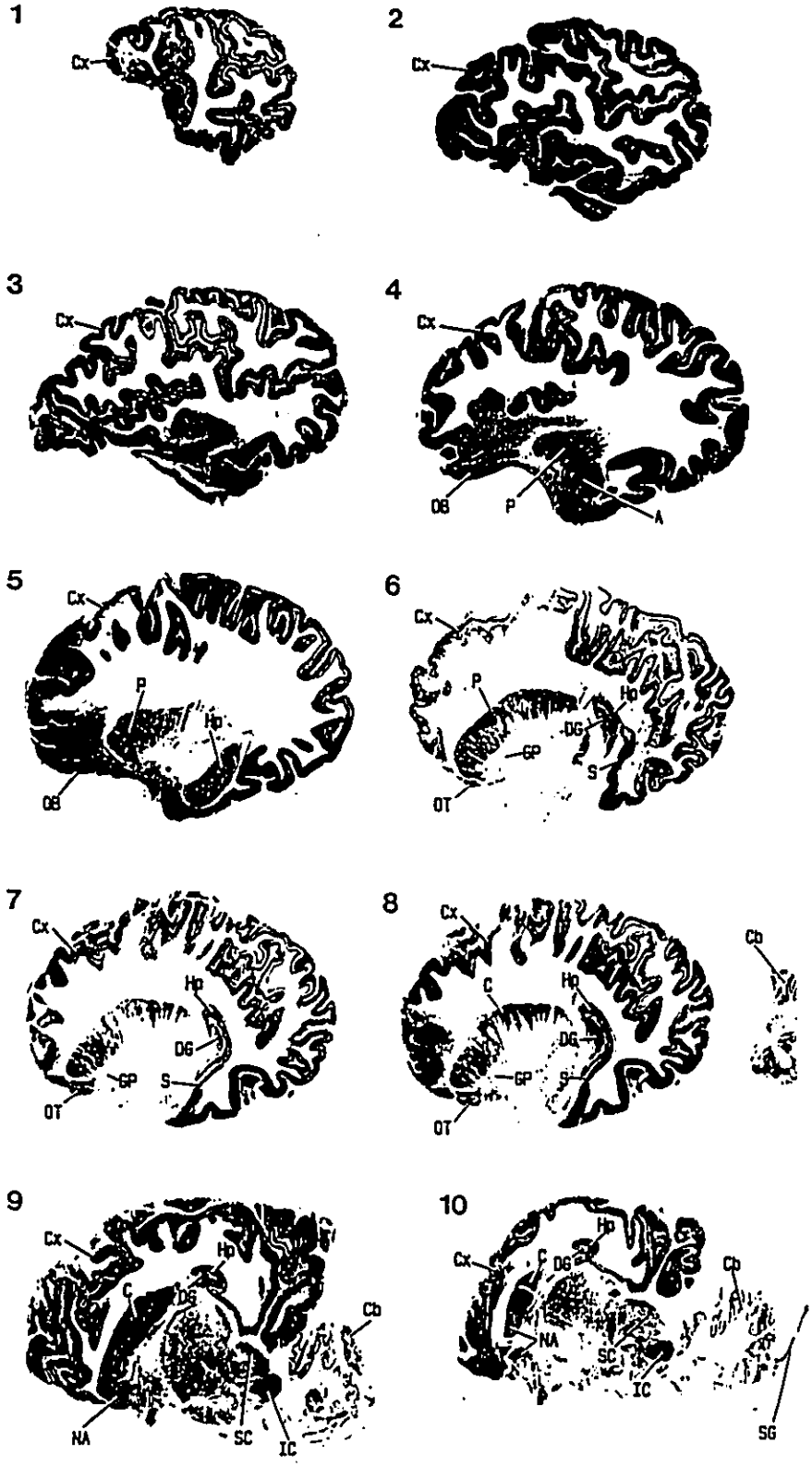
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TABLE XI

Microdensitometric Determination of [^{125}I]BH-CCK-8 Receptor Densities in Various Areas of the Canine Brain[1]

BRAIN AREA	Specific Binding	BRAIN AREA	Specific Binding
Cortex		Olfactory tract	7.0 ± 0.4
Frontal	8.7 ± 0.6	Thalamus	1.3 ± 0.4
Temporal	9.6 ± 0.4	Hypothalamus	0.4 ± 0.1
Parietal	10.2 ± 0.5	Cerebellum	
Occipital	12.8 ± 0.6	Molecular layer	11.3 ± 0.9
Basal Ganglia		Purkinje cell layer	7.5 ± 0.3
Caudate	8.9 ± 0.7	Granular layer	16.8 ± 1.0
Putamen	6.6 ± 0.4	White matter	0.1 ± 0.0
Nucleus accumbens	6.1 ± 0.4	Brainstem	
Globus pallidus	1.2 ± 0.2	Substantia nigra	0.6 ± 0.1
Hippocampus		Superior colliculus	0.5 ± 0.2
Presubiculum	1.4 ± 0.3	Inferior colliculus	2.4 ± 0.6
Subiculum	1.9 ± 0.5	Interpeduncular nucleus	0.2 ± 0.0
CA1	6.5 ± 0.3	Pons	1.1 ± 0.4
CA2-3	3.8 ± 0.8	Spinal cord	
Dendate gyrus	4.0 ± 0.2	Substantia gelatinosa	4.6 ± 0.3
Amygdala	3.7 ± 0.5	White matter tracts	0.3 ± 0.0

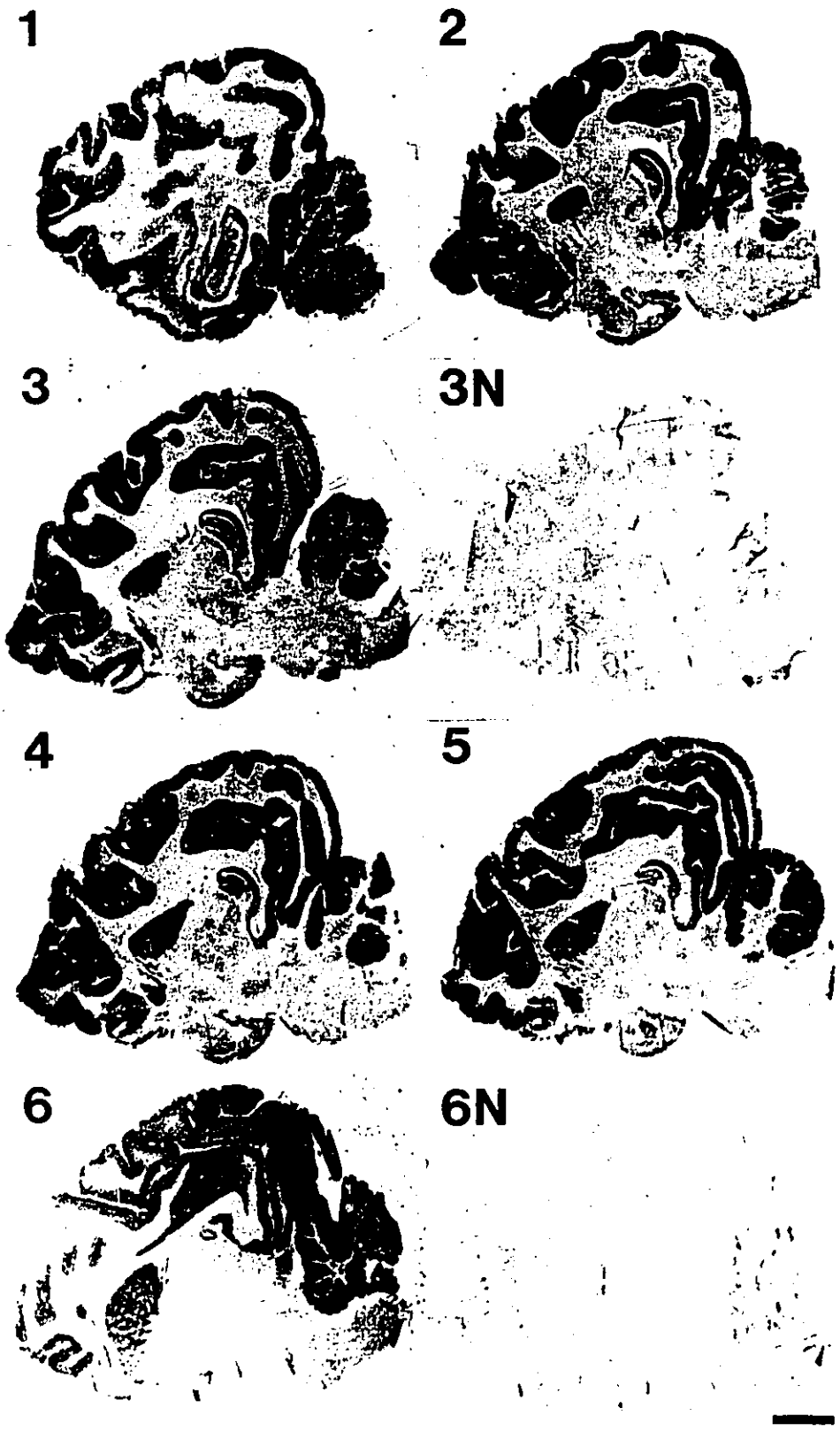
[1] All data are the mean (±SEM) receptor densities obtained with 100 pM [^{125}I]BH-CCK$_8$ in three animals. Receptor density estimations are expressed in fmol/mg. Brain regions were identified by comparing the Nissl-stained sections to those in the atlases of Adrianov & Mering (1964), Lim et al. (1960), and Meyer (1964).

Figure 17 Autoradiographic localization of [^{125}I]BH-CCK$_8$ receptors in sagittal sections from canine brain.

Quantitative autoradiography was done as described in the methods. Briefly, sections were incubated with 100 pM [^{125}I]BH-CCK$_8$ for 120 min at room temperature. Non-specific labelling was determined in adjacent sections with the addition of 1 μM CCK$_8$. Following the incubation, the sections were washed, dried, and apposed to Hyperfilm-^{3}H for 3–5 days with [^{125}I]micro-scales standards. Microdensitometric determinations of [^{125}I]BH-CCK$_8$ receptor densities are listed in Table XI. Bar = 1 cm.

FIRST PANEL: Sections are presented from the most lateral (1) to the most medial (6). Sections 3N and 6N represent non-specific labelling.

SECOND PANEL: Brain regions were identified by comparing Nissl-stained sections to those in the atlases of Adrianov & Mering (1964), Lim et al. (1960), and Meyer (1964) and some regions are indicated in this second panel. Abbreviations: A = amygdala; C = caudate nucleus; Cb = cerebellum; Cx = cortex; DG = dendate gyrus; GP = globus pallidus; Hp = hippocampus; IC = inferior colliculus; NA = nucleus accumbens; OB = olfactory bulb; OT = olfactory tract; P = putamen; S = subiculum; SC = superior colliculus; SN = substantia nigra.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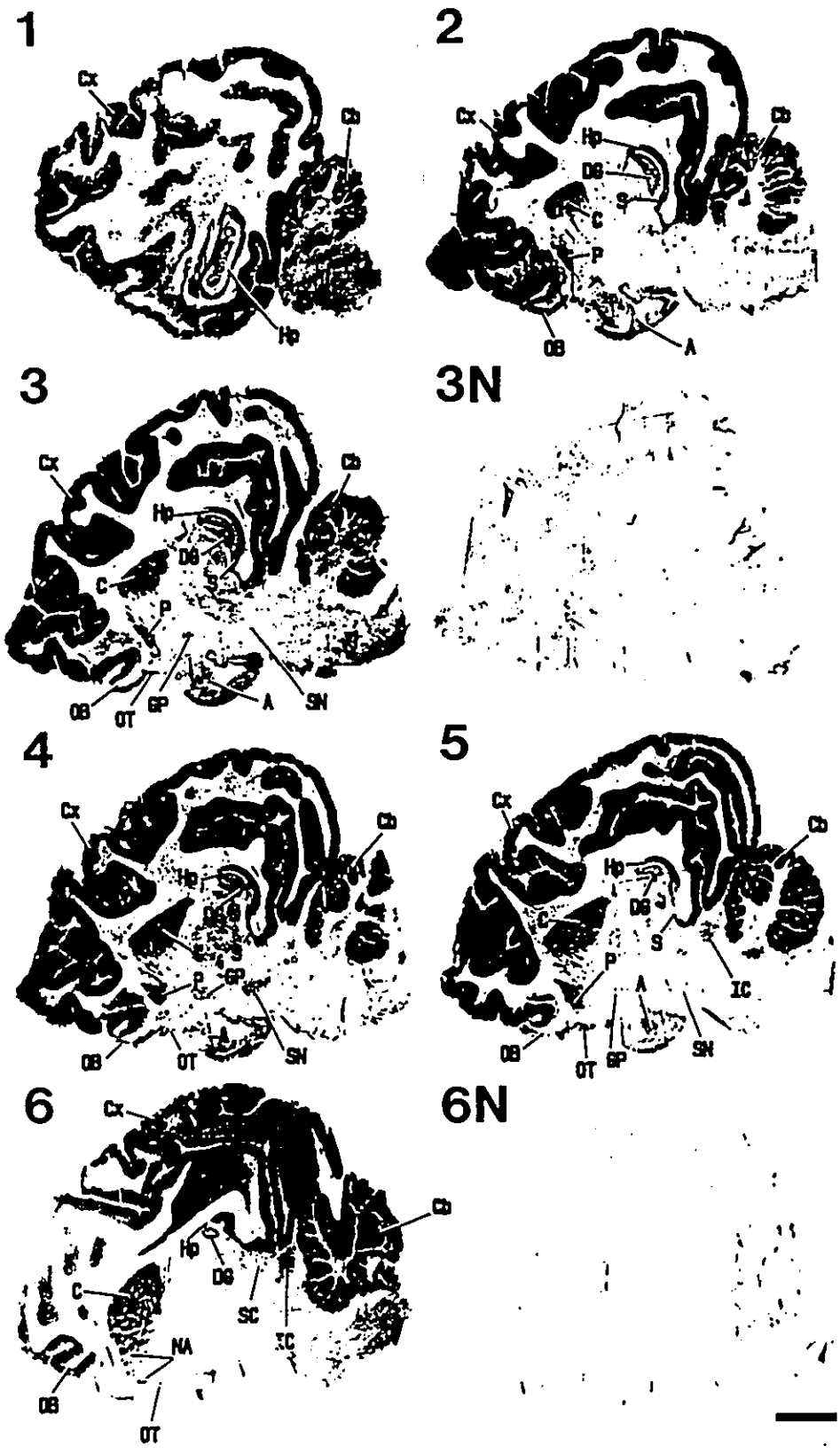
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TABLE XII

Microdensitometric Determination of [^{125}I]BH-CCK-8 Receptor Densities in Various Areas of the Rat Brain[1]

BRAIN AREA	Specific Binding
Cortex	
Frontal	17.2 ± 1.1
Temporal	14.1 ± 1.5
Parietal	16.5 ± 0.9
Occipital	19.8 ± 1.6
Basal Ganglia	
Caudate-putamen	17.2 ± 2.1
Nucleus accumbens	26.1 ± 1.9
Globus pallidus	0.8 ± 0.1
Hippocampus	
Presubiculum	10.0 ± 1.3
Subiculum	7.9 ± 0.9
CA1	2.4 ± 0.3
CA2-3	2.1 ± 0.3
Dendate gyrus	6.0 ± 0.5
Olfactory tract	17.7 ± 1.0
Amygdala	14.9 ± 1.7
Thalamus	
Reticular nucleus	8.1 ± 1.0
Hypothalamus	
Ventromedial nucleus	9.8 ± 1.1
Cerebellum	0.1 ± 0.0
Brainstem	
Substantia nigra	1.6 ± 0.3
Superior colliculus	3.2 ± 0.3
Inferior colliculus	2.4 ± 0.3
Intepeduncular nucleus	7.9 ± 0.6
Pons	2.0 ± 0.4
Spinal cord	
Substantia gelatinosa	4.2 ± 0.4
White matter tracts	0.2 ± 0.0

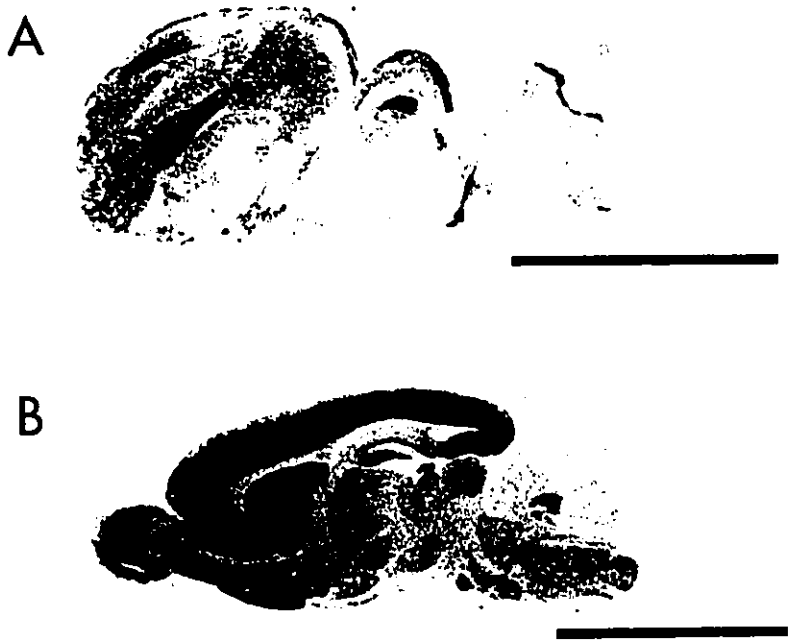
1 All data are the mean (±SEM) receptor densities obtained with 100 pM [^{125}I]BH-CCK$_8$ in three animals. Receptor density estimations are expressed in fmol/mg. Brain regions were identified by comparing the Nissl-stained sections to those in the atlases of König & Klippel (1960) and Paxinos & Watson (1986).

Figure 18 Distribution of [^{125}I]BH-CCK$_8$ receptors in sagittal sections from chicken (A), rat (B), canine (C), and bovine (D) brains.

Quantitative autoradiography was done as described in the methods. Briefly, sections were incubated with 100 pM [^{125}I]BH-CCK$_8$ for 120 min at room temperature. Non-specific labelling was determined in adjacent sections with the addition of 1 μM CCK$_8$. Following the incubation, the sections were washed, dried, and apposed to Hyperfilm-^{3}H for 3–5 days with [^{125}I]micro-scales standards. Microdensitometric determinations of [^{125}I]BH-CCK$_8$ receptor densities for various areas of the chicken, rat, canine, and bovine brains are listed in Tables XIII, XII, XI, and X, respectively. Bar = 0.5 cm.

FIRST PANEL: Representative sections from chicken (A), rat (B), canine (C), and bovine (D) brains are presented in this panel.

