THE NEUROPHARMACOLOGY AND BEHAVIOURAL EFFECTS OF CHOLECYSTOKININ

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CONTENTS

THESIS SUMMARY

Studies regarding the influences of cholecystokinin on anxiety, learning and memory, are rife with inconsistency. This thesis attempts to address many of these inconsistencies and to elucidate valid arguments for these.

1. Chlordiazepoxide decreases startle amplitudes without altering spontaneous locomotor activity in the acoustic startle chamber. Cholecystokinin forms, CCK-4 and CCK-8s attenuate the activity of chlordiazepoxide. A combination of CCK-4 and CCK-8s at half their maximally effective doses exerts no effect on startle amplitude. CCK_2 antagonists CI-988 and LY-288513 increase startle amplitudes in an inverse bell-shaped dose response profile, and at higher doses inhibit the effects of CCK-4 and CCK-8s.

2. A similar phenomenon is observed in the elevated plus-maze model of anxiety. Chlordiazepoxide increases open arm exploration indicative of an anxiolytic activity. Cholecystokinin forms and CI-988 inhibit chlordiazepoxide-induced increases in exploration with in an inverse bell shaped dose response profile. Combinations at half their maximally effective doses of CCK-4 and CCK-8us/8s exert no effects on these chlordiazepoxide-induced increases in open arm exploration. CI-988 and LY-288513 both attenuate the activity of cholecystokinin forms on chlordiazepoxide-induced increases.

These phenomena are explained by a hypothesis highlighting a subtle association between sub-populations of the CCK_2 receptor.

The effects of cholecystokinin with chlordiazepoxide and cholecystokinin antagonists alone on plus-maze behaviour of socially isolated animals are similar to that of those group housed. This implies that social isolation increases anxiety-like behaviour but does not alter cholecystokinin pharmacology specifically.

3. Characterisation of cholecystokinin activity within the two-trial plus-maze paradigm has shown that LY-288513 exhibits a similar profile to chlordiazepoxide. Scopolamine is able to prevent development of chlordiazepoxide insensitivity but not that of the CCK_2 antagonist. A similar profile was observed when testing acoustic startle amplitudes post plus-maze exposure. Maze naïve animals responded to chlordiazepoxide with a decrease in startle amplitudes. Exposure to the plus-maze negated this effect. LY-288513 exhibited no activity in altering startle amplitudes.

Cholecystokinin forms, CCK-4, CCK-8us and CCK-8s did not exhibit any activity in the fear potentiated startle paradigm of conditioned fear.

Experiments within this thesis have also attempted to provide a clear explanation for seemingly contradictory data implicating cholecystokinin in either amnestic or promnestic activity. The data and discussion therein has strongly refuted claims of activity for cholecystokinin in learning and memory of associative, non-appetitive tasks. It has been observed here that freezing-like behaviour occurs in cholecystokinin administered animals within the model. This is demonstrated by increases in both passive and active avoidance latencies.

Radioligand binding studies, however, failed to distinguish multiple binding sites / sub-populations of CCK_2 receptors. These have been observed in previous studies. The reasons for this failure are discussed within.

This thesis also highlights the complex nature of cholecystokinin activity regarding animal models of anxiety and fear. The inconsistency of putative anxiety-like activity between models has drawn attention to the multiplicity of procedural factors and subtle differences in neurotransmitter activities underlying minor changes in behaviour within these paradigms.

CHAPTER ONE - THE NEUROPHARMACOLOGY AND BEHAVIOURAL EFFECTS OF CHOLECYSTOKININ - INTRODUCTION

The Discovery and Characterisation of Cholecystokinin

The peptide, cholecystokinin, was first discovered in the gastrointestinal tract in 1928 (Ivy and Oldberg). In 1975, Vanderhaeghen *et al*., observed that the vertebrate brain contained a small peptide which showed immunoreactivity with gastrin antibodies. Subsequent studies revealed that this gastrin-like substance, which for the most part was cholecystokinin, which was able to cross react with gastrin antibodies due to the homology of the C-terminal sequence, Trp-Met-Asp-Phe-NH_2, in both molecular structures (Dockray, 1976; Rehfeld, 1977; Muller *et al*., 1977). The presence of small, discrete gastrin rich areas are also present within the brain, but by comparison are very limited (Rehfeld 1978). In addition, cholecystokinin is also expressed in the peripheral nervous system with particular abundance in the distal regions of the gut (Larsson and Rehfeld 1979). Cholecystokinin is generally held to be one of the most widespread and abundant peptide neurotransmitters in the central nervous system (Noble *et al*., 1999).

Synthesis of Cholecystokinin peptides in Neurones

Cholecystokinin peptides are encoded within a single gene containing three exons. At a transcriptional level it appears that alternate splicing of the cholecystokinin gene does not occur. A probable mechanism, by which production of the numerous molecular forms of cholecystokinin appears to be accomplished, is that of post-translational processing. The immediate gene product transcribed from the cholecystokinin mRNA is a 115 amino acid residue peptide sequence (Deschenes *et al*., 1984; Takahashi *et al*., 1985). This pre-pro-cholecystokinin contains an N-terminal sequence, a splicing region, containing each of the various bioactive forms from CCK-83 to CCK-4; and a C-terminal peptide sequence. Within the rough endoplasmic reticular organisation, the cleavage enzyme, signalase, removes a signal

sequence, and truncates these cholecystokinin forms. This cleavage yields the pro-cholecystokinin sequence, which is transported to the Golgi apparatus, where the enzyme tyrosyl-protein sulphotransferase confers *O*-sulphated tyrosine residues (Tyr-77, -92, and -95). The action of the enzyme, trypsin-like endopeptidase begins within the Golgi apparatus and continues within the small immature vesicle formations with subsequent transportation toward the axonal synapse. This enzyme produces proteolytic cleavage at multiple monobasic sites and a single dibasic site along the length of the pro-cholecystokinin peptide. Activity of these enzymes at these cleavage sites yields several fragments that subsequently undergo terminal processing in the mature synaptic vesicles. The synaptic vesicles contain the necessary precursor and enzymes for amidation of the peptide molecule. The enzymes, carboxypeptidase E-like exopeptidase and peptidylglycine α-amidating monooxygenase remove the glycoxylate group from the glycine extended precursor to the bioactive form. This yields the bioactive α-carboxyamidated peptides. This process has been characterised in several studies in the rat (Goltermann *et al*., 1980a; Goltermann *et al*., 1980b; Stengaard-Pedersen *et al*., 1984), in the pig (Eng *et al*., 1983; Rehfeld and Hansen, 1986), and has been reviewed in the periphery (Schwartz, 1990) and in the central nervous system (Rehfeld and Nielsen, 1995). Interestingly, Rehfeld and Hansen (1986) proposed that the brain contained three or more subpopulations of cholecystokinin neurones, each with distinct post-translational processing pathways.

Molecular forms of Cholecystokinin in the Central Nervous System

Cholecystokinin (CCK) exists within the central nervous system in several different molecular forms, each with specific transmitter activity. Each of the bioactive peptides present the same tetrapeptide amide derived structural sequence (Trp-Met-Asp-Phe-NH_2) at their C-terminus. It is evident that this sequence is central to binding affinity and possibly efficacy at cholecystokinin receptors (Rehfeld and Neilsen 1995).

The porcine cerebral cortex region of the central nervous system consists of several different molecular forms:

The octapeptide form, CCK-8, is the most abundant form with a mean concentration of 429.6 pmoles/g tissue. Of this amount, the predominant isoform (about 99%) is the sulphated-tyrosine species, CCK-8s [Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2] with a much lesser amount (less than 1%) of the nonsulphated-tyrosine form, CCK-8us [Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2]. The CCK-8us form is unlikely to be an artefact of the extraction process as desulphation by non-enzymatic processes only occurs under highly acidic conditions, not found in brain tissue. It appears that within porcine brain tissue there are three different forms of CCK-8s, which exhibit different net charges as demonstrated in their fractionation pattern on an ion-exchange chromatography analysis (Rehfeld and Hansen 1986).

The larger molecular forms, CCK-22 (4.7 pmol/g), CCK-33 and CCK-39 (37.9 pmol/g) and CCK-58 (142.1 pmol/g), also exhibit bioactivity (Rehfeld and Hansen 1986).

The pentapeptide form, CCK-5 [Gly-Trp-Met-Asp-Phe-NH_2], which differs from synthetic pentagastrin [BOC-Ala-Trp-Met-Asp-Phe-NH_2], occurs in smaller quantities, 102.6 pmoles/gram tissue wet weight. The tetrapeptide form, CCK-4 [Trp-Met-Asp-Phe-NH_2] appears to occur at yet even smaller quantities within the cerebral cortices, 13.3 pmoles/gram tissue wet weight.

It is as yet unknown whether CCK-5 and CCK-4 are specifically synthesised as active transmitter molecules or whether they are merely CCK-8s degradative products (Rehfeld 1985). According to Rehfeld (2000) there is little or no evidence of CCK-4 synthesis in the brain, and it is most likely that CCK-4 is an in-vitro degradation product of the closely related CCK-5.

Regional Distribution of Cholecystokinin in the Brain

Neurones containing cholecystokinin either as a co-transmitter or a singular transmitter are numerous and widespread within the brain. With the exception of the cerebellum, which shows very poor expression, cholecystokinin neurones exhibit an almost ubiquitous distribution within the brain (Rehfeld *et al*., 1992; Eng *et al*., 1983). Some regions of particularly high densities are networks present within the cortical areas; the neocortex and the entorhinal cortex. Within these regions, cholecystokinin peptide molecules are located chiefly in the thin terminal neuronal processes throughout all layers, with a slightly higher density in the molecular, rather than the deeper layers. Cortical staining for cholecystokinin neurones is thus broadly represented as a light staining of the entire region with a higher concentration in the entorhinal cortex than in the neocortex. Within the lateral and caudal hippocampus, cholecystokinin neurones are restricted for the most part to a thin band occupying an area beneath the main neuronal cell layer. In the medial and anterior hippocampal regions cholecystokinin neurones innervate pyramidal cell soma in the CA1 region. In addition the medial and anterior regions contain a small percentage of cholecystokinin neurone soma (about 1% of total soma in this region). Cholecystokinin neuronal soma and processes are also found within the dendate gyrus (Rehfeld and Neilsen 1995). The amygdaloidal region is also rich in cholecystokinin neurones with high density networks detected in the medial amygdaloidal nucleus. The caudate nucleus and the putamen contain fairly dense networks of cholecystokinin neurones with greater concentrations at the rostal level. The medial preoptic, the periventricular and the lateral areas of the hypothalamus are also particularly cholecystokinin dense as are regions including the lateral septal nuclei, the midbrain periaqueductal grey and the area postrema. There are several very low-density regions containing little or no cholecystokinin projections or soma. These include the cerebellum, the corpus callosum, the internal capsule, and the commissural organs (Rehfeld and Neilsen 1995).

Cholecystokinin as a candidate for a neurotransmitter

Several criteria necessary for a categorical definition of a neurotransmitter are required. CCK-8s appears to satisfy these criteria as described forthwith (Rehfeld, 1980; Rehfeld, 1985):

Neurotransmitters are localised in neurones

CCK-8 is localised in an extensive network of neurones in the central nervous system (CNS) in both soma and terminal regions (Larsson and Rehfeld 1979).

Neurotransmitters are concentrated in neuronal synapse terminals

Immunocytochemical techniques have revealed that cholecystokinin peptides are located in neuronal synapses. This is confirmed by subcellular fractionation of brain tissue whereby a four-fold increase in concentration of cholecystokinin peptides are observed in synaptosomes and synaptic vesicles as compared to other neuronal regions (Pinget *et al*., 1978; Emson *et al*., 1980).

Neurotransmitters exhibit demonstrable synthesis pathways

Radiolabel chasing techniques using [^{35}S]-methionine via intraventricular pulse administration in rat brains is found to be incorporated into large molecular forms of cholecystokinin (CCK-83 and CCK-58) prior to incorporation into the CCK-33-like fragment and then subsequently into the small peptide sequences, CCK-8 and CCK-5. Unlabelled methionine chasing revealed subsequent radiolabel dilution with the larger forms followed by CCK-8. The CCK-5 molecule appeared to be somewhat independent (Goltermann *et al*., 1980b).

Neurotransmitters are released by depolarisation

A calcium dependent release of a CCK-like material can be induced by potassium chloride superfusion onto brain slices. This implies that calcium dependent release underlies

cholecystokinin release from synaptic terminals which is a typical neurotransmitter release mechanism (Emson *et al*., 1980; Dodd *et al*., 1980).

Upon application, neurotransmitters mimic transmitter effects

Application of CCK-8 in the femtomole range at the post-synaptic membrane strongly excited hippocampal neurones in the rat (Dodd and Kelly, 1981).

Neurotransmitters are inactivated

Evidence of CCK-8 degrading enzymes have been found within brain tissue (Desholt-Lanckmann *et al*., 1981).

Neurotransmitter activity can be abolished by antagonists

A review of the many highly specific antagonists with affinity to the cholecystokinin receptor is detailed later in this chapter.

Cholecystokinin Receptors in the Central Nervous System

Cholecystokinin receptors exist as two major subtypes, CCK_1 and CCK_2. The CCK_1 receptor was first characterized in pancreatic acini (Sankaran *et al*., 1980). The CCK_2 receptor subtype was discovered in the brain that same year (Innis and Snyder, 1980). A third type of cholecystokinin receptor was thought to be the gastrin receptor, mediating gastric acid secretion (Song *et al*., 1993). However, subsequent molecular characterisation has revealed that these putative gastrin receptors share identical homology with CCK_2 receptors (Kopin *et al*., 1992; Wank, 1995). CCK_1 and CCK_2 receptors initially were assigned nomenclature of CCK-A and CCK-B respectively. This nomenclature was recently revised, especially given evidence for the presence of subpopulations of both receptors (Noble *et al*., 1999)

Cholecystokinin Receptor Distribution

CCK-binding sites were first described in the brain in the early nineteen-eighties (Hays *et al*., 1980; Innis and Snyder, 1980; Saito *et al*., 1980). Various studies using autoradiography, in-situ hybridisation and immunocytochemistry have investigated regional distribution using nonselective cholecystokinin specific ligands in numerous species. These studies demonstrated that despite similarities, there are also marked variations in the comparative distribution between species.