SECOND PANEL: Brain regions were identified by comparing Nissl-stained sections to those in the atlases of Adrianov & Mering (1964), Karten & Hodos (1967), König & Klippel (1960), Lim et al. (1960), Meyer (1964), Paxinos & Watson (1986), van Tienhoven & Juhasz (1962), and Yoshikawa (1968) and some regions are indicated in this second panel. Abbreviations: Bas = nucleus basalis; C = caudate nucleus; Cb = cerebellum; Cx = cortex; CP = caudate-putamen; DG = dendate gyrus; HA = hyperstriatum accessorium; HIS = hyperstriatum intercalatus superior; Hp = hippocampus; HV = hyperstriatum ventrale; IC = inferior colliculus; LPo = lobus paraolfactorius; NA = nucleus accumbens; NC = neostriatum caudale; NF = neostriatum frontale; NI = neostriatum intermedium; OB = olfactory bulb; OT = olfactory tract; PA = paleostriatum augmentatum; Rt = reticular nucleus; S = subiculum; SC = superior colliculus; SGF = stratum griseum et fibrosum superficiale; SGP = substantia grisea et fibrosa periventricularis; SN = substantia nigra.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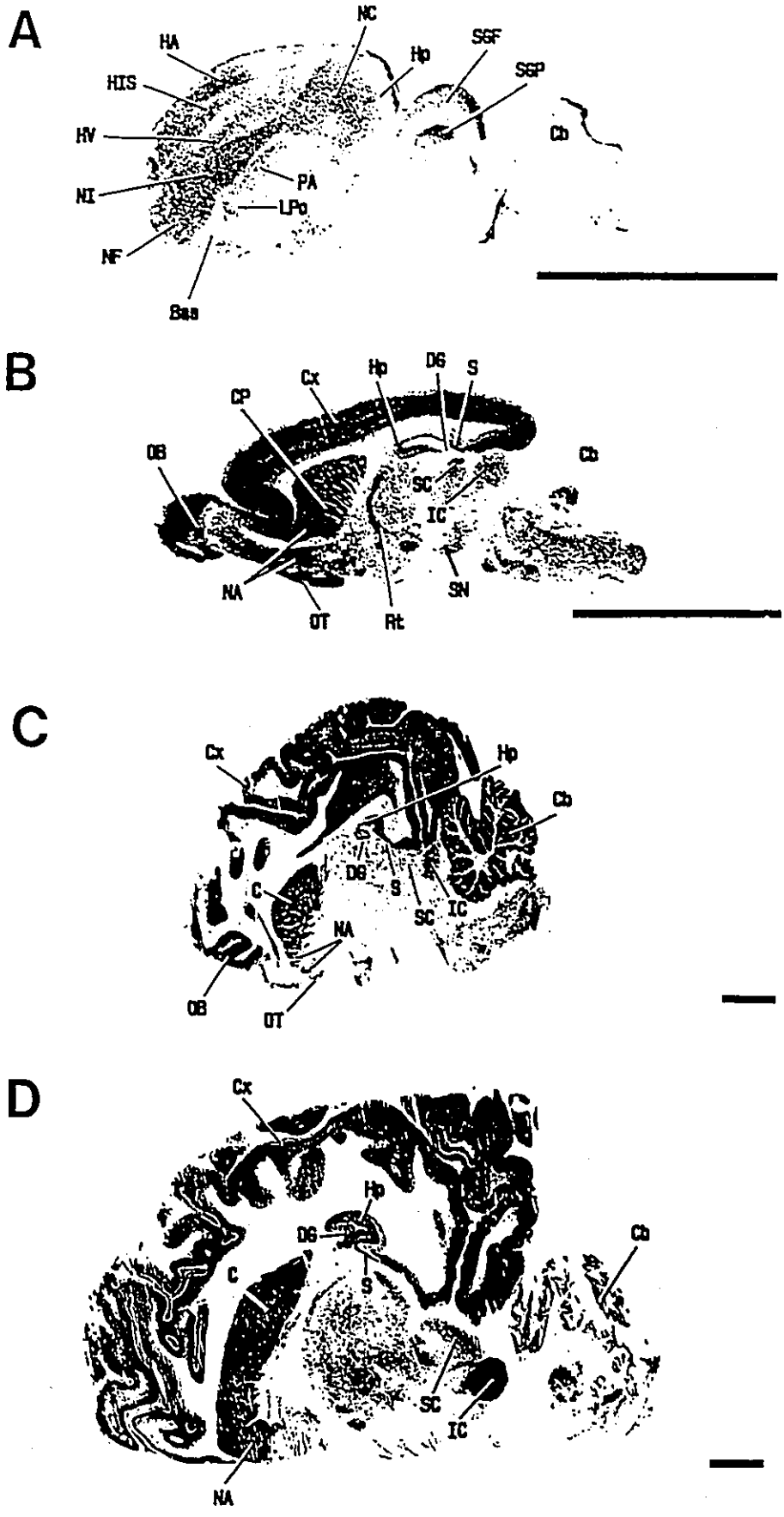
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TABLE XIII

Microdensitometric Determination of [^{125}I]BH-CCK-8 Receptor Densities in Various Areas of the Chicken Brain[1]

BRAIN AREA	Specific Binding	BRAIN AREA	Specific Binding
Hyperstriatum accessorium	2.6 ± 0.3	Ectostriatum	0.5 ± 0.1
Hyperstriatum dorsale	0.4 ± 0.0	Archistriatum, pars dorsalis	4.4 ± 0.2
Hyperstriatum intercalatus superior	8.6 ± 0.5	Archistriatum, pars ventralis	10.0 ± 1.2
Hyperstriatum ventrale	2.8 ± 0.1	Area Parahippocampalis	3.1 ± 0.7
Neostriatum	2.1 ± 0.1	Hippocampus	4.6 ± 0.3
Neostriatum caudale	3.2 ± 0.2	Cerebellum	0.0 ± 0.0
Neostriatum frontale	8.9 ± 0.9	Tractus olfactorium	0.8 ± 0.1
Neostriatum intermedium	11.3 ± 1.0	Stratum opticum	0.1 ± 0.0
Paleostriatum primitivum	0.3 ± 0.0	Stratum griseum et fibrosum superficiale	3.7 ± 0.5
Paleostriatum augmentatum	6.4 ± 0.4	Substantia grisea et fibrosa periventricularis	1.3 ± 0.3
Lobus paraolfactorius	5.1 ± 0.6	Nucleus basalis	0.1 ± 0.0

[1] All data are the mean (±SEM) receptor densities obtained with 100 pM [^{125}I]BH-CCK$_8$ in two animals. Receptor density estimations are expressed in fmol/mg. Brain regions were identified by comparing the Nissl-stained sections to those in the atlases of Karten & Hodos (1967), van Tienhoven & Juhasz (1962), and Yoshikawa (1968).

II-4.2.1 Cerebellar Cortex:

The most striking species-specific differences in CCK receptor distribution in the vertebrate brain were found in the cerebellum. No significant levels of specific binding were detected in the cerebellum of chickens and rats (Fig. 18). In contrast, moderate and very high densities of CCK binding sites were observed in the bovine (Fig. 16) and canine (Fig. 17) cerebellum, respectively. Indeed, the highest binding in the canine brain was found in the cerebellum (see Table XI). Similarly, an absence of CCK receptors has also been previously reported in the cerebellum of pigeons (Dietl & Palacios, 1989), rats (e.g., Mantyh & Mantyh, 1985; Niehoff, 1989; Sekiguchi & Moroji, 1986), and hamsters (Miceli & Steiner, 1989) contrasting with the higher densities observed in the cerebellum of guinea pigs, mice (e.g., Dietl & Palacios, 1989; Sekiguchi & Moroji, 1986), cats, monkeys, and humans (Cross et al., 1988; Dietl & Palacios, 1989; Dietl et al., 1987).

The moderate CCK receptor levels in the bovine cerebellum was uniformly distributed across cell layers. In contrast, CCK binding in the cerebellum of other mammalian species was layer specific. While higher levels in the granular than in the molecular layer have been observed in the canine (Table XI), guinea pig feline, and humans cerebellum (e.g., Dietl & Palacios, 1989; Dietl et al., 1987; Niehoff, 1989), the contrary was observed in the mouse and monkey cerebellum (Dietl & Palacios, 1989). Finally, the cerebellar Purkinje cell layer of the canine (Table XI), feline, guinea pig, and monkey cerebellum (Dietl & Palacios, 1989) contained low or very low CCK receptor levels in contrast to the higher levels found in the human cerebellum (Dietl & Palacios, 1989).

I'-4.2.2 Neocortex:

The distribution of CCK binding sites in the mammalian brain has been characterized by the presence of high densities in the neocortex (see Morency & Mishra, 1987). Indeed, autoradiographic studies indicated that the neocortex contained some of the highest densities found in the rat, canine, and bovine brain (see Fig. 18). In lower vertebrates where no neocortex can be defined, the 'external striatum' probably corresponds to the neocortex in mammals (Sarnat & Netsky, 1974). The 'external striatum' in the chicken brain presented low densities in most of its divisions. However, intermediate densities were distributed in some particular areas such as in the neostriatum (see Table XIII).

Furthermore, autoradiographic studies have demonstrated the presence of a laminar distribution in the mammalian neocortex, i.e., the binding was concentrated within particular cortical layers and was superimposed upon a background of diffuse label in other layers. Laminar distributions varied from area to area inside a given species and, as well, from species to species. For example, the lamination of CCK binding sites in the occipital cortex of rodents (Fig. 18; Dietl & Palacios, 1989; Miceli & Steiner, 1989; Niehoff, 1989; Sekiguchi & Moroji, 1986) is most prominent in the deeper layers (IV–VI) whereas the more superficial layers (II–III) were labelled in the canine occipital cortex (Fig. 17). In higher mammalian species, however, the lamination is more intricate and evident; very marked differences in the laminar distribution of CCK receptors are readily observed in the different cortical areas of the bovine (Fig. 16), primate (Kritzer et al., 1987) and human (Dietl et al., 1987) brain. In the bovine occipital cortex, for example, laminae III–IV were enriched in CCK binding sites

while laminae I–II and V presented low binding densities and lamina VI presented moderate binding densities (see Fig. 16).

II-4.2.3 Hippocampal Formation:

The most notable species-specific differences in the mammalian brain were found in the hippocampal formation. In the rodent (Table XII, Fig. 18; Köhler & Chan-Palay, 1988; Köhler et al., 1987; Niehoff, 1989), monkey (Köhler & Chan-Palay, 1988; Kritzer et al., 1988) and human hippocampus (Cross et al., 1988; Dietl et al., 1987; Köhler & Chan-Palay, 1988), high densities of CCK binding sites were located in the presubiculum and subiculum while Ammon's horn and dentate gyrus harboured relatively few binding sites. In contrast, the canine (Table XI, Fig. 17), and feline (Dietl & Palacios, 1989) hippocampus presented relatively low densities of CCK binding sites in the retrohippocampal region, whereas much higher densities were found in Ammon's horn and the dentate gyrus. Also in contrast to other mammalian species, the bovine hippocampal formation presented high densities of [^{125}I]BH-CCK$_8$ binding sites in the presubiculum, subiculum, and Ammon's horn but very low binding was detected in the dentate gyrus (Table X, Figs. 16 & 18).

Species-specific differences in the distribution of CCK receptors were also evident at the cellular level within hippocampal structure. For example, within the dentate gyrus of the rat, CCK binding was most prominent in the hilus (Fig. 20; Köhler & Chan-Palay, 1988; Köhler et al., 1987). However, labelling in the the dentate gyrus of the bovine (Figs. 16 & 18), canine (Figs. 17 & 18), feline (Dietl & Palacios, 1989), primate (Köhler & Chan-Palay, 1988; Kritzer et al., 1988), and human (Köhler & Chan-Palay, 1988; Dietl et al., 1987), a single band

of dense CCK binding sites was found in the granule cell layer and adjacent molecular layer, contrasting with the low densities found in the hilus.

Finally, the density of CCK binding sites in the hippocampus of lower vertebrates such as chicken (Table XIII, Fig. 18) and pigeon (Dietl & Palacios, 1989) was relatively low when compared to the densities observed in the mammalian hippocampal formation.

II-4.2.4 Basal Ganglia:

High densities of CCK binding sites were present in the nucleus accumbens, caudate, and putamen of the rat, canine, and bovine basal ganglia (see Fig. 18; Tables X, XI, and XII). In contrast to the heavy labelling observed in these structures, low to very low densities of CCK binding sites were present in the globus pallidus. A similar profile was reported in all the other mammalian species previously characterized (Cross et al., 1988; Dietl & Palacios, 1989; Dietl et al., 1987; Kritzer et al., 1990; Niehoff, 1989; Sekiguchi & Moroji, 1986) except in the mouse (Dietl & Palacios, 1989; Sekiguchi & Moroji, 1986) and hamster basal ganglia (Miceli & Steiner, 1989) where CCK binding was virtually absent.

In the avian brain, the lobus paraolfactorius and the paleostriatum augmentatum are thought to correspond to the mammalian caudate-putamen (Karten & Dubbeldam, 1973; Sarnet & Netsky, 1974). These structures presented relatively high densities of CCK binding sites in the chicken (Fig. 18; Table XIII) and pigeon brain (Dietl & Palacios, 1989). Nevertheless, these densities were low compared to the mammalian basal ganglia.

II-4.2.5 Other Brain Areas:

Marked differences in the distribution of CCK receptors were also evident in other areas of the rat, canine, and bovine brain. For example, whereas low levels of CCK binding sites were present in the inferior colliculus of rodents (Fig. 18, Table XII; Niehoff, 1989; Sekiguchi & Moroji, 1986) and canines (Fig. 17 & 18, Table XI), this structure was heavily labelled in the bovine brain (Fig. 16 & 18; Table X). Interestingly, in the guinea pig brain, it was the superior colliculus which presented high densities of CCK binding sites (Niehoff, 1989; Sekiguchi & Moroji, 1986). The thalamus is another brain area where differences were noted. Relatively high densities of CCK receptors were evident in the reticular nucleus of the rat thalamus (Fig. 18; Dietl & Palacios, 1989; Niehoff, 1989) whereas this nucleus was not labelled in the thalamus of bovine, canine (Fig. 18), or the other mammalian species (Dietl & Palacios, 1989; Miceli & Steiner, 1989). Finally, the interpeduncular nucleus was labelled in the rat (Table XII; Dietl & Palacios, 1989; Niehoff, 1989) and hamster (Miceli & Steiner, 1989) but not in the bovine, canine (Tables X & XI) or the other mammalian species (Dietl & Palacios, 1989).

II-4.2.6 CCK-A and CCK-B Receptors:

Using [^{3}H]L-364,718 membrane binding assays, Barrett et al. (1989) identified high densities of CCK-A receptors in membrane preparations of all six areas of the bovine and rat brain tested (i.e., nucleus accumbens, caudate, cortex, cerebellum, hippocampus, and brainstem). As previously described, this is not consistent with pharmacological characterization of CCK receptors in bovine cortical membranes which indicated that the CCK receptors were of the CCK-B subtype (Section II-2). In our laboratory, attempts to characterize CCK-A and

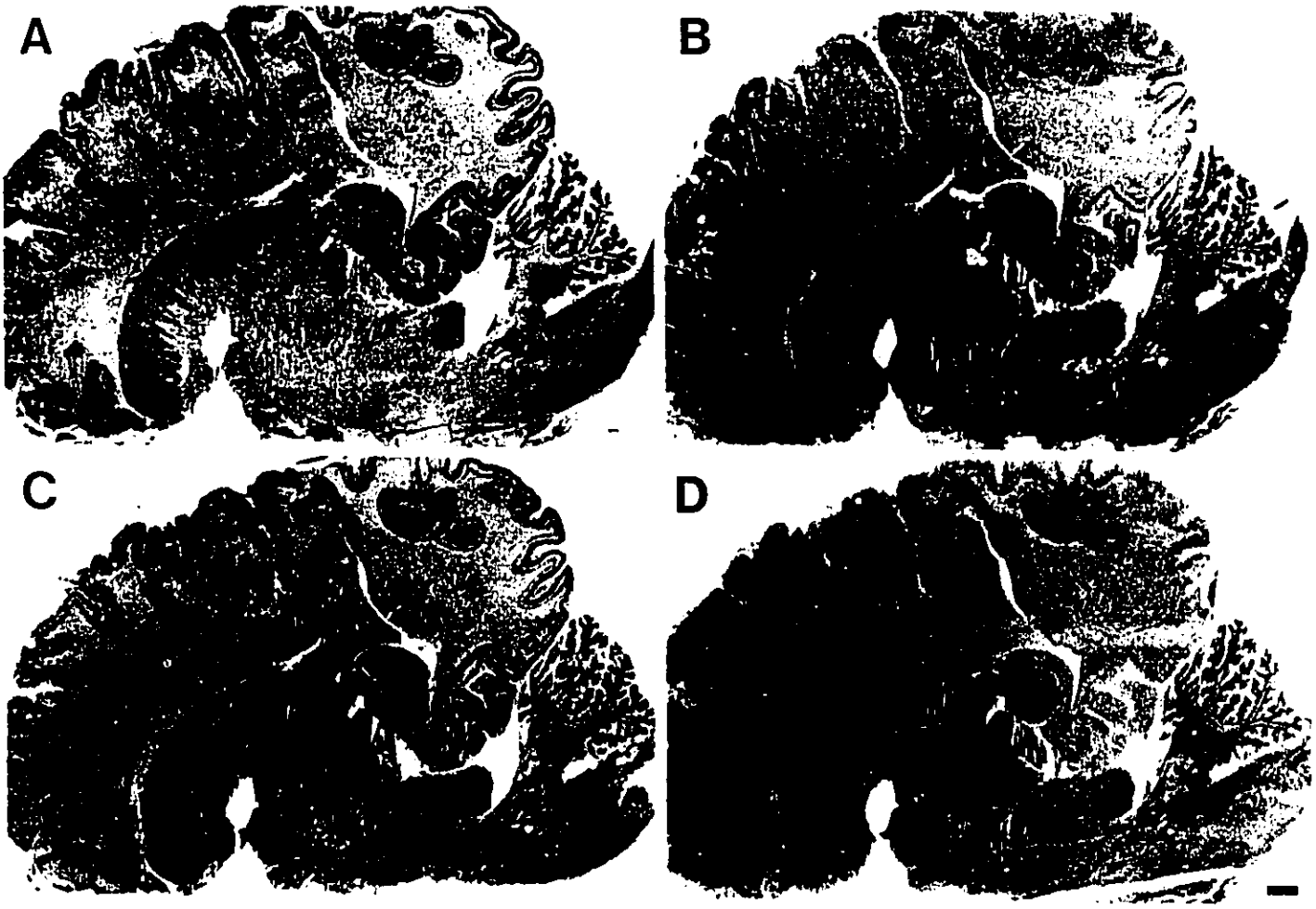
CCK-B receptors in bovine brain sections with [^{3}H]L-364,718 and [^{3}H]L-365,260 were unsuccessful; levels of non-specific labelling exceeded 90%. Therefore, the unlabelled CCK antagonists, L-364,718 and L-365,260, were used to characterize CCK receptors in bovine and rat brain sections by receptor autoradiography.

L-364,718 and L-365,260 were the most specific CCK-A and CCK-B antagonists available. However, as indicated by displacement studies, these antagonists only offer approximately 100-fold higher specificity for one subtype than the other (Section II.2.2.3, Table IX; see Chang & Lotti, 1988; Freidinger, 1989). Therefore, the concentration of CCK antagonist was titrated to obtain a significant displacement of [^{125}I]BH-CCK$_8$ binding from the specified receptor subtype without altering its interaction with the other. Stated simply, L-365,260 should be added at a concentration which displaces most of the [^{125}I]BH-CCK$_8$ at the CCK-B receptors without inhibiting the binding at CCK-A receptors and vice versa. Preliminary experiments indicated that a concentration of 30 nM was optimal (data not shown).

As depicted in Figure 19, the addition of the CCK-B antagonist L-365,260 inhibited the vast majority [^{125}I]BH-CCK$_8$ binding to the nucleus accumbens, caudate, cortex, cerebellum, hippocampus, and brainstem in rat and bovine brain sections (B). Although these sections are virtually identical to sections incubated with 1μM CCK$_8$ (D), some residual binding is visible probably resulting from the above-mentioned cross-specificity. In contrast, the addition of the CCK-A antagonist L-364,718 did not significantly inhibit [^{125}I]BH-CCK$_8$ binding to the nucleus accumbens, caudate, cortex, cerebellum, hippocampus, and brainstem in rat and bovine brain sections (C). In this instance, these sections are virtually

Figure 19 Autoradiographic localization of CCK-A and CCK-B receptors in bovine and rat brain sections.

The procedure was essentially the same as described in the methods except that bovine (upper panel) and rat (lower panel) brain sections were incubated with 100 pM [^{125}I]BH-CCK$_8$ for total binding (A) or in the presence of 30 nM L-365,260, a CCK-B antagonist (B), 30 nM L-364,718, a CCK-A antagonist (C), or 1 μM CCK$_8$ for non-specific labelling (D). Bar = 0.5 cm.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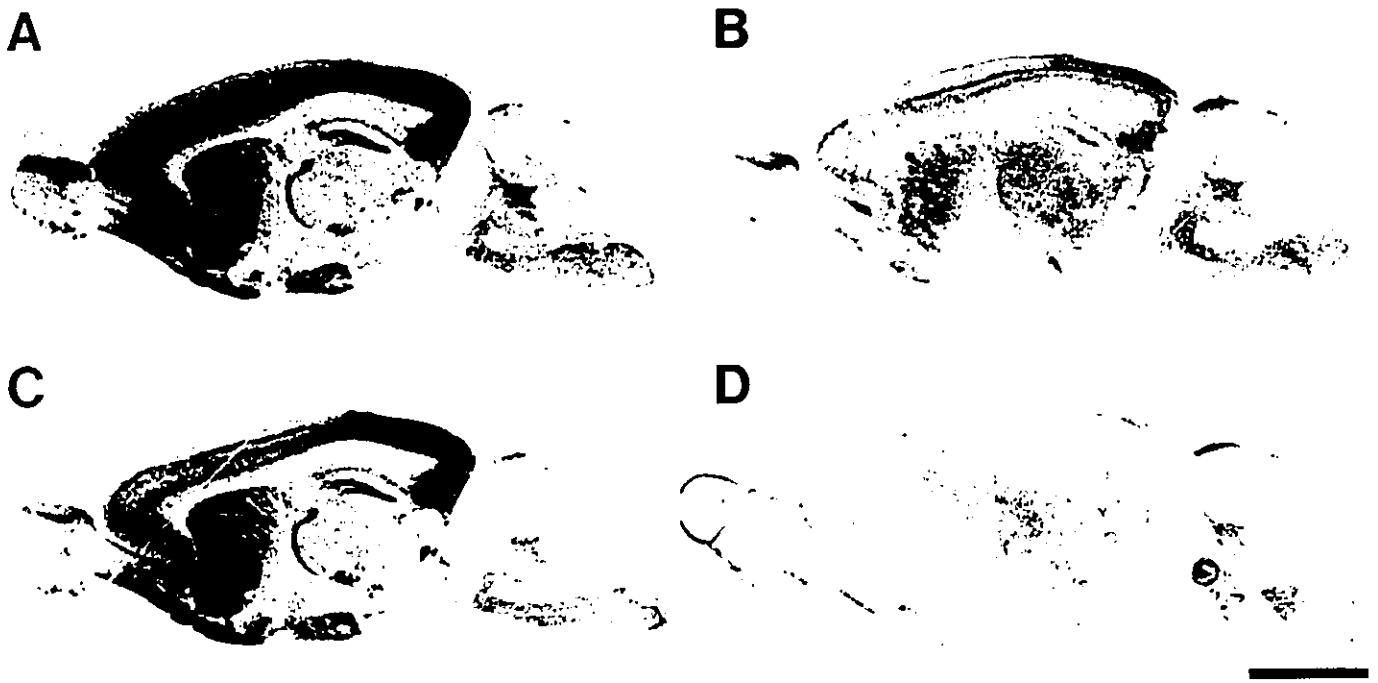
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identical to the total binding sections (A) although some displacement is apparent, especially in the cortex. Again, this slight displacement probably resulted from the cross-specificity of L-364,718. These results suggest that [^{125}I]BH-CCK$_8$ bound to CCK-B receptors in the nucleus accumbens, caudate, cortex, cerebellum, hippocampus, and brainstem of the rat and bovine brain. This conclusion is also supported by autoradiographical studies employing short tritiated CCK peptides such as [^{3}H]CCK$_4$ (Durieux et al., 1988) and [^{3}H]pentagastrin (Gaudreau et al., 1983ab) which have low affinities for CCK-A receptors. In these studies, low concentrations of these ligands labelled the above-mentioned structures in the rodent brain.

Thus, the only possible explanation which would reconcile these observations with the high densities of CCK-A receptors detected by Barrett et al. (1989) would be that [^{125}I]BH-CCK$_8$ binds exclusively to CCK-B receptors in these areas of the rat and bovine brain. In that case, Barrett et al. (1989) would have exclusively characterized CCK-A receptors with [^{3}H]L-364,718 and we would have exclusively characterized CCK-B receptors with [^{125}I]BH-CCK$_8$. However, this possibility seems unlikely. As previously discussed, brain CCK-A receptors have previously been characterized by displacing [^{125}I]BH-CCK$_8$ binding with selective antagonists (e.g., Chang et al., 1989; Hill & Woodruff, 1990; Hill et al., 1987a; 1990) or various CCK peptides (e.g., Hruby et al., 1990; Miceli & Steiner, 1989; Moran et al., 1986). Moreover, the research group at Merck Sharp and Dohme, the developers of L-364,718, did not observe CCK-A receptors in the above-mentioned areas of the rat brain using [^{3}H]L-364,718; they only reported specific binding in a few isolated nuclei (Hill et al., 1987a).

II-4.3 DISCUSSION

Although the distribution profiles of CCK binding sites in the bovine and canine brains were similar to those previously reported in other mammalian species, marked differences were observed in several brain areas. These species-specific differences contrast with the preservation of the pharmacological characteristics of these receptors. Estimates of binding parameters by Scatchard analysis in bovine and canine cortical membranes, the rank order of affinity for CCK peptides and antagonists as well as the linkage of CCK receptors with a G-protein in bovine cortical membranes were comparable to previously-reported values in other mammalian species.

Basically, there were two main types of species-specific differences in the distribution of CCK binding sites. The first one was the absence or presence of labelling in a given structure. The most notable example was the presence of CCK binding sites in the cerebellum of mouse, guinea pig, feline, canine, bovine, monkey, and human contrasting with the absence in the rat and avian cerebellum. Other examples were the paucity of CCK binding sites in the mouse and hamster basal ganglia, unlike the high densities found in every other mammalian species tested, and the exclusive presence of CCK binding sites in the rat thalamic reticular nuclei. A second type of difference were species-specific differences in CCK receptor distributions within a given structure. Examples of such differences were described in the cerebellum, neocortex, and hippocampus. The reasons for such species-specific differences in receptor distribution are not clear at the present time. Since related species (e.g., mouse, rat, and guinea pig) present significant differences in the distribution of CCK receptors (e.g.,

cerebellum and basal ganglia), these variations cannot be readily explained in terms of evolutionary changes. However, species differences in receptor distribution and/or pharmacology have also been reported for other transmitters, e.g., α_1 adrenoceptors, serotonin receptors, VIP receptors, and tachykinin receptors (Dietl & Palacios, 1988; 1989; Palacios et al., 1987; Pazos et al., 1984).

The physiological and behavioural consequences of these species-specific differences in the distribution of CCK receptors remain to be determined and should be defined as more information on the actions of this neuropeptide in various species become available. In the rat, where the actions of CCK have been most extensively studied, there is correspondence between the localization of CCK receptors and the observed physiological and behavioural actions of this peptide (see Zarbin et al., 1983). Briefly, the present study and results from previous reports (Dietl & Palacios, 1989; Gaudreau et al., 1983ab; 1985; Köhler et al., 1987; Mantyh & Mantyh, 1985; Niehoff, 1989; Sekiguchi & Moroji, 1986; Van Dijk et al., 1984; Zarbin et al., 1983) support a role for CCK in feeding (via receptors in the hypothalamus, amygdala, limbic system, cortex, and olfactory areas), sensory processing (via receptors on olfactory, auditory, and visual areas of the cortex and colliculi), regulation of motor behaviour (via receptors in motor areas of the cortex, basal ganglia, and, in some species, the cerebellum).

In the monkey brain, Kritzer et al. (1990) have determined that the distribution of CCK binding sites in the neostriatum coincide with regions innervated by association cortex of the frontal and temporal lobes. In addition, localization of CCK binding in the medial temporal lobe (i.e., amygdala, hippocampus, medial temporal cortex) revealed that CCK receptors are

anatomically coincident with the terminal fields of projection systems which interconnect the amygdala, hippocampal formation, and association cortices of the frontal, temporal, and parietal lobes (Kritzer et al., 1988). These finding suggest that CCK may have a particularly strong influence on cognitive function. It should be noted that many of the behavioural anomalies resulting from damage to some of these regions resemble those observed in schizophrenia and, thus, these areas have been implicated in the pathophysiology of this disorder (for review see Zec & Weinberger, 1986).

DA is another putative etiological pathophysiological component of schizophrenia. Because of the colocalization of CCK and DA in neurons of the ventral mesencephalon (Hökfelt et al., 1980ab), as well as data suggesting that CCK can modulate DAergic function (see Section I-4.3), it is interesting to note that several regions of the rat, canine, and bovine brains which contain high levels of CCK receptors are also projection sites for DAergic fibres and contain DA receptors. For comparative purposes, the distribution of DA receptors in the canine brain is presented in Appendix I. Briefly, in these species, CCK, DA D_1 and D_2 receptors are present in the nucleus accumbens, caudate-putamen, amygdala, olfactory tubercle, and cerebral cortex. In contrast, few CCK receptors are present in the ventral mesencephalon, where a high density of cells containing both CCK and DA have been demonstrated (Hökfelt et al., 1980ab).

CHAPTER III

CCK/DOPAMINE INTERACTIONS: ACUTE STUDIES

The discovery of the coexistence of DA and CCK in a subpopulation of mesencephalic DA neurons has been a major impetus for research into the interaction between these two neurotransmitters and the role of CCK in the CNS in general. In the present study, CCK/DA interactions were investigated using three measures of central DAergic function: Circling behaviour, DA-stimulated adenylate cyclase, and ligand binding at the DA receptor.

III-1 EFFECTS OF CCK ON CIRCLING BEHAVIOUR

Circling behaviour (rotational preference, turning preference) is one of the best understood behaviours in animals. It is, for the most part, DAergically mediated and related to asymmetry in DAergic activity between the right and left striatum (for review, see Pycock, 1978) or right and left frontal cortex (for review, see Beninger, 1988; Pycock, 1978); as a rule, animals circle away from the hemisphere with higher DAergic activity. Manipulations of other neurotransmitter systems can also produce circling. However, as Pycock & Marsden (1978) pointed out, "circling appears to depend ultimately on asymmetrical striatal DA receptor stimulation." For example, although circling is induced by anticholinergic drugs, it can be abolished by blockade of either postsynaptic DA receptors

with neuroleptics or by inhibition of DA synthesis with α-methyl-p-tyrosine (see Pycock, 1978; Pycock & Marsden, 1978). Indeed, the rodent circling model has been used for screening new neuroleptic and antiparkinsonism drugs (e.g. Crow & Gillbe, 1973; Glick & Shapiro, 1985; Ungerstedt, 1971).

Although the effects of CCK on circling behaviour had not been systematically investigated at the outset of this study, Mann et al. (1980) had described a syndrome of barrel rotations accompanied by contralateral postural asymmetry following ICV administrations of CCK_7; the unsulfated form, CCK_{7U}, induced none of the symptoms. With the high dose used in their study ($3x10^{-8}$ M or ~31 μg in 10 μL ICV), however, no spontaneous locomotor activity other than barrel rolling was observed. But other investigators had reported ambulation following ICV administrations of lower doses (0.01−2 μg) of CCK_8 (e.g., Hsiao et al., 1985; Katsuura & Itoh, 1986; Katsuura et al., 1985); hypolocomotion was noted at the high doses. Thus, the present study was undertaken to examine the effects of lower doses of CCK-peptides on circling behaviour. It was reasoned that if spontaneous locomotor activity is present after administration of lower doses of CCK, then the contralateral postural asymmetry reported with the high dose of CCK_7 (Mann et al., 1980) could be expressed as a contraversive circling bias.

III-1.1 METHODS

III-1.1.1 Animals:

Male Sprague-Dawley rats (200−250 g) were obtained from Charles River, Canada. Animals were individually housed in a climatically controlled

(21 ± 1°C) colony room on a 12 h light (2100–0900)/dark cycle. Food and water were continuously available in the home cages. Behavioural testing was performed during the dark cycle.

III-1.1.2 Drugs:

CCK peptides were dissolved in 0.9% saline with 0.5 N $NaHCO_3$. Proglumide was dissolved in a minimal amount of 1 N NaOH, adjusted to pH 7.35 with 1 N HCl, and diluted to the required concentrations with the CCK vehicle. Haloperidol was dissolved in a few drops of glacial acetic acid, diluted with distilled water and adjusted to pH 7 with 0.1 N NaOH. Proglumide sodium was purchased from Research Biochemicals Inc. (Wayland, MA, USA). CCK-peptides and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

III-1.1.3 Surgery:

The surgery was done essentially as described by Morency & Beninger (1986). Briefly, rats were anaesthetized with sodium pentobarbital (60 mg/kg, IP) and secured in a Kopf stereotaxic instrument. A stainless steel guide cannula (20 gauge) was implanted into one of the lateral ventricles at the following coordinates: 1.0 mm posterior to bregma, 1.7 mm lateral to the midline, and 2.5 mm ventral to the dura mater (König & Klippel, 1963). Cannulae were anchored to the skull with stainless steel screws and acrylic cement. They were protected between central injections with stainless steel obturator pins secured with silicone. Cannulae were implanted into the left lateral ventricle of half the animals and into the right lateral ventricle of the other half.

III-1.1.4 Apparatus:

Two Lafayette Instrument Co. 86010 (A-501) activity platforms, consisting of a 30 x 30 cm platform, an activity sensing transducer, enclosure (with Plexi-glass viewing window), and an amplifier were used as arenas for behavioural testing. These were attached to a multi-activity printout counter (Lafayette Instrument Co. 5711). The Gain control on the amplifier was adjusted to be responsive to gross motor activity and ambulation.

III-1.1.5 Central Injections:

Manual ICV microinjections of the drug or the vehicle were delivered by a 5 μL Hamilton microsyringe. Injection cannulae were constructed with 26 gauge stainless steel tubing cut to extend 1.0 mm beyond the tip of the guide cannula and attached to the microsyringe by a length of polyethylene tubing. Animals were hand-restrained during the insertion of the injection cannula into the guide cannula and were then placed in an activity box. At this point, the 1 μL injection was delivered in 20−30 sec and the injection cannula left in place for an additional 60 sec to ensure sufficient diffusion and avoid withdrawal of the drug during removal of the injection system. After the behavioural session, the obturator pin was reinserted into the guide cannula and secured with sealant.

III-1.1.6 Behavioural Testing:

Testing began 7 days after surgery. Each animal was tested 7 times during the study with 2 days between each test session. During each observation period, all complete turns, ipsiversive and contraversive to the side of the cannulae, were counted. A turn was defined as a 360° rotation without any turns in the opposite direction of more than 90°. Two animals were scored during

each 30 min session, observation periods being at 0–3, 6–9, 15–18, 21–24 and 27–30 min. The clock was stopped during the time taken to administer the central injections. Animals were started at staggered intervals of 3 min such that only 1 animal was being scored at any time. Thus, each animal was scored for a total of 15 min, in five 3 min blocks at approximately equal intervals throughout a 30 min session.

When investigating the effects of CCK-peptides on circling behaviour, each animal was tested 7 times as follows: (1) no central injection; (2) central injection of the vehicle; (3) each of the 3 drug doses with order of administration counterbalanced across rats over 3 sessions; (4) replication of vehicle; (5) replication of no central injection. The schedule of drug administration was modified slightly in experiments assessing the antagonism of CCK_8-induced circling behaviour. Rats were tested 7 times as follows: (1) central injection of CCK_8 and antagonist vehicles; (2) administration of antagonist vehicle and CCK_8 (5.0 ng, ICV); (3) each of the 3 doses of the antagonist with a counterbalanced order of administration and CCK_8 (5.0 ng, ICV); (4) replication of second session; (5) replication of first session.

Circling behaviour was expressed as the ratio of ipsiversive turns to the total number of turns (ipsiversive + contraversive). Ratio values of 0.5 indicate equal turning in both directions. Values greater or less than 0.5 indicate a tendency for ipsiversive or contraversive circling, respectively. The total number of turns and the activity counts for the session served as the other dependent measures.

III-1.1.7 Histology:

At the conclusion of behavioural testing, rats were administered an over-dose of sodium pentobarbital. A 1% Evans blue solution (5 μL) was injected into the guide cannula as a marker. Animal were decapitated, the brains were extracted and sectioned to verify cannula placement.

III-1.1.8 Statistical Analyses:

For all experiments, an arcsine transformation was conducted on the ratio data to satisfy the assumptions of the analysis of variance (ANOVA). One-factor, repeated measures ANOVAs were used to analyze drug effects (including the no-injection and vehicle conditions). Post hoc pairwise comparisons between treatment means were made with Duncan multiple range tests.

III-1.2 RESULTS

III-1.2.1 Effects of CCK-Peptides on Circling Behaviour:

Two t-tests for correlated measures were performed for each of the three dependent measures, one for the first and second no-injection scores and one for the vehicle-injection conditions. No significant differences were found for any of the experiments described below. Consequently, each of these conditions were combined for subsequent statistical analyses.

The two higher doses induced barrel rotations in some rats. However, as depicted in Figure 20 (top panel), only the highest CCK_8 dose (5000 ng) induced a slight reduction in activity. Indeed, the lower dose (500 ng) produced behavioural stimulation. A one-factor, repeated measures ANOVA confirmed a significant dose effect for total turns and activity counts, $F(4,36) = 4.57$ and

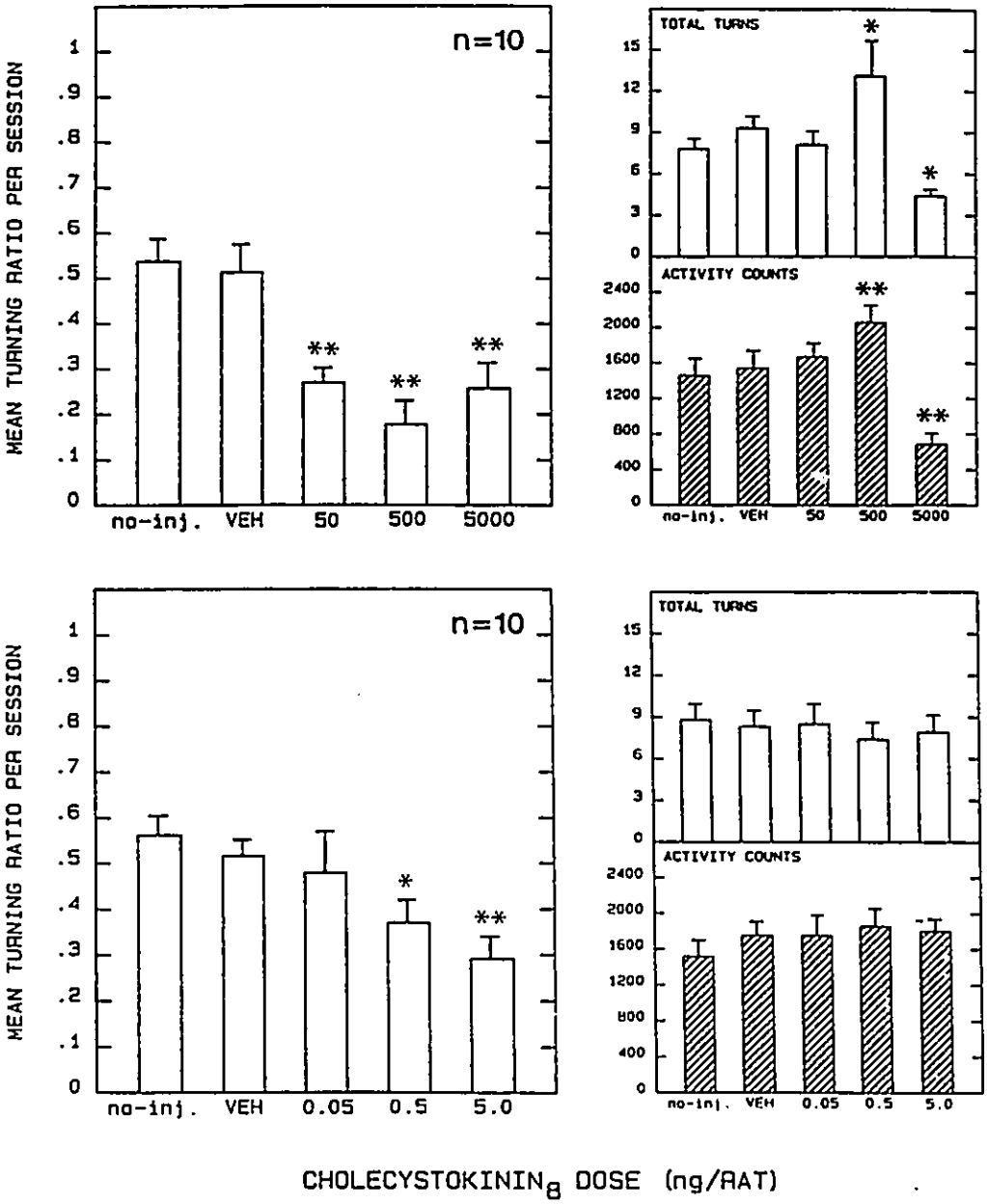
40.72, $p<0.005$, respectively. Post hoc pairwise comparisons between each condition and vehicle revealed that the total turns and activity counts induced by the 5000 ng dose were significantly lower than vehicle whereas they were significantly higher for the 500 ng dose.