CCK_1 receptors are located in the pancreatic acini, gastric mucosa, gallbladder, gastrointestinal tract and specific areas of the central nervous system. Rat CCK_1 receptors are located in the interpeduncular nucleus (Hill *et al*., 1988), area postrema, and medial nucleus tractus solitarius, with additional areas of binding found in the habenular nuclei, dorsomedial nucleus of the hypothalamus, and central amygdala (Moran *et al*., 1986; Hill *et al*., 1987; Moran and McHugh, 1988; Woodruff *et al*., 1991; Carlberg *et al*., 1991; Zajac *et al*., 1996). However, studies in primates demonstrated a much greater prevalence and broader distribution of CCK_1 receptors compared to that in rodents (Hill *et al*., 1988). In the primate, CCK_1 receptor-binding sites are located in the area postrema, nucleus tractus solitarius, hypothalamic dorsomedial nucleus, supraoptic nucleus, paraventricular nucleus, mammillary bodies, supramammillary region, infundibular region, dorsal motor nucleus of the vagus, and the neurohypophysis. Furthermore, the entire mesostriatal dopaminergic system of the primate, contains CCK_1 receptors (Hill *et al*., 1990).

Studies utilising in-situ hybridisation techniques, using mRNA probes, have demonstrated that CCK_1 receptor mRNA is distributed within the identical regions to those containing CCK_1 receptors in the rat (Honda *et al*., 1993). This study however revealed other areas containing CCK_1 receptor mRNA without receptor binding characteristics. Moderate levels were found in the forebrain, olfactory region, piriform cortex, neocortex, claustrum, throughout the

hippocampal formation, medial nucleus of the amygdala, lateral olfactory tract nucleus, lateral septal nucleus, stria terminalis bed nucleus, preoptic nucleus, thalamic reticular nucleus, various hypothalamic regions, the arcuate nucleus, and in lateral and posterior hypothalamic areas. Light staining was also found in the brainstem, dorsal motor nucleus of the vagus nerve and the interpeduncular, caudal linear raphe, and hypoglossal nuclei (Lanaud *et al*., 1989; Hökfelt *et al*., 1991).

CCK_2 receptors are located throughout the central nervous system with moderate to high densities. Certain distinct regions contain only low densities. Interestingly, the absence of cholecystokinin binding sites in the cerebellum is species dependent. Cerebellar cholecystokinin receptors are detected in the guinea pig, human, and mouse, but not in the rat (Sekiguchi and Moroji, 1986; Williams *et al*., 1986; Dietl *et al*., 1987).

Subsequent studies utilising in-situ hybridisation techniques, have demonstrated that CCK_2 receptor mRNA is distributed within regions corresponding to those containing CCK_2 receptors, in the rat (Honda *et al*., 1993; Hansson *et al*., 1998), and in the Mastomys natalensis (Shigeyoshi *et al*., 1994; Jagerschmidt *et al*., 1994).

This wide ranging morphological distribution of cholecystokinin receptors would seem to support the large number of functions attributed to cholecystokinin.

The Cholecystokinin Receptor Gene

Despite some evidence for further cholecystokinin receptor subtypes, only two genes have been cloned (Noble *et al*., 1999). The human CCK_1 receptor gene was first identified on chromosome 4 (Huppi *et al*., 1995). Subsequent analysis mapped the human CCK_1 gene to positions 4p15.1-p15.2 (Inoue *et al*., 1997). The mouse and rat CCK_1 receptor genes have also been mapped on chromosome 5; 5p16.2-p15.1; (Huppi *et al*., 1995) and on chromosome 14 (Takiguchi *et al*., 1997). The mouse gene again appears in a similar position on chromosome 7

(Huppi *et al*., 1995). The human CCK_2 receptor gene was first identified on chromosome 11 (Song *et al*., 1993) and further localised to position 11p15.4 (Huppi *et al*., 1995).

Interestingly the CCK_1 receptor gene is co-localised with the dopamine D5 receptor gene at 4p15.1-p15.3 (Sherrington *et al*., 1993) and the CCK_2 receptor gene with the dopamine D4 receptor gene at 11p15.4-p15.5 (Pisegna *et al*., 1992). This is particularly poignant given the coexistence of cholecystokinin and dopamine in midbrain neurons (Crawley and Corwin, 1994).

The genes encoding the CCK_1 receptor and the CCK_2 receptor in humans are organised in a similar manner consisting of five exons and four introns (Miller *et al*., 1995; Wank, 1995; Inoue *et al*., 1997; Song *et al*., 1993). Each exon encodes distinct regions of the receptor molecule:

1. The first exon sequence encodes the extracellular N-terminal domains.

2. The second exon sequence encodes from the transmembrane region I to the beginning of region II.

3. The third exon sequence encodes from the transmembrane region III to the beginning of region V.

4. The fourth exon sequence encodes from the transmembrane region V to the beginning of the third intracellular loop.

5. The fifth exon sequence encodes the remaining receptor structure.

Interestingly, Jagerschmidt *et al*., (1994) isolated several distinct CCK_2 receptor mRNA forms from rat brain tissue, including a truncated mRNA species. These forms typically exhibit variation at the 5' end. The precursor mRNA and mature form were located in the cerebral cortex, hypothalamus, and hippocampus in apparently differing proportions. This implies

that the expression of the CCK_2 receptor is modulated at a post-transcriptional level. Furthermore, the cerebellum contained only an unspliced mRNA form. This appears to agree with studies observing an absence of CCK_2 receptor-binding sites in the rat cerebellum (Pelaprat *et al*., 1987). This genetic organisation is similarly conserved between humans and animal species. Gene organisation for CCK_1 receptors in the mouse (Lacourse *et al*., 1997) and rat (Takata *et al*., 1995) and for CCK_2 receptors in the mouse (Nagata *et al*., 1996) and rabbit (Blandizzi *et al*., 1994) are organized similarly.

Cholecystokinin Receptors and Signal Transduction

Cloning of the CCK_1 receptor has revealed a seven-transmembrane receptor structure linked to a G-protein second messenger system (Wank *et al*., 1992a). CCK_1 receptor activity has been found to be unaffected by pertussis toxin, implying coupling with the G_q family of G proteins (Pang and Sternweiss, 1990). Piiper *et al*., (1997) subsequently demonstrated that CCK_1 receptors in the pancreas are coupled to either G_q or G_{11}, which activates phospholipase C-1. However, it has also been demonstrated that in rat pancreatic acini, the CCK_1 receptor is coupled to the phospholipase A2 enzyme, thus mediating the arachidonic acid messenger system (Yoshida *et al*., 1997b; Yoshida *et al*., 1997a). Furthermore, CCK_1 receptor activation in the pancreas has also been shown to activate the adenylyl cyclase signal-transduction cascade (Marino *et al*., 1993). Site-directed mutagenesis studies imply that the CCK_1 receptor is directly coupled with both G_s and G_q (Wu *et al*., 1997).

Pharmacological studies and subsequent cloning, have confirmed that CCK_2 receptors also belong to the seven-transmembrane receptor family structure, linked to a G-protein second messenger system (Wank *et al*., 1992b; Knapp *et al*., 1990; Durieux *et al*., 1992). The signal-transduction cascade for the CCK_2 receptor is however, less extensively characterised, due in part to technical considerations of working with isolated neurons and gastric mucosa cells (Noble *et al*., 1999).

Physiological studies using CCK_2 receptor cDNA transfected cells have demonstrated that the CCK_2 receptor is coupled to a pertussis-toxin insensitive G protein (Roche *et al*., 1990), possibly of the G_q/G_{11} family, which activates phospholipase C (Delvalle *et al*., 1992). It has been shown that CCK_2 receptors are also coupled to a phospholipase, inducing release of arachidonic acid, but interconnected through a pertussis-toxin sensitive G protein (Pommier *et al*., 1999) and to a MAP kinase pathway (Taniguchi *et al*., 1994).