As postulated above, with the presence of spontaneous locomotor activity, the contralateral postural asymmetry observed with a high dose of CCK_7 (Mann et al., 1980) was translated in a contraversive circling bias with lower doses. A one-factor repeated measures ANOVA confirmed the significance of this effects, $F(4,36) = 5.82$, $p<0.005$. Post hoc comparisons revealed that all three doses produced turning ratios significantly lower than the vehicle control (Fig. 20; top panel). Thus, three lower doses were tested in the second experiment. Again, a significant main effect of dose was observed, $F(4,44) = 4.50$, $p<0.005$. Post hoc comparisons revealed that 5.0 and 0.5 ng but not the 0.05 ng dose induced a significant contraversive circling bias (Fig. 20; lower panel). These three lower CCK_8 doses had no significant effect on total turns or activity counts.

As described in the previous chapters, the C-terminal tetrapeptide of CCK is the minimal structural requirement for binding to CCK receptors and the C-terminal heptapeptide with the sulfated tyrosine residue is necessary for full potency but CCK-B receptors are much less sensitive to these structural requirements than CCK-A receptors. In the present study, neither CCK_4 nor CCK_{8ns} (0.05–5000 ng) induced circling behaviour (data not shown); no significant main effect of dose was detected with the one-factor, repeated-measures ANOVA (Morency et al., 1987a).

Figure 20 Effects of CCK_8 on circling behaviour.

Data are mean (±SEM) turning ratio (left panel), total turns and activity counts (right panels) per session. Note that turning ratios less than 0.5 indicate tendencies for contraversive circling. * $p<0.05$, ** $p<0.01$ vs. vehicle control (VEH).


MEAN TURNING RATIO PER SESSION
n=10
no-inj.
VEH
50
500
5000
TOTAL TURNS
ACTIVITY COUNTS
MEAN TURNING RATIO PER SESSION
n=10
no-inj.
VEH
0.05
0.5
5.0
TOTAL TURNS
ACTIVITY COUNTS
CHOLECYSTOKININ8 DOSE (ng/RAT)

III-1.2.2 Effects of Proglumide on CCK_8-Induced Circling Behaviour:

Proglumide is a weak CCK antagonist which has a higher affinity for CCK-A receptors (see Chapter I). In the CNS, proglumide administered either peripherally or centrally blocks the behavioural (e.g., Barbaz et al., 1985; Crawley et al., 1986; Daugé et al., 1989b; Hsiao et al., 1985; Katsuura et al., 1984a; Vaccarino & Vaccarino, 1989) and electrophysiological actions (e.g., Chiodo & Bunney, 1983; Wang & White, 1985; White & Wang, 1984; see review Bunney, 1987) of CCK administered ICV, IC, or iontophoretically. Although more specific and potent CCK antagonists have recently been developed, proglumide was the best available at the time of this study and was therefore assessed for possible antagonistic effects on CCK_8-induced circling behaviour. Proglumide was co-injected with CCK_8 ICV.

As in the previous experiments, two t-tests for correlated measures were performed for each of the 3 dependent measures; one for sessions 1 and 7 and one for sessions 2 and 5. Since no significant difference were detected, these conditions were combined for subsequent analyses.

As depicted in Figure 21 (top left panel), the contralateral circling behaviour induced by CCK_8 (5.0 ng) was antagonized by proglumide in a dose-dependent manner, $F(4,28) = 29.36$, $p<0.0001$. Post hoc comparisons revealed that the 10 and 100 μg doses of proglumide significantly attenuated the CCK_8-induced circling. The co-administration of proglumide with CCK_8 had no significant effects on total turns and activity counts (Fig 21; top right panels).

III-1.2.3 Effects of Haloperidol on CCK_8-Induced Circling Behaviour:

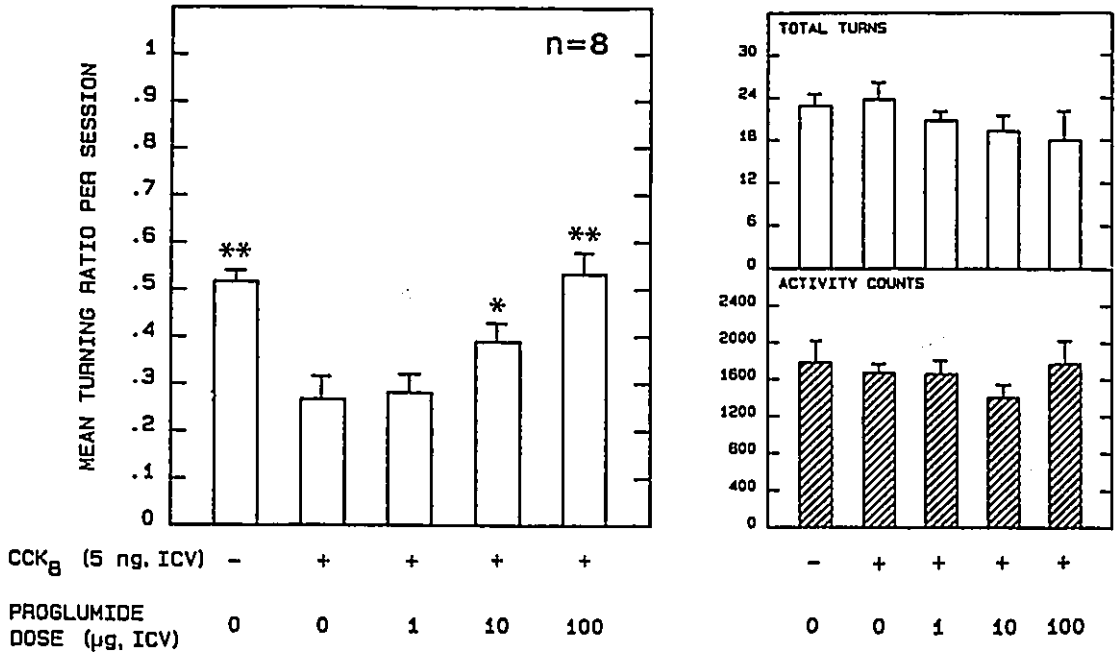
As described above, circling behaviour resulting from unilateral manipulation of central DA has commonly been used to assess this neurotransmitter's contribution to motor control; animals characteristically circle away from (contralateral to) the side of higher DA activity (see Pycock, 1980). In this respect, CCK_8-induced circling behaviour which was qualitatively similar to DA agonists. If this dopaminomimetic effect is induced through a unilateral facilitatory influence on DA neurotransmission, then it would be attenuated by the disruption of DA function. The effects of haloperidol, a DA receptor antagonist, on CCK_8-induced circling were assessed in the following experiment.

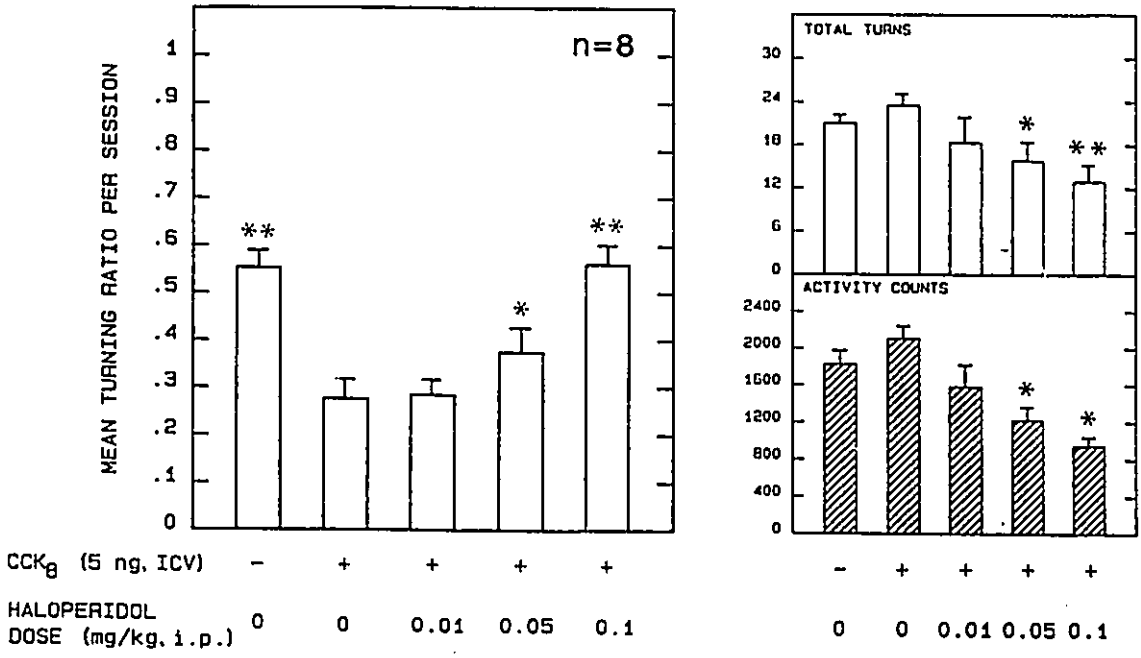
Haloperidol, when administered 45 min prior to the test session, resulted in a dose-dependent antagonism of the CCK_8-induced contraversive circling bias induced by CCK_8 (5.0 ng, ICV), $F(4,28) = 32.13$, $p<0.0001$. Post hoc comparisons revealed that the 0.05 and 0.1 mg/kg, IP doses significantly antagonized the CCK_8-induced contraversive circling bias (Fig. 21; lower left panel).

It has repeatedly been found that global interventions that decrease central DA function produce hypoactivity (see review Beninger, 1983; 1988). Indeed, a dose-dependent decrease in total turns and activity counts was observed in the present experiment, $F(4,28) = 4.24$ and 6.68, $p<0.01$, respectively. Post hoc comparisons revealed that the two higher doses of haloperidol (0.05 and 0.1 mg/kg, IP) significantly reduced the number of total turns and activity counts (Fig. 21; lower right panels).

Figure 21 Antagonism of CCK_8-induced circling behaviour.

Antagonism of CCK_8-induced circling behaviour by the CCK receptor antagonist, proglumide (upper panel), and by the DA D_2 receptor antagonist, haloperidol (lower panel). Data are mean (±SEM) turning ratio (left panel), total turns and activity counts (right panels) per session. * $p<0.05$, ** $p<0.01$ vs. CCK_8 + antagonist vehicle.


n=8
MEAN TURNING RATIO PER SESSION
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
**
*
**
TOTAL TURNS
30
24
18
12
6
0
ACTIVITY COUNTS
2400
2000
1600
1200
800
400
0
CCK8 (5 ng, ICV) - + + + +
PROGLUMIDE DOSE (µg, ICV) 0 0 1 10 100
- + + + +
0 0 1 10 100


n=8
MEAN TURNING RATIO PER SESSION
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
**
*
**
TOTAL TURNS
30
24
18
12
6
0
*
**
ACTIVITY COUNTS
2400
2000
1600
1200
800
400
0
*
*
CCK8 (5 ng, ICV) - + + + +
HALOPERIDOL DOSE (mg/kg, i.p.) 0 0 0.01 0.05 0.1
- + + + +
0 0 0.01 0.05 0.1

III-1.3 Discussion

Unilateral ICV microinjections of CCK_8 induced a dose-dependent contraversive circling bias. Absence of significant differences between the first and second administration of the no-injection and vehicle conditions indicates that, subsequent to the drug sessions, rats returned to baseline levels for all three behavioural measures. This illustrates the acute effects of the central drug injections of this study. Furthermore, as the vehicle scores were not significantly different from the no-injection scores, it is clear that the vehicle did not induce a directional bias. The possibility that the circling behaviour resulted from progressive damage due to repeated central injections seems unlikely as the order of dose was counterbalanced, yet a dose-dependent effect was induced by CCK_8. Furthermore, no circling bias was observed following unilateral ICV administrations of CCK_{8U} and CCK_4.

Thus, the sulfated tyrosyl residue of CCK_8 appeared to be an important structural requirement for this behavioural effect as CCK_{8U} and CCK_4 failed to elicit contraversive circling, possibly suggesting the involvement of CCK-A receptors. Further support for this conclusion was that proglumide, a weak CCK-A antagonist, attenuated the CCK_8-induced contraversive circling bias in a dose-dependent manner. The involvement of CCK-A receptors has been suggested for other behavioural effects of CCK in the CNS (e.g., Daugé et al., 1989b; Hagino et al., 1989; Katsuuras et al., 1984b; O'Neil et al., 1991; Soar et al., 1989). However, as was described in the previous chapters, apart from a few isolated nuclei, the vast majority of CCK receptors in the rodent brain are of the B-subtype. In the nucleus accumbens, for example, several investigators have

provided behavioural and electrophysiological evidence of rostral-caudal differences in CCK function (e.g., Crawley et al., 1985ab; Daugé et al., 1989a; 1990; Vaccarino & Rankin, 1989; Vaccarino & Vaccarino, 1989; White & Wang, 1984) and the use of highly potent and subtype-specific CCK drugs has supported the involvement of CCK-A receptors in the posterior-medial nucleus accumbens (Daugé et al., 1989; 1990). However, although such evidence supports a neuroanatomical heterogeneity in the distribution of CCK receptors in the nucleus accumbens, autoradiographic studies indicate the presence of only CCK-B receptor in this structure (see Chapter II, Fig. 19; Durieux et al., 1988; Gaudreau et al., 1983ab; Hill & Woodruff, 1990; Hill et al., 1987a; 1990). Matters are further complicated by behavioural studies in which CCK_{8U} or CCK_4 produce effects opposite to those produced by CCK_8 (e.g., Hsioa et al., 1985; Itoh & Katsuura, 1985; Katsuura & Itoh, 1982; Katsuura et al., 1985) or behavioural studies in which the rank order of potency observed in binding studies is violated, e.g., CCK_{8U} produces a behavioural effects but CCK_8 is ineffective (Kovács et al., 1981). Such results underscore the complexity of CCKergic systems in the CNS and the need for the development of more potent and selective pharmacological agents.

The discovery of CCK being co-localized with DA in some areas of the brain has generated considerable interest in potential CCK/DA interactions. Biochemical, pharmacological, electrophysiological and behavioural investigations have suggested a modulatory role for CCK on central DA function; however, considerable controversy exists in the literature as to whether CCK-peptides inhibit or facilitate DA function in the mammalian CNS (see Chapter I). Circling

behaviour has commonly been used to assess the contribution of DA to motor control. Animals characteristically circle away from the side of higher DA activity; thus, unilateral intracerebral microinjections of DA agonists in the striatum (see Pycock, 1980) and in the frontal cortex (Morency et al., 1985; 1987b; Stewart et al., 1985) have been reported to produce contraversive circling. In this respect, the circling bias induced by CCK_8 in the present study was qualitatively similar to that induced by DA agonists; animals circled away from the injection. In addition, this effect was blocked in a dose-dependent manner by the DA receptor antagonist haloperidol. These data suggest that, under the acute conditions employed in the present study, CCK_8 may induce contraversive circling by exerting a unilateral facilitatory influence on DA neurotransmission.

Shortly after these data were first presented (Morency et al., 1986), Worms et al. (1986) reported that unilateral intrastriatal injections of CCK_8 induced contraversive circling in mice. As was observed with ICV administrations in the present study, CCK_{8U} was ineffective and the CCK_8-induced circling behaviour was antagonized by the CCK antagonists, Z-CCK-(27−32)NH_2 and proglumide, as well as by the neuroleptic drug, haloperidol (Worms et al., 1986). This may suggest that, in the present study, CCK_8 injected in the lateral ventricle may have induced circling, at least in part, through its action in the striatum. However, this should be interpreted with caution since autoradiographical studies have revealed a virtual absence of CCK receptors in the mouse basal ganglia (see Section II-4).

Other studies employing ICV administrations of CCK-peptides have

suggested a facilitatory role for CCK_8. Thus, acute ICV administration of CCK peptides have been shown to down-regulate striatal DA D_2 receptors (Marshal et al., 1983) and potentiate apomorphine-induced behaviour (Ellinwood et al., 1983). Furthermore, intra-VTA injections of CCK potentiated amphetamine-induced conditioned place preference (Pettit & Mueller, 1989) and dopamine-induced hyperlocomotion (Crawley, 1989; Daugé et al., 1990) while injections of CCK peptides into the caudal nucleus accumbens potentiated amphetamine-induced hyperlocomotion (Crawley et al., 1985b; Vaccarino & Rankin, 1989) and apomorphine-induced stereotypy (Crawley et al., 1985a). In addition, in vitro studies have suggested that CCK_8 induces a marked enhancement of DA release from slices of rat (Hamilton et al., 1984; Starr, 1982) and mouse (Kovács et al., 1981) striatum. The present study provides further evidence for a facilitatory influence of CCK-peptides on central DA function.

When postulating the possible mechanism(s) underlying the modulatory effects of CCK on DA behaviours, investigators commonly cite studies demonstrating CCK-induced modulation of DA release, DA receptor binding, and/or DA-stimulated adenylate cyclase (e.g., Crawley, 1991; Vaccarino & Rankin, 1989; Weiss et al., 1989). As mentioned above, circling behaviour is related to asymmetry in DAergic activity between the right and left striatum or right and left frontal cortex (for review, see Beninger, 1988; Pycock, 1978). To further elucidate the mechanism(s) underlying CCK-induced circling behaviour, we have investigated the effects of CCK peptides on DA-stimulated adenylate cyclase and on ligand binding at DA receptors in the rat striatum and cortex (see the following two sections).

III-2 EFFECTS OF CCK ON DOPAMINE-STIMULATED ADENYLATE CYCLASE:

Recently, Studler et al. (1986) reported opposite effects of CCK_8 on DA-sensitive adenylate cyclase in two areas of the rat nucleus accumbens; the stimulatory effect of DA on adenylate cyclase was potentiated in the area innervated by mixed CCK/DA fibres (caudal part) while the opposite effect was seen in the rostral part where CCK is not present in DA fibres.

The striatum and frontal cortex are other areas where CCK and DA are localized in distinct fibres. Moreover, CCK-peptides have been reported to enhance cAMP efflux (Long et al., 1986) and to increase cAMP concentration in synaptosomes (Chowdhury et al., 1987) in the striatum. In addition, CCK was shown to stimulate DA synthesis in rat striatal synaptosomes by a cAMP-dependent mechanism (Chowdhury et al., 1987) whereas stimulation of DA D_1 receptors was reported to decrease the release of CCK from slices of rat neostriatum (Meyer et al., 1984). Therefore, it was of interest to examine the ability of CCK_8 to modulate basal and DA-stimulated adenylate cyclase activity in the striatum and frontal cortex.

III-2.1 METHODS

III-2.1.1 Chemicals:

α-[^{32}P]ATP (30–34 Ci/mmol) and [^{3}H]cAMP (31 Ci/mmol) were purchased from NEN/Dupont (Boston, MA, USA). The Dowex 50 ion exchange resin (AG 50W-XB 200–400 mesh, H^+ form) was purchased from Bio-Rad Laboratories (Richmond, CA, USA). All other compounds were obtained from

Sigma Chemical Co. (St. Louis, MO, USA).

III-2.1.2 Tissue Preparation:

Male Sprague Dawley rats (200−250 g) were sacrificed by cervical dislocation and the brains removed. The striata and frontal cortex were dissected and homogenized in 20 volumes of 2 mM Tris-maleate buffer (pH 7.35) containing 2 mM EGTA with 10 strokes of a manual Teflon-glass pestle.