Cholecystokinin receptor specific ligands

Non-selective cholecystokinin Receptor Agonists

The sulphated octapeptide CCK-8s binds to the CCK_1 receptor with a 500- to 1000-fold greater affinity than non-sulphated cholecystokinin or sulphated gastrin (Silvente-Poirot *et al*., 1993). The CCK_2 receptor binds pentagastrin, CCK-4, CCK-8us and CCK-8s with similar affinity (Saito *et al*., 1980).

CCK_1 Receptor Specific Ligands: Agonists

Cholecystokinin analogues have been developed with greater specificity for CCK_1 receptors using the following approaches:

1. A-71378 [des-NH_2-Tyr(SO_3H)-Nle-Gly-Trp-Nle-(NMe)Asp-Phe-NH_2], with an (NMe)Asp residue critical for CCK_1 receptor selectivity (Holladay *et al*., 1992).

2. A series derived through replacement of the methionine residue of Boc-CCK-4 with side chain-substituted Lys derivatives: Boc-Trp-Lys(X)-Asp-(NMe)Phe-NH_2, such as A-71623 and A-70874 (Lin *et al*., 1991).

3. A series of 1,5-benzodiazepines based CCK_1 receptor agonists (Aquino *et al*., 1996).

CCK_1 Receptor Specific Ligands: Antagonists

The first cholecystokinin antagonists were derived from a naturally occurring benzodiazepine, asperlicin, isolated from the fungus *Aspergillus alliacaeus* (Chang *et al*., 1985). Several studies support the concept that the natural ligand for the anti-anxiety benzodiazepine receptor is a peptide (Guidotti *et al*., 1983; Alho *et al*., 1985), suggesting that the 5-phenyl-1,4-benzodiazepine ring present in the structure of asperlicin and diazepam is in fact a chemical structure that recognizes a peptide receptor (Evans *et al*., 1986). Asperlicin is a nonselective antagonist for cholecystokinin receptors.

A range of highly potent and selective antagonists for the CCK_1 receptor has since been developed. These are summarised as follows:

1. An asperlicin derivative, L-364718 (otherwise known as MK-329 or devazepide) is a potent cholecystokinin antagonist developed with a high selectivity for CCK_1 receptors (IC_{50} $CCK_2/CCK_1 = 3750$); (Chang *et al*., 1985)

2. Glutamic acid derivatives, loxiglumide (CR-1505) or lorglumide (CR-1409), (Makovec *et al*., 1985).

3. Cholecystokinin C-terminal fragments, 2-naphthalenesulfonyl-1-aspartyl-(2-phenethyl) amide (2-NAP), a competitive antagonist at CCK_1 receptors (Hull *et al*., 1993).

4. Dipeptoids, such as PD-140548, which is a competitive antagonist with a high selectivity for the CCK_1 receptor (100:1) (Boden *et al*., 1993).

5. Synthetic peptides, such as JMV-179 [Tyr(SO_3H)-Ahx-Gly-D-Trp-Ahx-Asp-phenylethylester], are potent CCK_1 receptor antagonists (Lignon *et al*., 1987).

6. A serine derivative, (R)-1-[3-(3-carboxypyridine-2-yl)-thio-2-(indol-2-yl) carbonyl-

amino]propionyl-4-diphenylmethylpiperazine] (TP-680) is a highly selective and irreversible antagonist of CCK_1 receptors (Akiyama *et al*., 1996).

7. CCK-4 restricted analogues containing a 3-oxoindolizidine ring such as IQM-95333 with a very high selectivity for CCK_1 (8000:1) (Martin-Martinez *et al*., 1997).

CCK_2 Receptor Specific Ligands: Agonists

Several approaches have been devised in the design of selective agonists for CCK_2 receptors. A summary of these is as follows:

1. CCK-8 degradative enzyme-resistant forms. The first of which was BOC-[Nle28,31]CCK27-33 (BDNL) (Ruiz-Gayo *et al*., 1985). Several enzyme-resistant analogues have since been synthesised including BC-264 (Durieux *et al*., 1991).

2. A peptidase-resistant bioactive analogue [^{3}H]propionyl-BC-264 has been devised by replacement of the BOC with a tritiated propionyl group (Durieux *et al*., 1989).

3. CCK-8 appears to exist in a folded Asp1 and Gly4 linked configuration in solution (Fournie-Zaluski *et al*., 1986). Synthetic high affinity peptide-based CCK_2 receptor agonists, such as BC-197 and BC-254 utilised this property with amide bond formation between residues (Charpentier *et al*., 1988; Charpentier *et al*., 1989). A highly selective CCK_2 agonist (4000:1), SNF-8702, ([N-methyl-Nle28,31]CCK26-33) also relies on this derivation (Knapp *et al*., 1990).

CCK_2 Receptor Specific Ligands: Antagonists

A range of CCK_2 antagonists have been developed under four main chemical groups:

1. Peptide analogues, such as Boc-Trp-Orn(Z)-Asp-NH_2 (Gonzalez-Muniz *et al*., 1990).

2. Benzodiazepine derived ligands, such as L-364718 and L-365260 (Bock *et al*., 1989). A major drawback associated with early benzodiazepine-derived CCK_2 antagonists was a limited bioavailability (Noble *et al*., 1999). These flaws have been largely addressed with subsequent syntheses such as YM022 (Nishida *et al*., 1994).

3. Dipeptoids such as CI-988 (see Appendix A.1) are derived from the cholecystokinin tetrapeptide (Hughes *et al*., 1990). CI-988 exhibits 1600-fold selectivity for CCK_2 over CCK_1 receptors, although it also displays weak CCK_1 receptor agonist properties (Hocker *et al*., 1993). Clinical development of CI-988 is limited due to its poor bioavailability, which was attributed to poor absorption and efficient hepatic elimination in mice and rats (Trivedi *et al*., 1998). Later derivatives have addressed some of these flaws and await further evaluation.

4. Pyrazolidinone derived ligands are based on structural modification of the asperlicin structure (Yu *et al*., 1991). Pyrazolidinones such as LY-288513 (see Appendix A.1) however were prone to adverse effects in preclinical toxicological studies. Therefore development of this group has been discontinued (Howbert *et al*., 1992).

5. Ureidoacetamide derivatives, such as RP-73,870 are highly selective CCK_2 receptor antagonists with subnanomolar affinity for CCK_2 receptors with 100-1000-fold selectivity over CCK_1 receptors (Pendley *et al*., 1995). However bioavailability issues are once again prevalent.

Ligand-Receptor Interactions

Site-directed mutagenesis studies imply that cholecystokinin interacts with amino acid residues Trp39 and Gln40 at the extracellular segment of the first transmembrane sequence on the CCK_1 receptor. Some controversy arises in determination of residues at which CCK-8 binds. Different studies have indicated that either the N-terminal CCK-8 sequence (Kennedy *et al*., 1997) or the C- terminal CCK-8 sequence (Ji *et al*., 1997) interacts with hydrogen binding at

these residues. Furthermore, other studies suggest that Met195 (Gigoux *et al*., 1998) and Arg197 (Gigoux *et al*., 1999) interact with Tyr(SO_3H) of CCK-8s, and that Arg336 and Asn333 interact with the Asp8 and C-terminal fragment of CCK-9 (Gigoux *et al*., 1999). Other residues implicated in CCK-8 binding are generally based on those conferring tertiary structure; such as Ser131 (Kopin *et al*., 1995); residues 204-208, and Cys205 (which form a disulphide bridge with Cys127) (Silvente-Poirot and Wank, 1996). Maintenance of gastrin affinity is also attributed to residues at the outer section of the third transmembrane region (Wu *et al*., 1997) and of the outer two thirds of the fifth transmembrane section (Schmitz *et al*., 1996). Several residues are also important in maintaining CCK-8 affinity; His207, Leu103 and Phe107 (Silvente-Poirot *et al*., 1998).

Cholecystokinin antagonists appear to bind to distinct areas of the receptor. Chimeric and site-directed mutagenesis studies suggest that the outer third of the sixth and seventh transmembrane sections interact with benzodiazepine-derived antagonists, L-364718 and L-365260. In particular, residues Thr111 and His376 (Kopin *et al*., 1995) and His381 (Jagerschmidt *et al*., 1996) are important in maintaining high affinity. The lack of effect of these transmembrane VI and transmembrane VII residues on agonist affinity suggests that agonist- and antagonist-binding sites are, at best, only partially overlapping (Noble *et al*., 1999).

The Physiology of Cholecystokinin

Peripheral Physiology

Peripheral cholecystokinin receptors exert the following physiological effects:

1. Pancreatic CCK_1 receptors stimulate acinar cells to secrete the digestive enzyme pancreatic amylase (Jensen *et al*., 1989).

2. In the gallbladder, CCK_1 receptors stimulate gallbladder contraction (Chang *et al*., 1986).

3. Peripherally administered cholecystokinin produces satiation of feeding behaviour in rats (Gibbs *et al*., 1973); and in man (Sturdevant and Goetz, 1976; Pi-Sunyer *et al*., 1982). Furthermore, CCK_1 receptor antagonists increase food consumption and postpone satiety in rats (Corwin *et al*., 1991; Reidelberger *et al*., 1991; Moran *et al*., 1992); in rhesus monkeys (Moran *et al*., 1993) and in humans (Wolkowitz *et al*., 1990). Interestingly, despite some contrary evidence, this phenomenon is also produced by CCK_2 receptor antagonists in rats (Dourish *et al*., 1989). Lesions of the vagus nerve prevent the CCK-induced satiety phenomena (Smith *et al*., 1981). Furthermore, Moran *et al*., (1987) demonstrated that entry of food into the intestine triggers the release of endogenous cholecystokinin by the intestinal mucosa. These findings provide evidence for a hypothesis that cholecystokinin released from the intestine activates CCK_1 receptors on the vagus nerve to transmit satiation signals to the brain (Smith and Gibbs, 1992).

4. In the stomach, gastrin (Schubert and Shamburek, 1990) and cholecystokinin (Sandvik and Waldum, 1991) bind to CCK_2 receptors to stimulate gastric acid secretion. This effect is blocked by CCK_2 receptor antagonists (Bado *et al*., 1991; Pendley *et al*., 1995).

The behavioural profile of cholecystokinin

In line with its wide distribution in the brain, cholecystokinin is involved in the modulation/control of multiple central functions. In particular, numerous experimental and clinical studies have clearly shown that CCK, through its action at CCK_1 and CCK_2 receptors, participates in the neurobiology of anxiety, depression, psychosis, cognition, and nociception (Noble *et al*., 1999).

There is a significant correspondence between distribution of opioid receptors and of cholecystokinin receptors in the brain and spinal cord, particularly within areas associated with nociceptive pathways (Pohl *et al*., 1990). Enkephalin and the cholecystokinin octapeptide are co-localized within individual neurons and processes within discrete areas of rat midbrain and forebrain (Gall *et al*., 1987). Numerous studies have shown an antinociceptive effect of cholecystokinin agonists in various nociceptive models. In the hot-plate test Derrien *et al*., (1993) demonstrated that the non-selective cholecystokinin agonist, BDNL exhibited antinociceptive properties which were attenuated by the CCK_1 antagonist MK-329 and by the mu-opioid antagonist, naloxone. The selective CCK_2 agonist, BC-264 produced a slight decrease in lick latency, indicative of potentiation of nociception. Using the tail flick test, Rezayat *et al*., (2000) demonstrated that caerulein augmented antinociceptive effects of morphine in mice. However, administration of either CCK_1 or CCK_2 antagonists attenuated this effect (Zarrindast *et al*., 1998; Zarrindast *et al*., 1999). Faris *et al*., (1983) demonstrated that systemically or perispinally administered cholecystokinin, antagonised analgesia produced by foot shock or morphine. Furthermore, several studies demonstrate that CCK_2 receptor antagonists potentiate mu-opioid antinociceptive responses (Noble *et al*., 1995; Xu *et al*., 1996; Xu *et al*., 1997). Formation of hypotheses based on these seemingly conflicting findings, is problematic, however, differences in type of nociceptive stimulus in each model and species differences must be taken into account.

The discovery of the effects of cholecystokinin on anxiety disorders has generated much research interest in this area. This is further described in chapters two, three and four and in a preface following this section.

Furthermore effects of cholecystokinin on learning and memory processes have been studied in some detail. This is illustrated further in chapters four and five.

CHOLECYSTOKININ AND ANXIETY DISORDERS - AN INTRODUCTION

Anxiety Disorders afflict up to ten percent of the general population with a one-year prevalence (the disorder lasting for at least one year). Anxiety disorders encompass a wide range of symptoms, differentiated by specific criteria into separate conditions, such as panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder and generalised anxiety disorder. The symptomology of these conditions frequently overlap (Dubovsky, 1990).

Generalised anxiety disorder (GAD) accounts for over half of all anxiety diagnoses. GAD is characterised by symptoms of motor tension (fidgeting), autonomic hyperactivity (high startle), hypervigilance and scanning. Panic disorder afflicts almost two percent of the population. Patients with panic disorder undergo repeated bouts involving discrete episodes of intense anxiety. These episodes appear to occur spontaneously and may last from a few minutes to over an hour. Panic attacks are perceived as uncontrollable sensations including; palpitations, breathing difficulty, chest pain, tremors, paraesthesia, and hallucinations accompanied by immobility. Psychosensorial symptoms such as depersonalisation, fear of losing control and fear of dying are very common. Both GAD and panic disorder appear to be familial (Dubovsky, 1990).

Several neurotransmitter systems have been implicated in the pathogenesis of these anxiety disorders. Research, until quite recently, has concentrated on four major neurotransmitters and their interactions:

The dopaminergic mesocortical system appears to be involved in emotional behaviour, including that associated with anxiety. The dopaminergic regions of the amygdala and possibly the nucleus accumbens are implicated (Harro and Vasar, 1991b; Crawley, 1991).

Noradrenergic systems, focused primarily at the locus coeruleus, have been associated with arousal in response to danger signals. The underlying cause of these anxiety disorders may be

linked to altered perception of danger signals (Gray, 1978).

Serotonergic pathways, focused primarily at the dorsal raphe nuclei, have a variety of different effects that appear to influence arousal states. Serotonergic pathways are particularly responsive to punished behaviour. Punished behaviour is frequently anxiogenic (Salzman *et al*., 1993).