III-2.1.3 Adenylate Cyclase Assay:

Adenylate cyclase assays were performed as previously described (Kazmi & Mishra, 1987; Morency et al., 1988b). Briefly, adenylate cyclase activity was measured by conversion of α-[^{32}P]ATP into [^{32}P]cAMP. The enzyme activity was determined in 150 μL reaction mixture containing the following: 50 mM Tris-maleate (pH 7.35), 0.5 mM IBMX, 1 mM cAMP, 10 μM GTP, 1 mM unlabelled ATP, 1 μCi α-[^{32}P]ATP, 4 mM $MgSO_4$. 1 mM DTT, 1 mg/mL BSA, 20 mM phosphocreatine, 10 units of creatine phosphokinase, 100 μg/mL bacitracin (or various other peptidase inhibitors in the second experiment) and CCK_8 (0−100 μM). The reaction was initiated by the addition of membranes (−100 μg protein) and the assay was incubated for 7 min at 30°C. The reaction was terminated by the addition of 150 μL TCA (13%). Recovery of formed [^{32}P]-cAMP was monitored with the addition of 10,000 cpm of [^{3}H]cAMP in 300 μL of water. The separation of [^{32}P]cAMP from α-[^{32}P]ATP was accomplished by sequential elution over Dowex and alumina columns (White & Karr, 1978). Recovery was consistently greater than 75%.

III-2.1.4 Statistical Analyses:

Statistical analysis between concentration-response curves was determined

by analysis of variance. In the other experiment, Student's t-test was used to compare the effects of various peptidase inhibitors.

III-2.2 RESULTS

In the first experiment, the effects of CCK_8 (0−100 μM) on basal and DA-stimulated adenylate cyclase activity were investigated. Two concentrations of DA were used; 30 and 100 μM which resulted in approximately 60% and 70% increases in cAMP production over basal activity, respectively (see Figure 22). As summarized in Table XIV, CCK_8 (0−100 μM) did not significantly modulate basal nor DA-stimulated adenylate cyclase activity in the striatum and frontal cortex ($p>0.05$).

The negative results observed in the first experiment could have resulted from premature degradation of CCK_8. Thus, various peptidase inhibitors were added in another series of adenylate cyclase assays. As summarized in Table XV, soybean trypsin inhibitor, benzamidine and/or PMSF were all ineffective in inducing a modulatory effect of CCK_8 on DA-stimulated adenylate cyclase ($p>0.05$).

Furthermore, since the incubation was only 7 min, it is possible that CCK_8 did not have sufficient time for interaction with its receptor; approximately 100 min is required to reach equilibrium (see Chapter II). However, preincubation of the tissue in the presence of 1 μM CCK_8, 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitor, and 0.1 mM PMSF for 100 min prior to the assay did not alter the lack of effect of CCK_8 on DA-stimulated adenylate cyclase activity (Table XV).

Table XIV

Effects of CCK_8 on Basal and DA-Stimulated Adenylate Cyclase Activity[a]

$[CCK_8]$ (M)	Adenylate Cyclase Activity (% of control)		
	Basal	30 μM DA	100 μM DA
STRIATUM:			
0 (control)	100 ± 5	100 ± 8	100 ± 6
1×10^{-8}	102 ± 5	110 ± 7	93 ± 5
1×10^{-7}	97 ± 6	95 ± 8	103 ± 13
5×10^{-7}	94 ± 9	100 ± 7	96 ± 6
1×10^{-6}	102 ± 7	96 ± 6	101 ± 8
1×10^{-5}	100 ± 5	106 ± 10	99 ± 7
1×10^{-4}	96 ± 6	88 ± 11	103 ± 9
FRONTAL CORTEX:			
0 (control)	100 ± 8	100 ± 7	100 ± 6
1×10^{-8}	102 ± 6	96 ± 6	97 ± 8
1×10^{-6}	106 ± 8	98 ± 5	105 ± 9

[a] Data represents the mean (±SEM) of three determinations, each done in triplicates.

Table XV

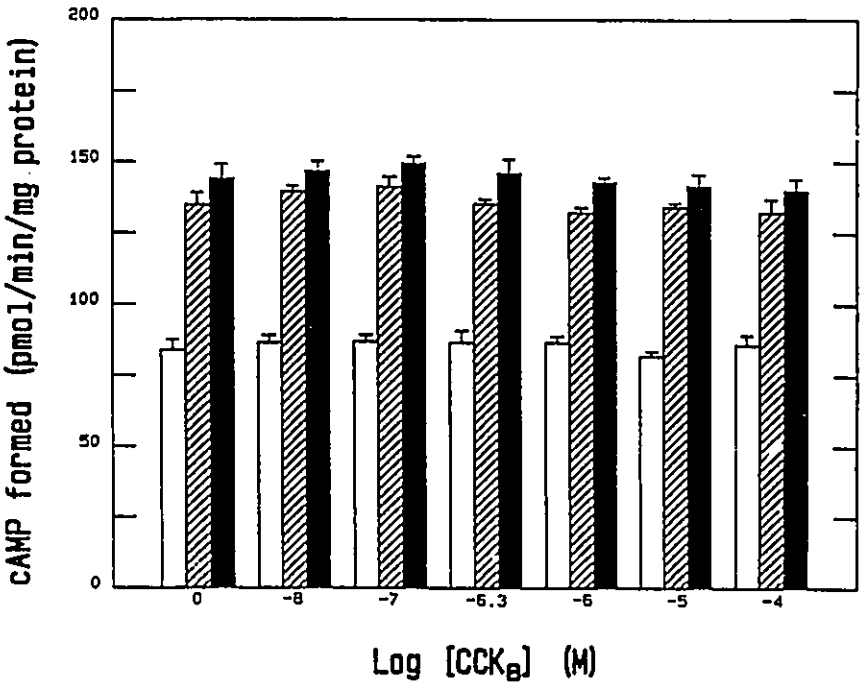
Effects of Various Peptidase Inhibitors and Preincubation on the Potential Modulatory Effects of CCK_8 on DA-Stimulated Adenylate Cyclase Activity[a]

Conditons	Adenylate Cyclase Activity[b] (% of control)[c]
Bacitracin (100 μg/mL)	100 ± 7
Soybean Trypsin Inhibitor (1 mg/mL)	93 ± 11
Benzamidine (0.1 mM)	107 ± 5
PMSF (0.1 mM)	98 ± 6
Benzamidine (0.1 mM) and PMSF (0.1 mM)	109 ± 9
PREINCUBATION: Striatal tissue was preincubated 100 min in the presence of 1 μM CCK_8, 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitor, and 0.1 mM PMSF	105 ± 9

[a] Data represents the mean (±SEM) of three determinations, each done in triplicates.
[b] $[CCK_8]$ = 1 μM and [DA] = 100 μM were used.
[c] Control = 100 μg/mL bacitracin (as was used in previous experiments).

Figure 22 Effects of CCK_8 on basal and DA-stimulated adenylate cyclase activity in the rat striatum.

Effects of CCK_8 on basal (open bars) and DA-stimulated (30 μM, striped bars; 100 μM, solid bars) adenylate cyclase activity. Data are mean (±SEM) of triplicate determinations from a representative experiment.


cAMP formed (pmol/min/mg protein)
200
150
100
50
0
0
-8
-7
-6.3
-6
-5
-4
Log [CCK$_B$] (M)

III-2.3 DISCUSSION

As stated above, Studler et al. (1986) reported opposite effects of CCK_8 on DA-sensitive adenylate cyclase in two areas of the rat nucleus accumbens; the submaximal stimulatory effect of DA (10 and 30 μM) on adenylate cyclase was potentiated in the caudal nucleus accumbens, an area innervated by mixed CCK_8/DA fibres, while the opposite was observed in the rostral area where CCK_8 is not co-localized with DA. In the present study, CCK_8 did not modulate the submaximal (30 μM) or maximal (100 μM) stimulatory effects of DA on adenylate cyclase activity in the rat striatum and frontal cortex, two other areas where both CCK and DA are present but CCK is not contained in DA fibres. Moreover, addition of various peptidase inhibitors and longer incubation of striatal tissue in the presence of CCK_8 did not alter these negative results, suggesting that the lack of modulatory effects was not due to enzymatic degradation of CCK_8 or inadequate length of incubation.

In addition, CCK_8 did not alter the basal level of adenylate cyclase activity in the present study. These data do not appear to be in agreement with the previously mentioned reports of increases in cAMP concentration in striatal synaptosomes (Chowdhury et al., 1987) and of CCK-induced enhancement of cAMP efflux form striatal slices (Long et al., 1986). However, in the Chowdhury et al. (1987) study, CCK_3, CCK_5, CCK_6, and CCK_8 were all shown to stimulate striatal DA synthesis but, curiously, only CCK_5 was reported to increase cAMP concentration in synaptosomes. Moreover, although Long et al. (1986) reported a CCK_8-induced enhancement of cAMP efflux in rat striatal slices, no consistent activation of adenylate cyclase were observed by these investigators in striatal

homogenates. In contrast, O'Shaughnessy & Bhoola (1986) reported that CCK_8 had no significant effect on cAMP levels in striatal slices. Thus, under the assay conditions employed in the present study, it would appear that CCK_8 has no significant influence on basal or DA-stimulated adenylate cyclase in the rat striatum and frontal cortex.

III-3 EFFECTS OF CCK ON DOPAMINE D_2 RECEPTOR BINDING

A considerable amount of interest in CCK/DA receptor-receptor interactions existed at the outset of this study. A summary of this literature, presented in Table XVI, reveals that the effects of various concentrations of several CCKergic drugs have been tested in vivo and in vitro for modulation of the binding of various tritiated DA agents. The numerous procedural discrepancies between studies make comparisons very difficult. For example, whereas ICV administration of CCK_8 has been reported to produce a 15% decrease in striatal B_{max} for [^{3}H]spiroperidol binding (Mashal et al., 1983), IP injections of CCK_8 or caerulein produced a 15% increase in striatal B_{max} for the same ligand (Dumbrille-Ross & Seeman, 1984). The last observation is interesting since animal studies have shown that CCK is quickly degraded in the bloodstream, and that little or no systemically administered CCK or caerulein is likely to reach the brain (Charpentier et al., 1988). When CCK_8 is added to the incubation in vitro, Agnati, Fuxe, and colleagues reported that this peptide significantly decreased the K_d and B_{max} of [^{3}H]spiroperidol in the striatum (Agnati et al., 1983b; Fuxe et al., 1981b; 1983). Conversely, Dumbrille-Ross & Seeman (1984) and Murphy (1985) reported that CCK_8 did not significantly alter [^{3}H]spiroperidol binding in the striatum. Interestingly, however, Murphy (1985) reported that the same concentration of CCK_{8U} decreased the K_d and B_{max} to such an extent that a meaningful saturation isotherm could not be obtained. Thus, in view of the inconsistencies found in this literature, the purpose of the present study was to investigate and further characterize the effects of CCK on in vitro binding of DA D_2 ligands in the rat striatum.

III-3.1 METHODS

III-3.1.1 Materials:

The materials were as previously described in Chapter II. In addition, [^{3}H]spiroperidol (23 Ci/mmol) and [^{3}H]NPA (57 Ci/mmol) were purchased from NEN/Dupont (Boston, MA, USA), ketanserine was a gift from Janssen Pharmaceutica, and (+)-butaclamol was obtained from Research Biochemicals Inc. (Wayland, MA, USA).

III-3.1.2 Membrane Preparation:

Membranes were prepared fresh prior to each experiment. Male Sprague-Dawley rats (200−250 g) were sacrificed by cervical dislocation, the brains removed, and the striata dissected. The tissue was homogenized using a Brinkmann Polytron (setting 6/10, 10 sec) in 50 vol of ice-cold Tris-HCl buffer (pH 7.4) and centrifuged at 50,000 g for 10 min. The pellet was resuspended in cold buffer and recentrifuged. The final pellet was resuspended in the indicated assay buffer (10 mg wet wt/mL). Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard.

III-3.1.3 Ligand Binding:

As in previous studies, receptor affinities (K_d) and densities (B_{max}) were evaluated using the tritiated D_2 receptor antagonist [^{3}H]spiroperidol and the tritiated D_2 receptor agonist [^{3}H]NPA as radioligands. The binding of these radioligands to the membrane preparation was assayed in triplicate in 1.0 mL of the indicated assay buffer containing either [^{3}H]spiroperidol or [^{3}H]NPA and ~200 µg of membrane protein to start the reaction. Non-specific binding was determined in parallel assays in the presence of 1 µM (+)-butaclamol.

Table XVI

Interaction of CCK with Dopaminergic Function: Acute Pharmacological Studies.

Dopamine ligand (tissue)	Species (route)	K_d	B_{max}	Effects	Study
[^{3}H]dopamine (striatal homogenates)	Rat (in vitro)	↓	↓	CCK_8 (10^{-6}M): 75%↓ K_d and 55%↓ B_{max}. CCK_{8U} and CER: 4-fold less active.	Murphy & Schuster (1982)
[^{3}H]NPA (striatal homogenates)	Rat (in vitro)	–	↓	CCK_8 (10^{-6}M): Unaffected K_d but ↓ B_{max}.	Fuxe et al. (1981b)
	Rat (in vitro)	–	–	CCK_8 (10^{-8}M): Unaffected K_d and B_{max}.	Fuxe et al. (1983)
	Rat (in vitro)	↑	↑	CCK_8 (10^{-8}M): 20%↑ K_d and 8%↑ B_{max}. CCK_4 (10^{-8}M): 20%↑ K_d and unaffected B_{max}.	Agnati et al. (1983a)
[^{3}H]spiroperidol (striatal homogenates)	Rat (in vitro)	↓	↓	CCK_8 (10^{-8}M): 35%↓ K_d and 6%↓ B_{max}. CCK_8 (10^{-10}M): no effect. CCK_{8U} (10^{-8}M): As effective as CCK_8.	Fuxe et al. (1981a)
	Rat (in vitro)	↓	↓	CCK_8 (10^{-8}M): 17%↓ K_d and 14%↓ B_{max}. CCK_4 (10^{-8}M): No significant effect.	Agnati et al. (1983b) Fuxe et al. (1983)
	Rat (in vitro)	–	–	CCK_8 (10^{-6}M): No significant effects.	Dumbrille-Ross & Seeman (1984)

	Rat (in vitro)	–	–	CCK_8 (2.5×10^{-7}M): No significant effects. CCK_{8U} (2.5×10^{-7}M): K_d and B_{max} were both so decreased that a meaningful saturation isotherm could not be obtained. PROG (up to 10^{-4}M) and benzotript (up to 3×10^{-8}M): No significant effects.	Murphy (1985)
	Rat (SC)	–	–	PROG (10-40 mg/kg): No significant effects 1 day after injection.	Bean et al. (1985)
	Old rats (in vitro)	–	↑	CCK_8 (10^{-8}M): Unaffected K_d and 8%↑ B_{max}.	Agnati et al. (1984)
	Rat (ICV)	–	↓	CCK_8 (100 ng): Unaffected K_d and 15%↓ B_{max} 1 hr after injection.	Mashal et al. (1983)
	Rat (IP)	–	↑	CCK_8 or CER (50 μg/kg): Unaffected K_d and 15%↑ B_{max} 3 hr after injection, maintained up to 14 days later.	Dumbrille-Ross & Seeman (1984)
[^{3}H]NPA (subcortical limbic - mainly OT/NAcc homogenates)	Rat (in vitro)	↑	↑	CCK_8 (10^{-8}M): 40%↑ K_d and 10%↑ B_{max}.	Agnati & Fuxe (1983)
[^{3}H]spiroperidol (NAcc homogenates)	Rat (in vitro)	–	↑	CCK_8 (10^{-6}M): Unaffected K_d and 20%↑ B_{max}.	Dumbrille-Ross & Seeman (1984)
	Rat (IP)	–	↑	CCK_8 or CER (50 μg/kg): Unaffected K_d and 20%↑ B_{max} 3 hr after injection, maintained up to 14 days later.	Dumbrille-Ross & Seeman (1984)

Abbreviations: CER = caerulein; NAcc = Nucleus accumbens; OT = Olfactory tubercle; PROG = proglumide.

Ketanserine (50 nM) was included in the assays for Scatchard analysis of [^{3}H]spiroperidol binding to block binding at serotonergic sites. Although not used by prior investigators, the addition of a serotonergic antagonist to the [^{3}H]spiroperidol assay is critical for investigations of the modulation of binding at the D_2 receptor since this ligand binds to both serotonin and DA receptors. Indeed, the modulation of [^{3}H]spiroperidol binding by CCK peptides in the rat cerebral cortex has been linked to serotonin receptors (Agnati et al., 1983b; Fuxe et al., 1983).

Following incubation the contents were rapidly filtered through Whatman GF/B filters using a Brandel Cell Harvester (M-24RI). Filters were rapidly washed four times with 5 mL ice-cold Tris-EDTA buffer (50 mM Tris-HCl buffer containing 1 mM EDTA at pH 7.4) and soaked overnight in a scintillation cocktail (Ready Safe, Beckman Canada). Radioactivity bound to the filters was determined in a Beckman liquid scintillation spectrometer (Model 5800).

Since the majority (~70%) of the reports in the literature are from the laboratory of Agnati, Fuxe, and colleagues (see Table XVI), their assay conditions were employed in an initial series of experiments (see Fuxe et al., 1983). Briefly, for [^{3}H]NPA binding, 15 mM Tris-HCl containing 0.01% ascorbic acid was used at a pH of 7.6. The modulation of [^{3}H]NPA binding by CCK peptides was evaluated by preincubation of the specified CCK peptide or vehicle for 10 min on ice before the addition of [^{3}H]NPA (0.05–3.0 nM). The samples were incubated at room temperature for 30 min before being rapidly filtered through Whatman GF/B filters. For [^{3}H]spiroperidol binding, the assays were done in the presence of 10 μM bacitracin and 0.05% BSA. Again, the striatal membrane preparations

were preincubated with the specified CCK peptide or vehicle for 10 min on ice before the addition of [^{3}H]spiroperidol (0.03–2.0 nM). The samples were incubated at 37°C for 10 min before being rapidly filtration.

In a second series of experiments, the assay was modified in order to incorporate some of the optimal conditions previously determined (see Section II-1). First, 130 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 100 µg/mL bacitracin, 5 µg/mL soybean trypsin inhibitors, and 0.1 mM PMSF was included in the Tris (50 mM) buffer and BSA was omitted. The incubation for [^{3}H]spiroperidol and [^{3}H]NPA binding was 60 min at 25°C as described by Srivastava et al. (1988) instead of 10 min at 37°C and 30 min at room temperature, respectively (see above). Finally, the preincubation was extended to 60 min at 25°C. In addition to CCK peptides, the effects of the subtype-specific CCK antagonists, L-364,718 and L-365,260, were also investigated using this modified assay.

III-3.1.4 Data Analysis:

Scatchard analysis was performed on an IBM-PC using BDATA (EMF software, Knoxville, TN, USA). Since equilibrium binding studies were always done in the absence (control) or presence of CCK peptides in parallel assays using the same membrane preparation, the binding parameters estimated by Scatchard analysis were compared by paired Student's t-tests.

III-3.2 RESULTS

As summarized in Table XVII, when using the assay conditions of Agnati, Fuxe, et al., CCK peptides (10^{-8} M) did not significantly alter [^{3}H]NPA and [^{3}H]spiroperidol binding in striatal homogenates. Higher concentrations (up to

10^{-6} M) were equally ineffective (data not shown). Although these CCK peptides did occasionally produce changes in binding parameters of similar magnitude to these previously reported by Agnati, Fuxe, et al. (see Table XVI), these fluctuations were not consistent and did not reach statistical significance. The inclusion of some of the conditions required for optimal binding of CCK peptides to CCK receptors did not change this negative outcome (see Table XVII). Finally, the subtype-specific CCK antagonists, L-364,718 and L-365,260, also failed to modulate [^{3}H]NPA and [^{3}H]spiroperidol binding in striatal homogenates (see Table XVII).

III-3.3 DISCUSSION

As was previously observed by Dumbrille-Ross & Seeman (1984) and Murphy (1985), CCK_8 did not significantly modulate in vitro [^{3}H]spiroperidol binding in the rat striatum. In contrast to reports of Fuxe et al. (1981a) and Murphy (1985), CCK_{8U} was ineffective in altering [^{3}H]spiroperidol binding in the present study; CCK_4 was also without effect (Table XVI). Similarly, these peptides failed to significantly modulate [^{3}H]NPA binding (Table XVI). Although Agnati, Fuxe, et al. published three papers on the modulation of CCK_8 on [^{3}H]NPA binding, the effects reported were not consistent. While 10^{-6} M CCK_8 decreased B_{max} without affecting K_d (Fuxe et al., 1981b), 10^{-8} M CCK_8 significantly increased both the K_d and B_{max} (Agnati et al., 1983a). In a third report by these same investigators, 10^{-8} M CCK_8 failed to produce significant changes (Fuxe et al., 1983).