GABA (γ-amino-butyric acid) gated pathways have a multiplicity of inhibitory effects on other pathways. The benzodiazepine group of anxiolytic drugs, through their own receptors, exert action upon GABA receptors (to increase the inhibitory action of GABA). Benzodiazepines exert general anti-convulsant, anti-anxiety, and sedative effects. Despite currently being the most effective treatment of anxiety disorder they are, due to both their sedative effects and their abuse potential, far from ideal (File *et al*., 1996; Olivier *et al*., 1996).

Cholecystokinin and Anxiety

The observation that cholecystokinin may have anxiogenic properties arose, from a study in 1979, of its satiety effect on sheep. Infusion of the cholecystokinin agonist, pentagastrin into the lateral ventricle produced an abnormal series of behaviours. These included 'foot stamping' and 'vocalisation', i.e.; those behaviours characteristic of an ovine response to fear (Della-Fera and Baile, 1979). Fekete *et al*., (1984) subsequently reported anxiogenic effects with CCK-8, injected into the amygdaloid central nucleus of rats.

A subsequent study also showed that CCK-8s induced excitation of the hippocampal pyramidal neurones in rats, is attenuated by a range of benzodiazepine receptor agonists. In addition, this effect was reversed by prior administration of the benzodiazepine antagonist, flumazenil. The dose-response profile, exhibited by these intravenously administered benzodiazepines in this study, was analogous to that of their clinical effect in the treatment of anxiety disorders (Bradwejn and de Montigny, 1984).

Cholecystokinin in Clinical Studies

Clinical studies demonstrated that a bolus injection of CCK_2 receptor agonists, CCK-4 or pentagastrin, induces panic attacks in patients with panic disorder (Bradwejn *et al*., 1990). These can be attenuated by antipanic pharmacological agents such as antidepressants (van Megen *et al*., 1997) and by CCK_2 antagonists such as L-365260 (Bradwejn *et al*., 1994). Panic attacks and anxiety symptoms were also induced in healthy human subjects by CCK-4 (Shlik *et al*., 1997; de Montigny, 1989; Bradwejn and Koszycki, 1991) and by pentagastrin (McCann *et al*., 1994-1995). The CCK_2 antagonist, L-365260 was able to prevent pentagastrin-induced anxiogenesis in healthy individuals (Lines *et al*., 1995).

Sensitivity to CCK-4 and pentagastrin is enhanced in panic disorder patients relative to healthy volunteers (Bradwejn *et al*., 1991). Furthermore, cerebrospinal fluid concentrations of CCK-8s are significantly decreased in patients suffering from panic disorder (relative to controls) (Lydiard *et al*., 1992).

The anxiogenic/panicogenic behavioural effects of CCK-4 in humans are accompanied by marked biological alterations including; robust increases in heart rate, blood pressure, and minute ventilation (Bradwejn *et al*., 1992; Koszycki *et al*., 1998), increased levels of plasma cortisol and prolactin (de Montigny, 1989), an immediate increase in plasma levels of adrenaline and noradrenaline, with delayed onset increases in dopamine plasma levels (Jerabek *et al*., 1999), and increased levels of neuropeptide Y (Boulenger *et al*., 1996). In addition, pentagastrin can induce large (up to 520% increases over the baseline) and very rapid, dose-dependent elevations in adrenocorticotropin (ACTH) and cortisol levels in healthy human subjects (Abelson and Liberzon, 1999). Whether these alterations in neurochemistry are reproducing those found within spontaneous panic episodes is as yet unclear, however, it would appear ostensibly that the cholecystokinin system in patients with panic disorder is altered compared to healthy subjects.

Studies using CCK_2 receptor antagonists have failed to provide consistent evidence of a potential in treatment for panic disorder. In using cholecystokinin antagonists, CI-988 (Adams *et al*., 1995) and L-365260 (Kramer *et al*., 1995), problematic pharmacokinetic profiles were observed. Other antagonists have shown toxicological concerns. Given the recent development of several different classes of CCK_2 antagonists, other suitable agents shall undoubtedly be examined in the near future.

Despite functional imaging studies having been performed in healthy individuals (Benkelfat *et al*., 1995), no studies involving patients with panic disorder have been completed to date. This is significant given that panic disorder patients clearly respond dissimilarly to cholecystokinin in addition to actually, exhibiting spontaneous panic episodes. Shlik *et al*., (1997) proposed that structures within the brainstem nuclei, thought to control regulation of respiratory and cardiopulmonary function, are significant sites of action for exogenous CCK-4. Animal studies have shown that cholecystokinin interacts with brainstem structures to modulate respiratory and cardiopulmonary activity, and blood pressure (Denavit-Saubie *et al*., 1985). Furthermore this region has close anatomical and functional links with the locus coeruleus, a brain region thought to be involved in the expression of fear and anxiety. Therefore it may be proposed that anxiogenic/panicogenic symptoms evoked by CCK-4 may rise from direct activation at brainstem structures leading to subsequent activation or inhibition of higher CNS regions mediated through neuronal projections (Noble *et al*., 1999).

A study of polymorphisms in genes encoding for cholecystokinin pre-pro hormone, cholecystokinin peptides and both receptor subtypes were examined in patients with panic disorder (according to DSM-IV classification). While cholecystokinin peptide, and CCK_1 receptor gene polymorphisms showed no association, a CCK_2 receptor gene polymorphism, showed a significant association with panic disorder patients (Kennedy *et al*., 1999).

Although CCK_2 receptors are certainly a major component in cholecystokinin inducible panicogenesis, it is probable that the range of symptoms observed within panic episodes are achieved through interactions between cholecystokinin and other neurotransmitter systems.

Clinical studies have revealed interactions between cholecystokinin induced anxiogenesis and benzodiazepines (de Montigny, 1989), serotonin (Bradwejn and Koszycki, 1994a; van Megen *et al*., 1997), and noradrenaline (Bradwejn and Koszycki, 1994a; Le Melledo *et al*., 1998). Subsequent investigations have revealed that CCK_2 receptor agonists are also active in generalized anxiety disorder (Brawman-Mintzer *et al*., 1997), social phobia (van Vliet *et al*., 1997), obsessive compulsive disorder (de Leeuw *et al*., 1996), and premenstrual dysphoric disorder (Le Melledo *et al*., 1995). The physiological properties of cholecystokinin in maintenance of appetite and behavioural implications with various anxiety disorders would imply a strong underlying role in anorexia and bulimia nervosa. This link was confirmed by Lydiard *et al*., (1993) with measurement of significantly lower CCK-4 levels in CSF of bulimic patients. cholecystokinin has also been implicated in anorexia nervosa (Stricker, 1984).

The proposal by Lydiard (1994) that panic episodes can result from a cholecystokinin imbalance of increased CCK-4 levels and decreased CCK-8 levels can be given some credence, but is drawn into question given more recent suggestions that CCK-4 is not in fact an endogenous form of cholecystokinin (Rehfeld, 2000). This article proposed that CCK-4 is actually an artefact derived from CCK-5, although whether the properties of CCK-5 are transferred to those observed with CCK-4 is unresolved.

Cholecystokinin in Animal Models of Anxiety

Concurrently with clinical trials, various animal studies have demonstrated a similar anxiogenic activity in several animal models and in several different animal species. However, this area of study is rife with inconsistency. Bradwejn and Vasar (1995) proposed that the conflicting data reported in the animal literature are attributable in part to the failure to address the various factors that potentially influence susceptibility to the anxiogenic-like effects of CCK. This is proposed with evidence that rats with low exploratory behaviour (i.e., "anxious" rats) have been reported to exhibit a higher density of cholecystokinin receptor-binding sites in the frontal cortex and hippocampus, relative to that in rats with high exploratory behaviour (i.e., "non-anxious" rats); (Harro *et al*., 1990a). Subsequently, effects of cholecystokinin compounds might suffer inconsistency in effect, due to differing endogenous levels of anxiety displayed by rats in each study. Moreover, anxiety levels have shown to vary markedly with minor changes in procedure (see chapters two, three and four).

Given that CCK-8s is a non-specific agonist (at CCK_1 and CCK_2 receptors), and CCK-4 is specific for CCK_2 receptors, Bradwejn and Koszycki, (1994b) proposed that an imbalance between CCK_1 and CCK_2 receptors could result in the pathology of anxiety. The administration of CCK_1 agonists however, has been shown to have little or no effects on anxiety related behaviour, although the intra-accumbal anxiogenic-like effect of CCK-8s can be blocked by a specific CCK_1 receptor antagonist (Dauge *et al*., 1989a). Furthermore, despite inactivity by most CCK_1 antagonists, one notable exception is that of a highly CCK_1 selective antagonist, IQM-95333 which exerts anxiolytic activity (Ballaz *et al*., 1997; Singh *et al*., 1995).

The anxiogenesis produced by CCK-8s administration is however, blocked by many CCK_2 receptor antagonists. In addition, most (but not all) CCK_2 agonists can induce anxiety-like symptoms in both animal models and in clinical studies.

Animal studies have demonstrated interactions between cholecystokinin induced anxiogenesis and; benzodiazepines (Singh *et al*., 1992; Chopin and Briley, 1993); serotonin (Vasar *et al*., 1993b; Rex *et al*., 1994; Bickerdike *et al*., 1994; Bickerdike *et al*., 1995; Rex *et al*., 1997; To and Bagdy, 1999); noradrenaline (Harro *et al*., 1995); dopamine (Biro *et al*., 1997; Nutt *et al*., 1998); corticotrophin-releasing factor (Biro *et al*., 1993); and opioids (Koks *et al*., 1998; Koks *et al*., 1999).

The Neuroanatomy of Cholecystokinin Induced Anxiety in Animals

Cholecystokinin is known to exhibit interactions with a variety of other neurotransmitters in the central nervous system:

1. Cholecystokinin is co-localised with GABA in the rat hippocampal interneurones and is found to enhance potassium evoked GABA release in the rat cerebral cortex (Raiteri *et al*., 1996).

2. CCK-8s induced excitation of the hippocampal pyramidal neurones in rats is attenuated by a range of benzodiazepine receptor agonists (Bradwejn and de Montigny, 1984).

3. Cholecystokinin is also found to co-exist with dopamine in the nucleus accumbens. The cholecystokinin antagonist, CI-988 has been shown to inhibit dopamine overflow release in the rat cerebral cortex (Corwin *et al*., 1995).

4. CCK-8 and BC-264 has been demonstrated to increase the basal release of endogenous glutamate from rat hippocampal slices. L-365260 reversed these effects (Migaud *et al*., 1994).

5. CCK_2 receptor activation, however, reduces glutamate-induced depolarisation in slices of rat cerebral cortex (Harro *et al*., 1993).

6. The CCK_2 receptor agonist BOC-CCK-4 produced 'anxious' behaviour and potentiated the rise in cortical 5-HT observed on exposure to the X-maze. L-365260 produced 'anxiolytic' behaviour and decreased basal extracellular cortical 5-HT (Rex *et al*., 1994).

7. Chemical lesioning of the noradrenergic, locus coeruleus produces an upregulation of cholecystokinin receptors in the frontal cortex and the hippocampus (Harro *et al*., 1992).

The septohippocampal system (SHS) has been implicated due to its' high cholecystokinin content. The SHS has also been proposed to form a fundamental network for the basis of anxiety, gating a behavioural inhibition system. Gray (1978) proposed that anxiety is caused by a mismatch in comparison of expected to actual events, which is thought to function within this system.

The midbrain region, the periaqueductal grey (PAG) has also been implicated in anxiety. Electrical stimulation of the PAG region in humans produces intense fear-like symptoms (Tasker, 1982). Stimulation of the dorsal PAG in animals is reflected by abrupt escape and flight reactions (Di Scala *et al*., 1987). Studies utilising immunohistochemical techniques, have shown a large distribution of cholecystokinin neurones in the periaqueductal grey region of the rat (Liu *et al*., 1994). Distribution is predominantly in the dorsolateral division, where the population of CCK-like immunoreactive neurones are largely heterogeneous. These neurons are subsequently activated by cholecystokinin application, which is then inhibited by antagonists, proglumide and CR1409 (Liu *et al*., 1994). Furthermore, infusion into the PAG of CCK-4 produces intense anxiety-like flight behaviour (Mongeau and Marsden, 1997).

Bradwejn and Koszycki (1994b) speculated that the opposing effects of CCK_1 and CCK_2 agonists on the brain stem region, the nucleus tractus solitarius (NTS), when exhibiting an imbalance, might induce anxiety and/or panic episodes. Given that it has not yet been determined whether CCK-4 is permeable to the blood brain barrier, the relative permeability of the NTS region is thus highlighted as a possible area of cholecystokinin action in anxiety. In

addition, the NTS contains a relatively high density of cholecystokinin receptors. This area is densely innervated and contains projections to the locus coeruleus (an area implicated in panic disorder and part of the septohippocampal system).

The precise neuroanatomical basis of cholecystokinin-induced anxiety states is thus unclear. The probability that all these areas interact, in order to produce the various types of anxiety disorder, is high. It is also probable that different interacting regions exhibit a variant activity dependent upon the symptomology of the various types.

Although a role for cholecystokinin in anxiety and panic disorder is evident, the mechanism by which this effect is produced is also still undefined. This thesis attempts to elucidate at least with regard to certain animal models, the underlying mechanisms controlling the induced anxiety-like behaviour. A necessary part of the ensuing hypotheses will be in the detailed analysis of preceding studies, in order to encompass and thus explain, both my own findings and those of others.

Aims

Studies regarding the influences of cholecystokinin on anxiety, learning and memory are rife with inconsistency. These discrepancies are described in the following chapters. This thesis attempts to address many of these inconsistencies and to elucidate valid arguments for these differences. Experimental research within this thesis is devoted to study of hypotheses connecting these seemingly contrary pieces of evidence. It is hoped that this thesis has gone some way in this endeavour.

CHAPTER TWO – ANIMAL MODELS OF GENERALISED ANXIETY: THE ACOUSTIC STARTLE REFLEX PARADIGM

Introduction

The acoustic startle reflex paradigm is a highly sensitive model of anxiety and fear related behaviour. The acoustic startle response is a short latency motor response to a loud and unexpected noise. The response involves a rapid sequential activation of muscles along the length of the body. The latency of the acoustic startle reflex in the rat is 8-milliseconds, measured from tone onset to the beginning of the electromyographic response in the hindleg. This extremely short latency may indicate that limited organisations of synapses are involved in the acoustic startle response circuit (Frankland *et al*., 1997).