Table XVII

Effects of CCK Peptides and Antagonists on [^{3}H]NPA and [^{3}H]Spiroperidol Binding in the Rat Striatum[a].

ASSAY CONDITIONS	[^{3}H]NPA			[^{3}H]spiroperidol		
	N	K_d (nM)	B_{max} (fmol/mg protein)	N	K_d (nM)	B_{max} (fmol/mg protein)
REPLICATED ASSAY[b]						
CONTROL	8	0.35 ± 0.03	62.5 ± 5	10	0.37 ± 0.03	375 ± 37
CCK_8	8	0.36 ± 0.03	56.3 ± 6	10	0.35 ± 0.03	366 ± 39
CCK_{8U}	4	0.32 ± 0.04	65.2 ± 10	5	0.39 ± 0.04	359 ± 46
CCK_4	4	0.38 ± 0.04	57.7 ± 12	5	0.40 ± 0.04	384 ± 44
MODIFIED ASSAY[c]						
CONTROL	5	0.23 ± 0.02	143 ± 9	5	0.34 ± 0.02	442 ± 42
CCK_8	5	0.23 ± 0.03	135 ± 12	5	0.35 ± 0.03	485 ± 34
L-365,260	5	0.24 ± 0.03	148 ± 11	5	0.32 ± 0.02	400 ± 49
L-364,718	5	0.25 ± 0.02	135 ± 14	5	0.36 ± 0.03	435 ± 35

[a] The concentration of CCK peptides added to assays presented in this table was 10^{-8} M.

[b] The assay conditions of Agnati, Fuxe, and colleagues (see Fuxe et al., 1983) were employed in this series of experiments. Briefly, for [^{3}H]NPA binding, 15 mM Tris-HCl containing 0.01% ascorbic acid was used at a pH of 7.6. The modulation of [^{3}H]NPA binding by CCK peptides was evaluated by preincubation of the specified CCK peptide or vehicle for 10 min on ice before the addition of [^{3}H]NPA (0.05-3.0 nM). The samples were incubated at room temperature for 30 min before being rapidly filtered through Watman GF/B filters. For [^{3}H]spiroperidol binding, the assays were done in the presence of 10 μM bacitracin and 0.05% BSA. Again, the striatal membrane preparations were preincubated with the specified CCK peptide or vehicle for 10 min on ice before the addition of [^{3}H]spiroperidol (0.03-2.0 nM). The samples were incubated at 37°C for 10 min before rapid filtration.

[c] In a second series of experiments, the assay was modified in order to incorporate some of the previously determined optimal conditions. First, 130 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitors, and 0.1 mM PMSF was included in the Tris (50 mM) buffer and BSA was omitted. The incubations for [^{3}H]spiroperidol and [^{3}H]NPA binding were 60 min at 25°C. Finally, the preincubation was extended to 60 min at 25°C.

It should be noted that in the present study, CCK peptides did occasionally produce changes in binding parameters of similar magnitude reported by these investigators. However, these fluctuations were not consistent and did not reach statistical significance. Since relatively little information on the interaction of CCK with its CNS receptors was available at the time of these early studies, it was reasoned that these inconsistencies might arise from the less than optimal conditions in the binding assay (e.g., lack of protease inhibitors, short incubations at higher or lower than optimal temperatures). Thus, the assay was modified in a second series of experiments incorporating some of the recently determined optimal conditions (see Section II-1). Although the results were more consistent, CCK peptides failed to significantly modulate [^{3}H]NPA and [^{3}H]spiroperidol binding.

The effects of subtype-specific CCK antagonists on [^{3}H]NPA and [^{3}H]spiroperidol binding were also assessed in the present study. As had been previously reported with the weak CCK antagonists proglumide and benzotript (Bean et al., 1985; Murphy, 1985), the potent subtype-specific antagonists, L-364,718 and L-364,718, did not significantly alter binding at the striatal DA D_2 receptor (see Table XVII).

It is interesting to note that, although a considerable amount of attention was given to the acute effects of CCK on DA binding in the early 1980s, the last of such report was published in 1985 (see Table XVI). Indeed, the most prolific group, Agnati, Fuxe, et al., published their last report in 1984. One would have thought that these investigators would have taken advantage of the novel, more potent and specific CCK analogues and antagonists to characterize their reported modulatory effects of CCK on DA binding.

CHAPTER IV

CCK/DOPAMINE INTERACTIONS: CHRONIC STUDIES

As mentioned in Chapter I, the majority of clinical studies evaluating the putative antipsychotic properties of CCK cited evidence obtained in animals studies as the basis for their investigations. However, the vast majority of these studies were of an acute nature (for review, see Nair et al., 1986). CCK peptides, when administered acutely in schizophrenic patients, have failed to show significant antipsychotic effects in properly controlled studies (see Section I-5.3). Properly controlled clinical trial using longer treatment periods are still awaited. Moreover, if CCK peptides are to be eventually used as antischizophrenic drugs, it would be important to determine the effects of longer administrations on central DA function. Thus, the present study sought to investigate effects of long-term CCK_8 administration on DA D_2 receptor binding and the expression of DA D_2 receptor mRNA.

Finally, as was noted in Section I-5.3, all but three clinical studies were performed on neuroleptic-treated schizophrenics. Since chronic neuroleptic treatment remains the therapeutic strategy in schizophrenia, it would be worthwhile to examine the effects of long-term blockade of DA D_2 receptors on CCK function. Thus, the effects of long-term administration of haloperidol on CCK binding was also examined.

IV-1 EFFECTS OF CHRONIC CCK_8 ADMINISTRATIONS ON DOPAMINE D_2 RECEPTOR BINDING AND ON THE EXPRESSION OF DOPAMINE D_2 RECEPTOR mRNA

There has been surprisingly few studies investigating the effects of long-term administrations of CCK peptides or antagonists on DA receptor binding. Dumbrille-Ross & Seeman (1984) reported a significant increase in [^{3}H]spiroperidol binding in the nucleus accumbens of rats receiving a continuous infusion of CCK_8 in the lateral ventricle for 14 days. A small, non-significant increase was also observed in the striatum. The increased binding was due to an increased receptor density (B_{max}); the affinity (K_d) was unaltered. Interestingly, chronic administrations of the CCK receptor antagonist proglumide have also been reported to significantly increase [^{3}H]spiroperidol binding ($\uparrow B_{max}$) in the nucleus accumbens (Csernansky et al., 1987) and in the striatum (Bean et al., 1985) of rats.

The above-described studies were all performed on tissue homogenates. Thus, quantitative autoradiography was used in the present study to investigate the effects of chronic administration of CCK_8 on [^{3}H]spiroperidol binding. Although it would have been very interesting to study the effects of subtype-specific CCK antagonists, the quantities of L-364,718 and L-365,260 available to us were not sufficient for such in vivo studies.

The cDNA and gene for rat and human DA D_2 receptor have recently been cloned and sequenced (Bunzow et al., 1988; Grandy et al., 1989; Selbie et al., 1989). In both species, two isoforms of the receptor, resulting from alternative RNA splicing, have been detected (Chio et al., 1990; Dal Toso et al., 1989; Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989; Selbie et al., 1989). With

appropriate probes, it is now possible to follow the changes in D_2 receptor mRNA levels in various brain regions. Given the previously reported modulation of D_2 receptor density by chronic administrations of CCK_8 and proglumide, it would be interesting to use in situ hybridization to test the hypothesis that chronic CCK_8 administrations may alter the level of expression of the D_2 receptor gene in discrete brain regions.

IV-1.1 METHODS

IV-1.1.1 Materials:

The materials were essentially as described in previous chapters except that [α-^{32}P]ATP (6000 Ci/mmol) was obtained from NEN/Dupont (Boston, MA, USA) and [^{3}H]spiroperidol (>100 Ci/mmol) was obtained from Amersham (Arlington Hgts., IL, USA). Alzet osmotic minipumps were purchased from Alza Corp. (Palo Alto, USA). T_4 polynucleotide kinase was purchased from Pharmacia (Baie d'Urfe, Qué), proteinase K from Boehringer Mannheim Canada (Laval, Qué), and NACS PREPAC column from BRL (Burlington, Ont). All other chemicals required for in situ hybridization were of molecular biology grade and obtained from BRL or Sigma Chemicals Co. (St Louis, MO, USA).

IV-1.1.2 Surgery:

The surgery was done essentially as described in Section III-1.1.3 except that the cannula was attached to an Alzet minipump (Model 2002). Briefly, rats were anaesthetized with sodium pentobarbital (60 mg/kg, IP) and secured in a Kopf stereotaxic instrument. A stainless steel guide cannula (20 gauge) was attached to a length of polyethylene tubing (PE 60 size) and flushed with the

content of the pump. The cannula was then implanted into one of the lateral ventricles (coordinates: 1.0 mm posterior to bregma, 1.7 mm lateral to the midline, and 2.5 mm ventral to the dura mater; König & Klippel, 1963) and anchored to the skull with stainless steel screws and acrylic cement. Cannulae were implanted into the left lateral ventricle of half the animals and into the right lateral ventricle of the other half. The pump, which contained either saline vehicle or CCK_8 (1 or 25 µg/day), was attached to the other end of the polyethylene tubing and was placed subcutaneously on the back of the rat. Animals were sacrificed following 14 days of continuous infusion. The brains were removed, prepared, and sectioned as described in section II-4.1.2 except that sections were not dried overnight at 4°C in a desiccator after sectioning (in order to prevent mRNA degradation for in situ hybridization study). Sagittal and coronal brain sections (20 µm) were cut, thaw-mounted onto gelatin-coated slides, and immediately stored at −80°C.

IV-1.1.3 Quantitative Autoradiography:

The day prior to the experiment, the appropriate sections were selected and dried overnight at 4°C in a desiccator under vacuum. [^{3}H]spiroperidol binding was done essentially as described by Boyson et al. (1986). Sections were incubated for 80 min at room temperature in 50 mM Tris buffer containing 1 mM EDTA, 154 mM NaCl, and 10 mg/L BSA (pH 7.4) in the presence of 1 nM [^{3}H]spiroperidol. Non-specific binding was determined on adjacent sections in the presence of 1 µM (+)-butaclamol. Following the incubation, the sections were transferred through four 20 min washes in ice-cold buffer and dipped 10 sec in ice-cold distilled water to remove excess salts. Sections were then rapidly

dried under a stream of cold air and stored for 24 hours in a desiccator under vacuum prior to apposition to Hyperfilm-^{3}H together with radioactive [^{3}H]micro-scales standards. Exposures were approximately 7−10 days.

IV-1.1.4 In Situ Hybridization:

The probe used for in situ hybridizations was a 39-mer oligonucleotide complimentary to nucleotides 28−72 of the coding region of rat D_2 receptor cDNA, and recognizing both isoforms of the receptor. The probe was labelled fresh prior to each hybridization by Dr. Lalit K. Srivastava as previously described (Srivastava et al., 1990b; 1992). Briefly, the oligonucleotide was 5'-end labelled with [γ-^{32}P]ATP and T_4 polynucleotide kinase to a specific activity of approximately 1−2x10^{8} cpm/μg and purified through a NACS PREPAC column.

On the day of use, brain sections were fixed in 1% glutaraldehyde in PBS (60 min on ice), rinsed 2 x 30 min in ice-cold PBS and digested with proteinase K (1 μg/mL) for 30 min at 37°C. After a brief rinse in water, the sections were acetylated with 0.25% acetic anhydride in 0.1 triethanolamine/0.15 M NaCl (pH 8.0) for 10 min at room temperature. The sections were then washed 2 x 5 min in 2 x SSC, dehydrated and delipidated in an ethanol/chloroform series. The air-dried sections were then hybridized by placing 100 μL of hybridization mixture containing the labelled oligonucleotide probes (1 x 10^{6} cpm), 5 x SSC, 50% formamide, 2 x Denhardt's solution, 10% dextran sulphate, 100 μg/mL salmon sperm DNA and 50 μg/mL yeast tRNA. The sections were cover-slipped, sealed with rubber-cement, and incubated at 42°C for 15−18 hours in a humidified box. The sections were washed 2 x 30 min in 2 x SSC at room temperature, 4 x 15 min in 0.1 x SSC at 58°C, and finally, 1 x 15 min in 0.1 x SSC at room tempera-

ture. After a brief rinse in ethanol containing 0.3 M ammonium acetate (pH 7), the sections were air-dried and apposed to βmax film (Amersham). The autoradiograms were analyzed on an MCID image analyzer.

IV-1.1.5 Statistical Analysis:

For both the binding data and in situ hybridization data, a one-way, between-subject ANOVA was performed for each brain area. In instances of a statistically significant ANOVA, Dunnett tests were used for post hoc comparisons between the control (i.e., vehicle) and the experimental (i.e., 1 or 25 μg CCK_8) conditions (Keppel, 1982).

IV-1.2 RESULTS

As summarized in Table XVIII, chronic administrations of CCK_8 failed to significantly alter binding at the D_2 receptors or the levels of D_2 receptor mRNA. All ANOVAs on the quantitative autoradiography and in situ hybridization data failed to reach the statistical significant level of $p<0.05$. Thus post hoc analyses were not performed. Examples of quantitative autoradiography and in situ hybridization data are presented in Figures 23 and 24, respectively.

[^{125}I]BH-CCK_8 binding was also assessed in brain sections from all chronically CCK_8-treated rats (data not shown). Although a decrease in CCK receptor density was observed in most brain areas, it only reached statistical significance in the hippocampus (vehicle = 9.11 ± 0.40, CCK_8 (1 μg/day) = 8.77 ± 0.37, and CCK_8 (25 μg/day) = 4.56 ± 0.31; F (2,27) = 47.32, $p<0.05$). Dunnett tests revealed that only the rats receiving the highest dose of CCK_8 had a significantly lower CCK receptor density in the hippocampus than rats receiving

the vehicle.

IV-1.3 DISCUSSION

The studies above were designed to test the hypothesis that long-term CCK_8 administration influences central DAergic function by altering the density of functional D_2 receptors and/or by regulating the expression of the gene for D_2 receptors. The results from quantitative autoradiography and in situ hybridization suggest that 14 days of continuous ICV infusion of CCK_8 did not significantly alter the D_2 receptor densities nor the levels of D_2 receptor mRNA in any of the brain areas tested.

Several technical limitations could account for these negative data. First, only two doses of CCK_8 were studied, 1 and 25 µg per day ICV. These doses were selected to correspond to doses previously reported to induced modulatory and behavioural effects when injected ICV in rats (e.g., Altar & Boyar, 1989; Katsuura & Itoh, 1985; 1986; Widerlöv et al., 1983b). However, these doses were much higher than the 2 ng/hour doses used by Dumbrille-Ross & Seeman (1984) and, as such, could be the source of discrepancy.

A second limitation to be considered is that only one time point was studied (14 days). For example, Dumbrille-Ross & Seeman (1984) observed a much greater effect after 24 hours of continuous ICV infusion than after 14 days. Indeed, the striatal D_2 receptor density was significantly increased after 24 hours but not after 14 days of CCK_8 administration. Thus, it is possible that a transient alteration in D_2 receptor density or mRNA levels occurred in the first two weeks in our study but was not detected.

Figure 23 Comparative [^{3}H]spiroperidol binding in control rats and long-term CCK_8-treated rats.

[^{3}H]spiroperidol binding was done essentially as described by Boyson et al. (1986). Sections were incubated for 80 min at room temperature in 50 mM Tris buffer containing 1 mM EDTA, 154 mM NaCl, and 10 mg/L BSA (pH 7.4) in the presence of 1 nM [^{3}H]spiroperidol. Non-specific binding was determined on adjacent sections in the presence of 1 μM (+)-butaclamol. Following the incubation, the sections were washed, dried, and apposed to Hyperfilm-^{3}H with radioactive [^{3}H]micro-scale standards. Exposure was 7−10 days.

Autoradiograms were analyzed using an MCID image analyzer. The brain images reproduced in the figure are in pseudocolour. The image is actually digitized as a range of grey levels. To create colours, the MCID system assigns each level of grey a specific combination of red, green, and blue colour values.

For ease of comparison between treatment groups, one hemisphere of total binding section was combined with the opposite hemisphere of a non-specific binding section. Sections from three different levels are presented: Top = A 9410 μ; middle = A 8920 μ; and bottom = A 2580 μ (König & Klippel, 1963). Microdensitometric determinations of [^{125}I]BH-CCK_8 receptor densities are listed in Table XVIII.

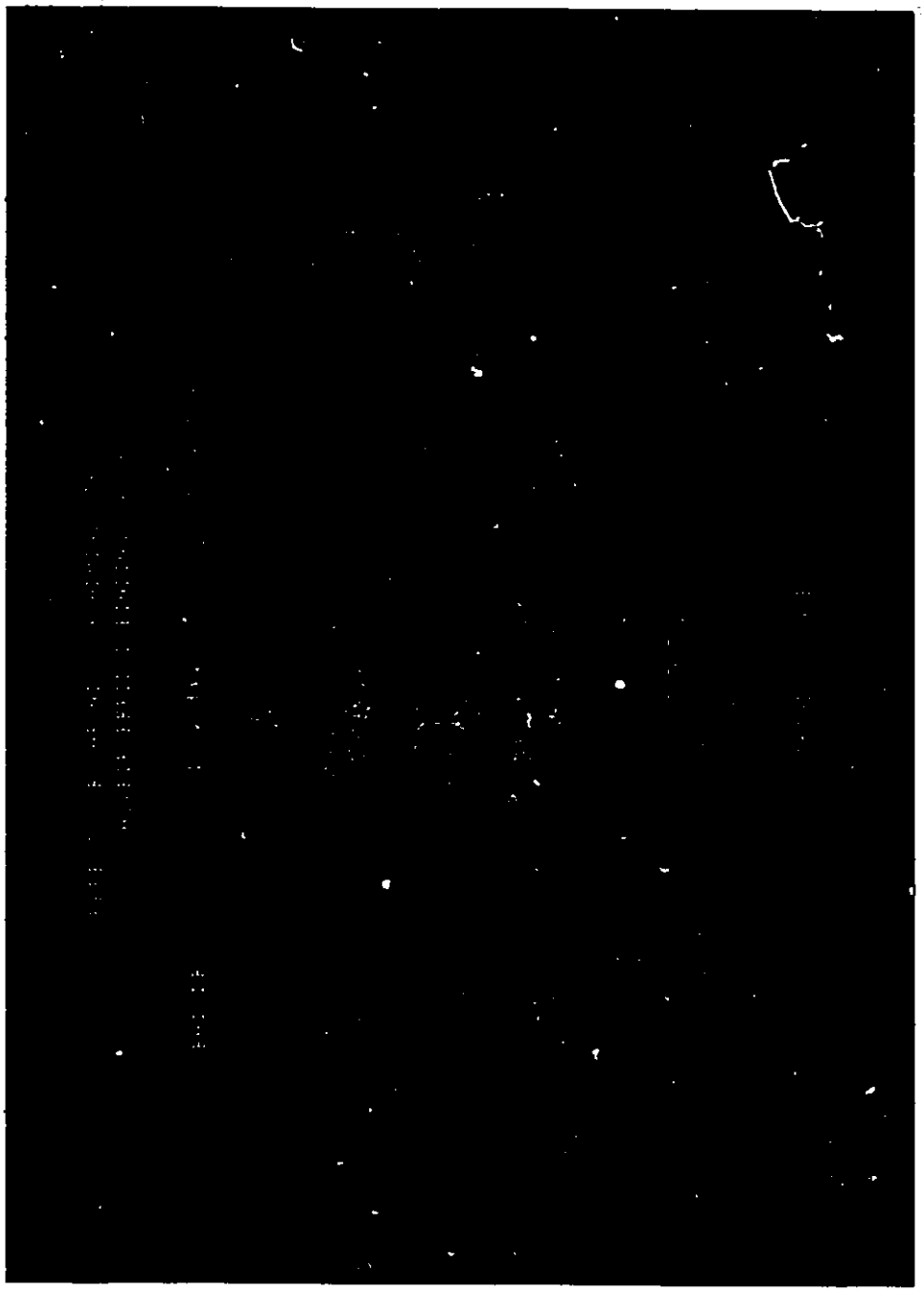


Table XVIII

Effects of Chronic CCK_8 Administrations on [^{3}H]spiroperidol Binding and on the Expression of Dopamine D2 Receptor mRNA in the Rat Brain

	Quantitative autoradiography - [^{3}H]spiroperidol binding[a]			In situ hybridization - Dopamine D_2 receptor mRNA[b]		
BRAIN AREA	VEHICLE[c]	CCK_8 (1 µg/day)	CCK_8 (25 µg/day)	VEHICLE[c]	CCK_8 (1 µg/day)	CCK_8 (25 µg/day)
Caudate-putamen	255 ± 19	249 ± 17	262 ± 22	0.224 ± 0.004	0.231 ± 0.004	0.229 ± 0.003
Nucleus accumbens	133 ± 14	139 ± 14	152 ± 17	0.180 ± 0.003	0.178 ± 0.004	0.173 ± 0.003
Olfactory tubercle	85 ± 10	68 ± 9	77 ± 11	0.174 ± 0.003	0.171 ± 0.003	0.174 ± 0.004
Substantia nigra	33 ± 7	37 ± 7	21 ± 6	0.129 ± 0.003	0.133 ± 0.002	0.135 ± 0.003

a Results are the mean ± SEM expressed in fmol/mg. Two estimates of binding were obtained from each animal (1 per hemisphere). N=5 for all groups.

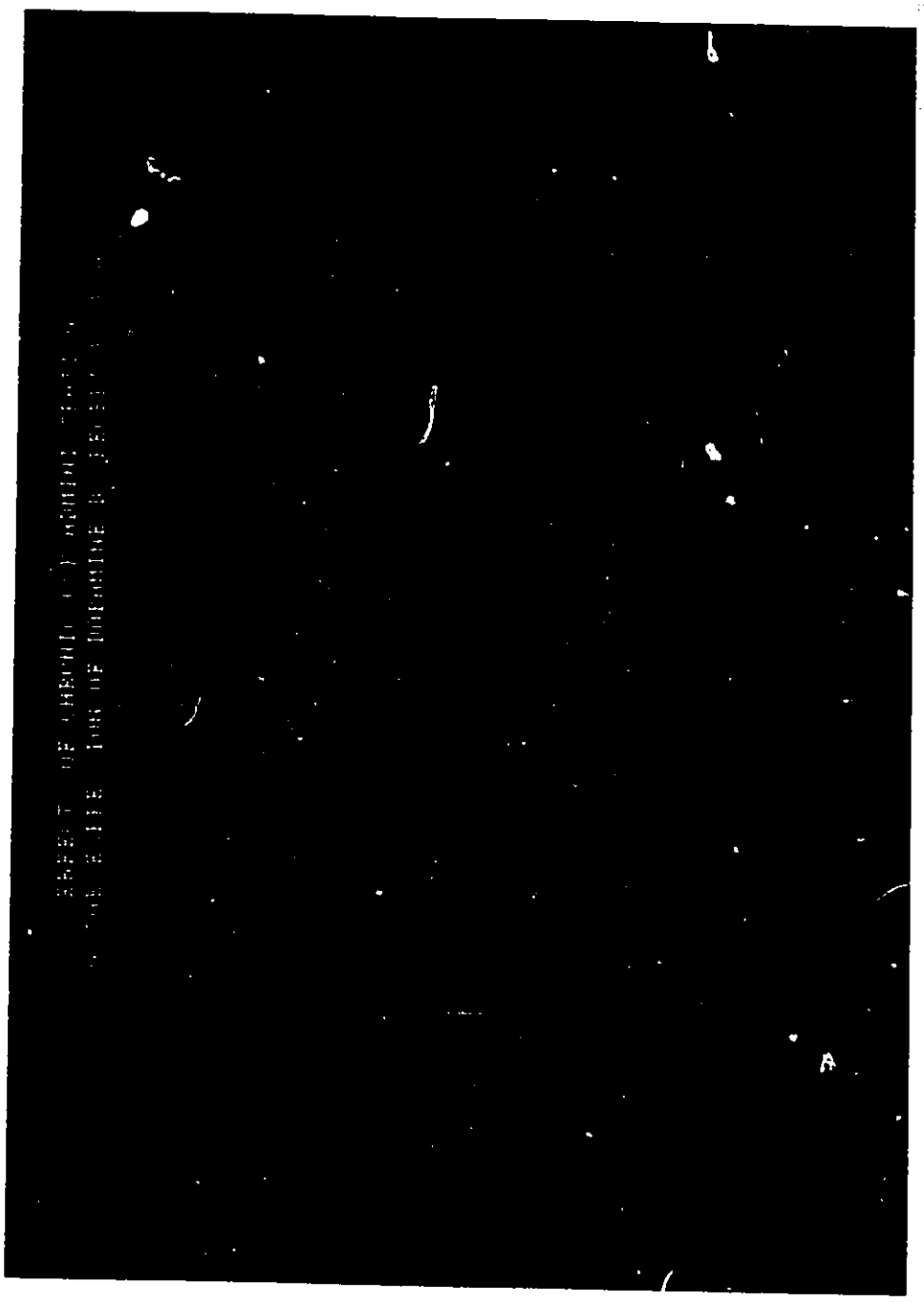
b Results are the mean ± SEM expressed in relative optical densities (ROD). The MCID system operates with 0-256 gray levels and ROD = $\log_{10}$ (level/256)$^{-1}$.

c A one-way, between-subject ANOVA was performed for each brain areas on the binding data and in situ hybridization data. All ANOVAs failed to reach the statistical significant level of $p<0.05$. Thus post hoc analyses were not performed.

Figure 24 Comparison of the expression of dopamine D_2 receptor mRNA in long-term CCK_8-treated rats.

For in situ hybridization, the sections were fixed in 1% glutaraldehyde in PBS (60 min on ice), rinsed 2 x 30 min in ice-cold PBS and digested with proteinase K (1 µg/mL) for 30 min at 37°C. After a brief rinse in water, the sections were acetylated with 0.25% acetic anhydride in 0.1 triethanolamine/0.15 M NaCl (pH 8.0) for 10 min at room temperature. The sections were then washed 2 x 5 min in 2 x SSC, dehydrated and delipidated in an ethanol/chloroform series. The air-dried sections were then hybridized by placing 100 µL of hybridization mixture containing the labelled oligonucleotide probes (1 x 10^6 cpm), 5 x SSC, 50% formamide, 2 x Denhardt's solution, 10% dextran sulphate, 100 µg/mL salmon sperm DNA and 50 µg/mL yeast tRNA. The sections were cover-slipped, sealed with rubber-cement, and incubated at 42°C for 15–18 hours in a humidified box. The sections were washed 2 x 30 min in 2 x SSC at room temperature, 4 x 15 min in 0.1 x SSC at 58°C, and finally, 1 x 15 min in 0.1 x SSC at room temperature. After a brief rinse in ethanol containing 0.3 M ammonium acetate (pH 7.0), the sections were air-dried and apposed to βmax film (Amersham).

Autoradiograms were analyzed using an MCID image analyzer. Again, the brain images reproduced in the figure are in pseudocolour. The image is actually digitized as a range of grey levels. To create colours, the MCID system assigns each level of grey a specific combination of red, green, and blue colour values. In these autoradiograms, pink represents background labelling whereas hybridization of the oligonucleotide probe to DA D_2 receptor mRNA is in blue. Sections from three different levels are presented: Top sections = A 9410 µ; middle sections = A 8920 µ; and bottom sections = A 2580 µ (König & Klippel, 1963). Microdensitometric determinations of [^{125}I]BH-CCK_8 receptor densities are listed in Table XVIII.



As an aside, I thought it was curious that both CCK_8 (Dumbrille-Ross & Seeman, 1984) and proglumide, a CCK antagonist, (Bean et al., 1985; Csernansky et al., 1987) were reported to induce an increase in D_2 receptor density in the striatum and nucleus accumbens. As mentioned above, Dumbrille-Ross & Seeman (1984) reported the CCK_8-induced increase in density after 24 hours and 14 days of continuous ICV infusion. In contrast, Mashal et al. (1983) reported a significant reduction in the density of striatal D_2 receptors when estimated one hour following an ICV injection of 100 ng CCK_8. Similarly, Murphy & Schuster (1982) reported a significant decrease in the density of striatal D_2 receptors following four daily systemic injections of CCK_8 (20 μg/kg). The reasons for such discrepancies remain to be determined.

Another limitation to be considered is the possible lack of sensitivity of the technique employed in the present study. Thus, it could be argued that CCK_8 failed to alter D_2 receptor density and D_2 receptor mRNA levels in our study because our techniques were not sensitive enough to detect the changes present. However, in my opinion, this was not the reason for the negative data obtained in the present study. First, the autoradiographic estimations of the density of D_2 receptors and distribution profiles obtained in the present study were quantitatively and qualitatively similar to those obtained by other investigators with [^{3}H]spiroperidol (e.g., Altar et al., 1985; Palacios et al., 1981) or other D_2 ligands (e.g., Boyson et al., 1986; Charuchinda et al., 1987). Moreover, altered densities of D_2 receptors induced by chronic haloperidol administrations (see next section) and the neuropeptide PLG (unpublished observations) have been detected using our assay.

Similarly, although our in situ hybridization technique did not reveal any altered levels of D_2 receptor mRNA following chronic administrations of CCK_8 (Table XVIII and Fig. 24), it was sufficiently sensitive to detect a differential developmental profile of D_2 mRNA for major DAergic brain regions in rats aged one day to one year old (Srivastava et al., 1992). Furthermore, the striatal profile obtained by in situ hybridization in our previous studies have been confirmed by Northern and solution hybridization, as well as by polymerase chain reaction (PCR) analysis (Srivastava et al., 1990b; 1992).

In conclusion, results from the present study suggest that continuous ICV infusions of CCK_8 (1 and 25 μg/day) for 14 days does not alter the density of D_2 receptors nor the levels of D_2 receptor mRNA in any of the brain regions studied. Although differences in doses administered precludes a direct comparison with their study, we were unable to reproduce the CCK_8-induced increase in D_2 receptor density reported by Dumbrille-Ross & Seeman (1984) using higher doses of the peptide. It should be noted, however, that two other groups of investigators reported a decrease in D_2 receptor density following acute or short-term administrations of CCK_8 (Mashal et al., 1983; Murphy & Schuster, 1982), evidence that contradicts some of the observations made by Dumbrille-Ross & Seeman (1984). Finally, it has also been reported by two other groups of investigators that chronic administrations of proglumide, a CCK antagonist, produces an increase in D_2 receptor density (Bean et al., 1985; Csernansky et al., 1987). It is difficult to understand why both the agonist and antagonist would produce the same modulatory effect. There is clearly a need for further research on the effects of long-term administrations of CCK on central DA function.

IV-2 EFFECTS OF CHRONIC HALOPERIDOL ADMINISTRATIONS ON CCK RECEPTOR BINDING

As with studies investigating the effects of chronic CCK administrations, there have been relatively few studies investigating the effects of long-term administrations of DA agonists or antagonists on central CCK function.

Chronic administration (2-3 weeks) of haloperidol has been reported to increase the concentration of CCK in the substantia nigra and ventral tegmental area (Radke et al., 1989). However, chronic haloperidol did not significantly alter the levels of CCK mRNA in the substantia nigra and ventral tegmental area (Cottingham et al., 1990). Again, conflicting results are evident in the literature. CCK concentration in the major DA projection areas, the striatum and nucleus accumbens, have been shown to be decreased (Fukamauchi et al., 1987), unchanged (Gysling & Beinfeld, 1984), or increased (Frey, 1983; Radke et al., 1989) by chronic haloperidol or sulpiride administration.

In addition, chronic administration of haloperidol has also been reported to increase the density of CCK binding sites in tissue homogenates of the frontal cortex and mesolimbic area (Chang et al., 1983; Morency & Mishra, 1987). Although this effect was recently replicated using brain sections (Debonnel et al., 1990; Fukamauchi et al., 1987), these investigators did not present any autoradiograms and receptor densities were only given for a few brain areas (Debonnel et al., 1990; Fukamauchi et al., 1987). Thus, quantitative autoradiography was used in the present study to provide a more detailed anatomical study of the effects of chronic haloperidol administration on CCK binding.

IV-2.1 METHODS

IV-2.1.1 Materials:

The materials were as described above and in Chapter II. Haloperidol was a gift from McNeil Canada.

IV-2.1.2 Surgery:

Rats were anaesthetized with sodium pentobarbital (60 mg/kg, IP) and an Alzet osmotic minipump (Model 2ML2) was implanted subcutaneously. Animals were sacrificed following 14 days of continuous infusion. The brains were removed, prepared, and sectioned as described in section VI-1.1.2.

IV-2.1.3 Quantitative Autoradiography:

The day prior to the experiment, the appropriate sections were selected and dried overnight at 4°C in a desiccator under vacuum. Labelling of CCK receptors with [^{125}I]BH-CCK$_8$ and of D_2 receptors with [^{3}H]spiroperidol was done as described Sections II-4.1.3 and VI-1.1.3, respectively.

IV-2.2 RESULTS

The effects of chronic haloperidol on [^{125}I]BH-CCK$_8$ binding in the rat brain are presented in Figure 25. Microdensitometric determinations and statistical analyses for several brain areas are listed in Table XIX. Continuous SC infusions of haloperidol administrations significantly increased [^{125}I]BH-CCK$_8$ binding in the frontal cortex, nucleus accumbens, olfactory tubercle, and caudate putamen. CCK receptor densities were not significantly affected in the hippocampus, amygdala, parietal, temporal, and occipital cortices. Similar results were obtained in animals receiving daily IP injections of haloperidol (data not shown).

Table XIX

Comparative [^{125}I]BH-CCK$_8$ Binding in Control and Haloperidol-treated Rats[a]

BRAIN AREA	Control	Haloperidol[b]	t value[c] (df=18)	Statistical Significance
Frontal cortex	20.2 ± 1.4	32.5 ± 2.5	4.34	$p<0.0005$
Parietal cortex	21.7 ± 1.3	24.0 ± 1.2	1.29	n.s., $p>0.05$
Caudate-putamen	22.0 ± 1.1	31.2 ± 1.8	4.41	$p<0.0005$
Nucleus accumbens	28.0 ± 1.4	49.3 ± 3.5	5.72	$p<0.00005$
Olfactory tubercle	18.4 ± 1.0	25.4 ± 1.6	3.81	$p<0.005$
Hippocampus	13.7 ± 1.2	11.3 ± 1.0	1.18	n.s., $p>0.05$

[a] Binding data were derived from quantitative autoradiography and are expressed in fmol/mg. Two estimates of binding were obtained from each animal (1 per hemisphere). N=5 for both groups.

[b] Animals were administered a continuous IP infusion of haloperidol (~1 mg/kg/day) for 14 days with an Alzet osmotic minipump (Model 2ML2). Control rats received an equivalent volume of vehicle (0.5% acetic acid).

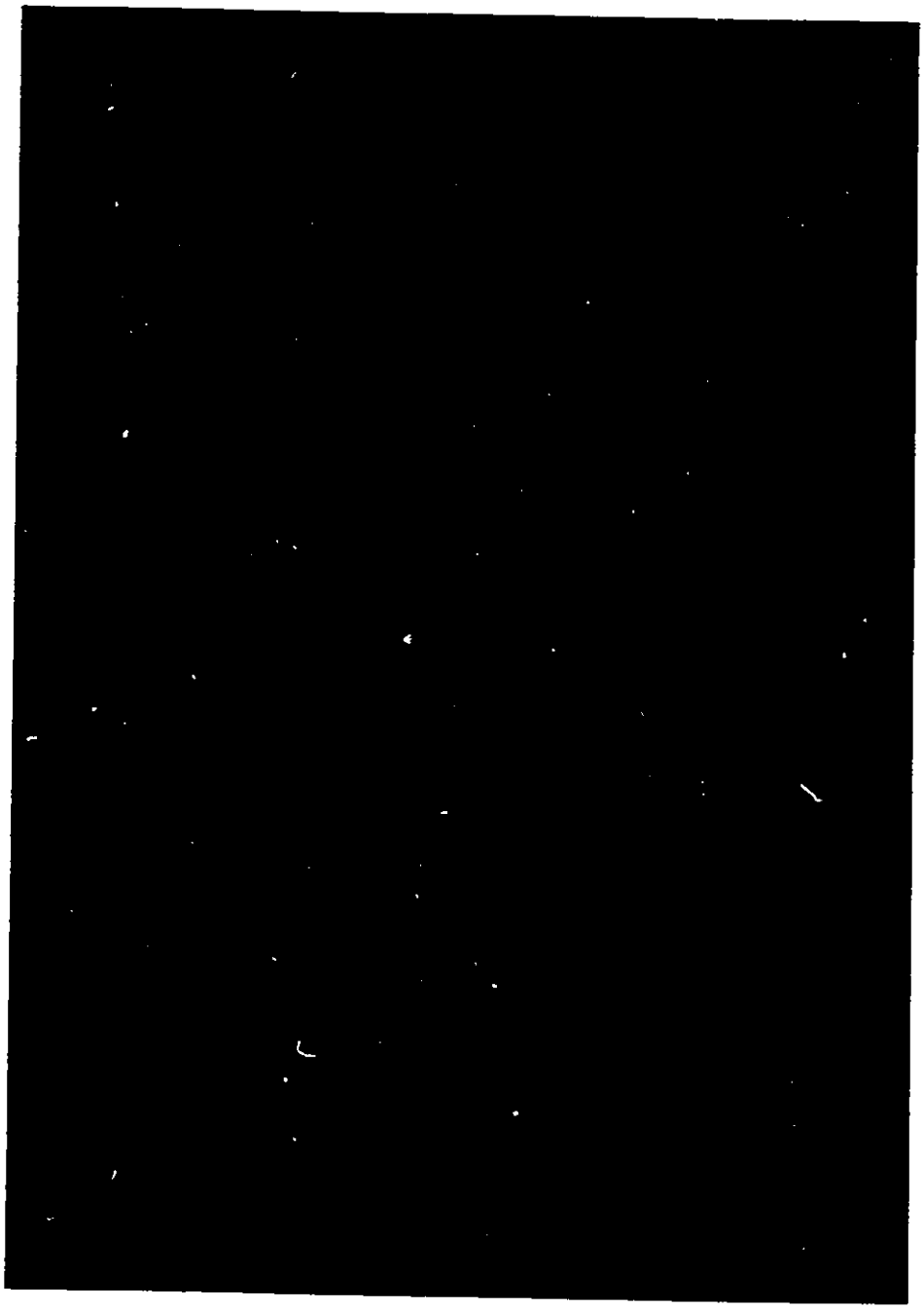
[c] Student's t-test. (n.s. = non-significant)

Figure 25 Comparative [^{125}I]BH-CCK$_8$ binding in control rats and long-term haloperidol-treated rats.

Quantitative autoradiography was done as described in Section II-4.1.3. Sections were incubated with 100 pM [^{125}I]BH-CCK$_8$ for 120 min at room temperature. Non-specific labelling was determined in adjacent sections with the addition of 1 μM CCK$_8$. Following the incubation, the sections were washed, dried, and apposed to Hyperfilm-^{3}H for 3–5 days with [^{125}I]micro-scales standards.

Autoradiograms were analyzed using an MCID image analyzer. The brain images reproduced in the figure are in pseudocolour. The image is actually digitized as a range of grey levels. To create colours, the MCID system assigns each level of grey a specific combination of red, green, and blue colour values.

For ease of comparison between treatment groups, one hemisphere of total binding section was combined with the opposite hemisphere of a non-specific binding section. Coronal sections from three different levels are presented: Top = A 9410 μ; middle = A 8920 μ; and bottom = A 2580 μ (König & Klippel, 1963). Microdensitometric determinations of [^{125}I]BH-CCK$_8$ receptor densities are listed in Table XIX.



Several research groups have reported that chronic administration of a variety of neuroleptic drugs to laboratory animals leads to an increase in the density of DA D_2 receptors (e.g., Boyson et al., 1988; Burt et al., 1977; Chiu et al., 1981; Hess et al., 1988; Mackenzie & Zigmond, 1985). As expected, chronic haloperidol administrations produced DA receptor supersensitivity in the present study. [^{3}H]spiroperidol binding was increased by 30-40% in the striatum and nucleus accumbens of haloperidol-treated rats (data not shown).

IV-2.3 DISCUSSION

In the present study, chronic haloperidol administrations significantly increased CCK receptor densities in the nucleus accumbens, olfactory tubercle, and frontal cortex, confirming previous reports by Chang et al. (1983), Debonnel et al. (1990), Fukamauchi et al., 1987, and Morency & Mishra (1987). Interestingly, Debonnel et al. (1990) also reported that chronic haloperidol induced a marked increase in the responsiveness of accumbens neurons to CCK_8 but not DA. They concluded that DA may still be released in amounts sufficient to maintain normal responsiveness, whereas the release of CCK is possibly decreased to a greater extent, resulting in an enhanced responsiveness of neurons to this peptide.

Contrary to these previous reports, however, a significant increase in [^{125}I]BH-CCK_8 binding was also observed in the caudate-putamen in the present study. This result was observed following both chronic continuous infusion and chronic daily injections of haloperidol. One possible reason for this discrepancy is the use of [^{125}I]BH-CCK_8 in the present study as opposed to [^{125}I]BH-CCK_{33} in

some of the previous studies. The former is preferred because CCK_8 possesses a higher affinity for CCK binding sites than does CCK_{33} and is less susceptible to proteolytic enzymatic cleavage. Since the ^{125}I is in the N-terminal region of the CCK_{33} radioligand and not within the biologically critical C-terminal octapeptide, proteolytic cleavage generates unlabelled, biologically active fragments (see Morency & Mishra, 1987). The resulting higher non-specific binding may have masked any effects in the caudate-putamen in these previous studies. Other factors which may have contributed to this discrepancy are the limited anatomical resolution of binding assays on tissue homogenates (Chang et al., 1983; Morency & Mishra, 1987) or of tissue scraped from brain sections (Fukamauchi et al., 1987). Finally, Debonnel et al. (1990) used much lower doses of haloperidol; 4 mg/kg, IM once a week instead of 1 mg/kg, IP daily in the present study.

CCK receptor densities in the hippocampus, amygdala, parietal, temporal, and occipital cortices were not altered by chronic administrations of haloperidol.

CHAPTER V

CONCLUSIONS

The growing evidence implicating abnormal DA function in patients with schizophrenia in conjunction with the demonstrated CCK/DA co-existence and interactions in animal studies provided a theoretical basis for investigating the involvement and potential therapeutic value of CCK as an antipsychotic. Animal studies providing physiological, pharmacological, biochemical, and behavioural evidence that CCK down-regulated DA function were cited in the numerous clinical trials conducted during the early 1980s. However, little was known about CCK in the brain of higher mammalian species. For example, a review of the literature on CCK receptors completed in 1986 revealed that brain CCK receptors had only been characterized in rodents and non-mammalian species (Morency & Mishra, 1987) and significant species differences in regional distribution were already apparent. Thus, the first objective of the present study was to characterize CCK receptors in the brains of higher mammalian species and to provide a detailed comparison of the regional distribution of CCK receptors in various species.

Binding to bovine cortical membranes was found to be of high affinity, specific, reversible, and time- and temperature-dependent. Saturation isotherms in equilibrium binding studies revealed that the binding was saturable at

10-20 nM. Scatchard transforms of these binding isotherms were linear, indicating a single population of CCK receptor sites. Estimates of the dissociation constant revealed the presence of high affinity CCK receptors (≈1 nM). Comparable estimates were also observed in the bovine caudate, and in the canine and rat cortex in the present study. Similar values had previously been reported in the rodent cortex using a variety of ligands (Table VII and, more recently, Durieux et al., 1988; Gut et al., 1989; Niehoff, 1989; Wennogle et al., 1988). Finally, competitive inhibition studies of [^{3}H]pCCK$_8$ binding to bovine cortical CCK receptors with various CCK agonists and antagonists revealed a pharmacological profile consistent with CCK-B receptors previously characterized in the mammalian brain.

As reviewed in Chapter I, there has been relatively little biochemical characterization of brain CCK-B receptor when compared to pancreatic CCK-A receptor. Indeed the latter has recently been purified to homogeneity (Duong et al., 1989; Szecowka et al., 1989). Future biochemical characterization of solubilized and native membrane-bound brain CCK-B receptor should include the glycoprotein nature of the receptor, estimated molecular size (Stokes Radius), characterization of G-protein interactions, and determination of which second messenger system is linked to brain CCK-B receptor.

Although the pharmacological characteristics of CCK receptors appear to have been well preserved in the mammalian brain, marked differences were observed in the distribution of CCK receptors. In the present study, the distribution profile of CCK receptors was determined by quantitative autoradiography in the bovine, canine, rat, and chicken brain and compared to previously

reported distributions in other species. There were basically two main types of species-specific differences. The first one was the absence or presence of CCK receptors in a given structure. The most notable example was the presence of CCK binding sites in the cerebellum of mouse, guinea pig, feline, canine, bovine, monkey, and human contrasting with the absence in the rat and avian cerebellum. Other examples were the paucity of CCK binding sites in the mouse and hamster basal ganglia, unlike the high densities found in every other mammalian species tested, and the exclusive presence of CCK binding sites in the rat thalamic reticular nuclei. A second type was species-specific differences in CCK receptor distributions within a given structure. Examples of such differences were described in the cerebellum, neocortex, and hippocampus. The reasons for such species-specific differences in receptor distribution are not clear at the present time. However, this evidence does caution against simple extrapolation of data obtained in animal models directly to clinical applications.

As mentioned above, animals studies providing evidence that CCK down-regulates DA function were cited in the numerous clinical trials investigating the potential therapeutic value of CCK as an antipsychotic. However, there was ample conflicting evidence in this literature (see Chapter I). Whereas some reports suggested an inhibitory effect of CCK on DA function, which would be compatible with a potential antischizophrenic action, others reported a lack of modulation or an enhancement of DA function.

Circling behaviour was used in the present study to investigate the effects of CCK on DA function because it is one of the best understood behaviours in

animals and is commonly used to assess DA function. Interestingly, a circling bias has been observed in unmedicated and never-medicated schizophrenic patients (Bracha, 1987). Unilateral ICV microinjections of CCK_8 induced a dose-dependent contraversive circling bias in rats. This CCK_8-induced contraversive circling bias was attenuated by the DA receptor antagonist haloperidol. These data suggest that, under the acute conditions employed in the present study, CCK_8 may induce contraversive circling by exerting a unilateral facilitatory influence on DA neurotransmission.

In a subsequent report by Worms et al. (1986), unilateral intrastriatal injections of CCK_8 induced contraversive circling in mice. This suggests that, in the present study, CCK_8 injected in the lateral ventricle may have induced the circling behaviour, at least in part, through its action in the striatum. To further elucidate the mechanism(s) underlying this CCK-induced circling behaviour, we investigated the effects of CCK peptides on DA-stimulated adenylate cyclase and on ligand binding at DA receptors in the rat striatum. Under the assay conditions employed in this study, it would appear that CCK_8 has no significant acute effects on binding at the DA receptor or on DA-stimulated adenylate cyclase. It is possible that CCK induced its acute facilitatory influence on DA function in the circling behaviour paradigm by altering DA turnover or release.

The vast majority of animal studies characterizing CCK/DA interactions have been of an acute nature. But if CCK is to be eventually used as an anti-schizophrenic drug or as an adjunct to neuroleptics, it would be useful to investigate CCK/DA interactions in chronic studies. Under the conditions used in the present study, DA D_2 receptor binding and the expression of DA D_2 receptor

mRNA were not significantly altered by long-term administration of CCK_8. However, several technical limitations could account for these negative data. Future studies should examine the effects of a wider range of dose and longer periods of administration. Furthermore, in view of the conflicting evidence in literature on CCK/DA interactions, it would also be worthwhile to investigate the effects of chronic administrations of the subtype-specific CCK antagonists L-364,718 and L-365,260 on DA function.

Since chronic neuroleptic treatment remains the therapeutic strategy in schizophrenia and most of the clinical trials evaluating the antipsychotic properties of CCK were carried out on neuroleptic-treated schizophrenics, the effects of long-term administrations of haloperidol, a DA D_2 antagonist, on CCK binding were evaluated in rats. Chronic administrations of this neuroleptic significantly increased CCK receptor densities in the nucleus accumbens, olfactory tubercle, and frontal cortex but not in the hippocampus, amygdala, nor in the parietal, temporal, and occipital cortices.

The results of the present study indicate that care must be taken when extrapolating evidence on CCK function obtained in animals to clinical application. Moreover, further studies on CCK function in the CNS and CCK/DA interactions are required before this peptide can be considered for further clinical trials in schizophrenic patients.

Future studies could aim at identifying the exact nature of CCK/DA interactions. Does CCK alter DA synthesis, release, uptake, metabolism? Does it alter the dynamics of the receptors or their interactions with their second messenger systems? Future studies could also investigate potential interactions of

CCK on DA through genetic control of tyrosine hydroxylase, signal transduction, or the expression of DA receptors, autoreceptors, or uptake sites. Furthermore, 6-OHDA lesions could be used to assess whether such CCK/DA interactions are unicellular or polycellular in nature.

Molecular biology is providing new approaches to probe some of these questions. Recombinant DNA techniques has resulted in the cloning and sequencing of five subtypes: two members of the D_1 receptor subfamily, D_1 and D_5, as well as three members of the D_2 receptor subfamily, D_2, D_3 and D_4, (for review, see Cooper et al., 1991; Sibley & Monsma, 1992). These receptor subtypes present different affinities for dopamine, clozapine, and butyrophenones and may be functionally linked to different second messengers. Interestingly, they are expressed in different brain regions. Whereas the D_1 receptor is enriched in the caudate/putamen, olfactory tubercle, and nucleus accumbens, the D_5 receptor is expressed mainly in the frontal cortex, hippocampus, and hypothalamus. Similarly, unlike the D_2 receptor which is abundant in the caudate/putamen, olfactory tubercle, and nucleus accumbens, the D_3 receptor is expressed predominantly in limbic brain areas including the olfactory tubercle, nucleus accumbens, islands of Calleja, and hypothalamus and the D_4 receptor is found predominantly in the frontal cortex, amygdala, medulla, and midbrain. Finally, the genes for the D_2 receptor subfamily are also unique among the G-protein-linked receptors in that they contain introns within the coding region and can thus generate alternative transcripts via alternative splicing mechanisms (see Cooper et al., 1991; Sibley & Monsma, 1992). If chronic administrations of CCK agonists or antagonists are found to induce an effect on DA receptors, it

would be of interest to determine which DA receptor subtype(s) are affected, the location of the interaction, and whether the ratio of the different D_2, D_3 and D_4 isoforms has been altered. In addition, as mentioned in Chapter I, McVittie et al. (1990) recently presented evidence that they have cloned a CCK/gastrin-responsive G-protein-coupled receptor. Cloned CCK-B receptors could be co-expressed with individual DA receptor subtypes in order to investigate potential CCK/DA receptor interactions as well as interactions at the level of the second messengers.

APPENDIX

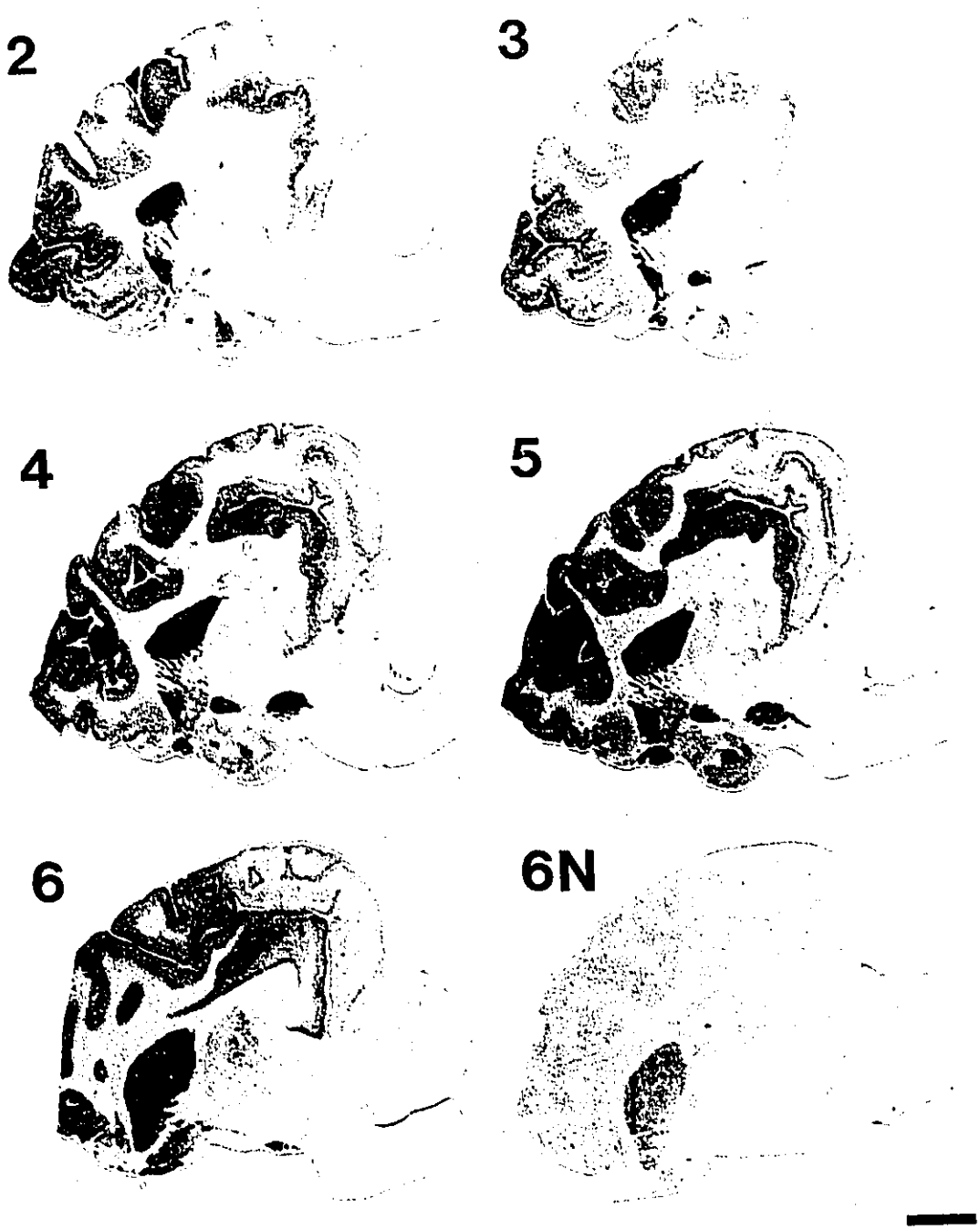
Autoradiographical Localization of Dopamine D_1 and D_2 Receptors in the Canine Brain.

[^{3}H]SCH 23390 and [^{3}H]spiroperidol were used to localize the DA D_1 and D_2 receptors, respectively. Quantitative autoradiography was done essentially as described by Boyson et al. (1986). Sections were adjacent to the ones presented in Fig. 17. They are presented from the most lateral (2) to the most medial (6). Section 6N represents non-specific labelling.

FIRST PANEL - [^{3}H]SCH 23390 binding: Sections were incubated for 80 min at 37°C in 50 mM Tris buffer containing 1 mM EDTA, 154 mM NaCl, and 10 mg/L BSA (pH 7.4) in the presence of 2 nM [^{3}H]SCH 23390. Non-specific binding was determined on adjacent sections in the presence of 1 μM cis-flupentixol. Following the incubation, the sections were washed twice for 10 min in ice-cold assay buffer and dried.

SECOND PANEL - [^{3}H]spiroperidol binding: Sections were incubated for 80 min at room temperature in 50 mM Tris buffer containing 1 mM EDTA, 154 mM NaCl, and 10 mg/L BSA (pH 7.4) in the presence of 1 nM [^{3}H]spiroperidol. Non-specific binding was determined on adjacent sections in the presence of 1 μM (+)-butaclamol. Following the incubation, the sections were washed 4 x 20 min in ice-cold assay buffer and dried.

Sections were then apposed to Hyperfilm-^{3}H with radioactive [^{3}H]micro-scale standards. Exposure was 7–10 days. Autoradiograms were analyzed using an MCID image analyzer. Autoradiographic images were obtained by using the autoradiograms as negatives and producing positive prints on Transtar photographic paper. Thus, dark areas have high densities and light areas have low densities of DA receptors. Bar = 1cm.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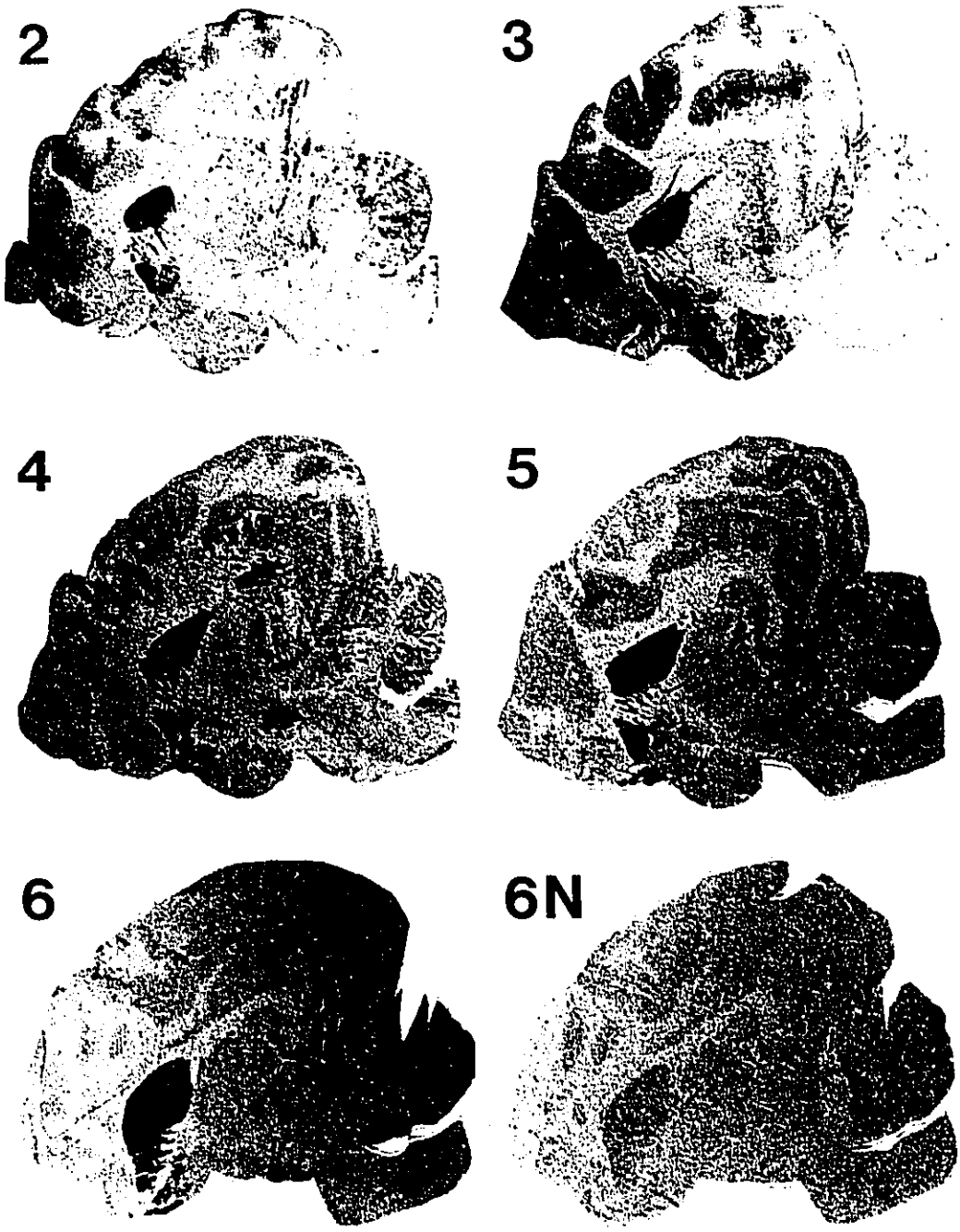
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