Neuroanatomical Organisation

In a study by Davis *et al*., (1982) bilateral lesions of the ventral cochlear nucleus, which receives the primary auditory input, appeared to abolish the acoustic startle reflex response. In addition, they observed that a single pulse electrical stimulation of the ventral cochlear nucleus elicits startle-like responses with a latency of about 7 milliseconds. Bilateral lesions of the dorsal and ventral nuclei of the lateral lemniscus, which receive direct input from the ventral cochlear nuclei, also abolished acoustic startle. Electrical stimulation of these nuclei elicited startle-like responses with a latency of about 6 milliseconds. Bilateral lesions of ventral regions of the nucleus reticularis pontis caudalis, which contain cell bodies that give rise to the reticulospinal tract, again abolishes acoustic startle. Electrical stimulation of these points elicits startle-like responses with a latency of about 5 milliseconds. Reaction product from horseradish peroxidase, iontophoresed into this area is found in the nuclei of the lateral lemniscus. This study suggested that a primary acoustic startle circuit in the rat consists of; the auditory nerve, ventral cochlear nucleus, nuclei of the lateral lemniscus, nucleus reticularis pontis caudalis, spinal interneuron, lower motor neuron, and muscles. Hence, five synapses,

plus the neuromuscular junction, are probably involved (Davis *et al*., 1982). The modification of this reflex circuit, by a more obscure neuronal organisation gating mood and anxiety state, may be via either a deficit of inhibitory, or intensification of excitatory modulating influences on signal transmission, within the acoustic startle circuitry (Krupina *et al*., 1994). Lee and Davis (1997) observed that the NMDA lesions of the bed nucleus of the stria terminalis (BNST) completely blocked corticotropin releasing hormone (CRH) enhanced startle, whereas chemical lesions of the ventral hippocampus and the amygdala failed to block CRH-enhanced startle.

Validity of Acoustic Startle as a Model of Anxiety

Animal models of human clinical conditions are required to satisfy particular validities in order to be established as models of the condition itself, or of some aspects of the condition.

Face Validity:

Face validity, in the case of animal models of anxiety, implies that the model produces a fear-like reaction, guided by anxiety-like states in animals, that are analogous to abnormal behaviour in clinical anxiety states. Without a general consensus with regard to classification of human anxiety, it is difficult to determine the face validity of any particular animal model of anxiety. Whether the subjective experience underlying the anxiety-like reactions in animals within these models, are analogous to the subjective anxiety experienced by anxious humans, is probably an irresolvable question.

An acoustic startle paradigm, however, probably more than any other animal model of anxiety, appears to satisfy face validity criteria. A spontaneous startle reflex following a loud and unexpected noise is prevalent across many species. Acoustic startle is observed clinically to be increased in amplitude in patients with a range of anxiety disorders (Shalev *et al*., 1998; Rodriguez-Fornells *et al*., 1999). The observation of a very similar phenomenon in a rodent

acoustic startle model may provide a dependable rationale for development of this paradigm as a model of generalised anxiety.

Furthermore, rodent ultrasonic vocalizations (USV) at 22 kHz in response to acoustic startle stimuli may perhaps provide additional evidence of the face validity of this model of anxiety, in addition to the actual reflex movement (Kaltwasser 1990). An inhibition of vocalisations is observed with rats administered diazepam, flunitrazepam, and ipsapirone after induction by either acoustic startle or electric shock. FG-7142 had no activity (Kaltwasser 1991). In addition, startle-induced USV are sensitive to the "anxiogenic-like" effects of withdrawal from chronic diazepam exposure (Vivian *et al*., 1994).

Plappert *et al*., (1993) observed that a group of rats are divided according to their responses to startle-eliciting stimuli into two groups, with different emotional states. About half of the female rats showed long-lasting freezing behaviour after 1-8 stimuli (10 kHz, 110 dB). In freezing rats the startle amplitude was higher than in non-freezing rats, throughout the entire sequence. This finding demonstrates that the anxiety state of these animals before the first startle-eliciting stimulus, and not just the aversiveness of the stimulus, contributes to freezing behaviour. The dichotomous variation in freezing behaviours, coupled to a synchronous and matching distribution in acoustic startle responses, again provides further evidence towards the face validity of this model of anxiety. In another study by Krupina *et al*., (1994) sensorimotor response was measured by acoustic startle reflex in male rats with innate high and low levels of anxiety. The levels of anxiety were determined using a complex multiparameter method for evaluating anxiety-phobic states in rats by a ranged scale. Amplitude and prepulse inhibition (PPI; see paragraph below) of the acoustic startle response were increased, but latency of the startle reflex was decreased in rats with intrinsically high levels of anxiety as compared with those with intrinsically low levels.

Predictive Validity:

Predictive validity, or correlation, refers to the sensitivity of the model to clinically active pharmacological intervention, and insensitivity to other clinically non-active intervention. An example of such is where a model of generalised anxiety can be manipulated by using clinically active benzodiazepine anxiolytics, and no effects are observed using a clinically inactive tricyclic antidepressant treatment. Hijzen *et al*., (1991) showed that the benzodiazepine agonist, midazolam, attenuated acoustic startle sensorimotor amplitude dose-dependently. This phenomenon has also been observed with anxiogenic pharmacological manipulations, whereby the anxiety level is increased, resulting in increased startle response amplitudes in rats (Rassnick *et al*., 1992; Rasmussen *et al*., 1993; Vivian *et al*., 1994). Krupina *et al*., (1994) observed that in rats with an intrinsically high level of anxiety, intraperitoneal injection of subconvulsive doses of pentylenetetrazol (10 and 15 $mg.kg^{-1}$) resulted in an increase of the amplitude of the acoustic startle response. Swerdlow *et al*., (1986) observed that intracerebroventricular (i.c.v.) administration of the stress hormone, corticotropin-releasing factor significantly potentiated acoustic startle amplitude. These effects were attenuated dose-dependently by pre-treatment with the benzodiazepine, chlordiazepoxide. Doses of chlordiazepoxide, that antagonized CRF-potentiated ASR, did not lower startle baseline. Liang *et al*., (1992) also observed that intracerebroventricular infusion of CRF produced a significant dose-dependent increase in the magnitude of the acoustic startle reflex in rats. This corresponds with observations that CRF levels are decreased by benzodiazepines in human subjects with high anxiety levels (Gram and Christensen 1986).

The eye-blink response following sudden acoustic noise bursts is part of the startle reflex in both animal models and in humans. In clinical studies the magnitude of the startle response can be attenuated by presentation of a weak stimulus before the startle-eliciting stimulus (prepulse inhibition, PPI). The magnitude of PPI in normal human subjects is decreased by increasing doses of the benzodiazepine agonist, midazolam. During infusion of flumazenil and in the

presence of midazolam, the magnitude of PPI increased, which is consistent with the mode of action of flumazenil as a benzodiazepine antagonist (Schachinger *et al*., 1999).

Construct validity:

Construct validity implies homology, or direct correspondence, at a physiological/neurological level, between the animal model and the condition being modelled (Rodgers and Cole 1994). Indeed, attempts to assess construct validity are compromised by our incomplete understanding of human anxiety (Lister 1987). However, where the septohippocampal system and the amygdaloid regions have been implicated in anxiety-like behaviour clinically, Decker *et al*., (1995) observed in rats that both septal and fimbria-fornix lesions had marginal effects on prepulse inhibition and baseline startle. Amygdaloid lesions markedly impaired prepulse inhibition of acoustic startle.

Cholecystokinin and Acoustic Startle

It has been observed that systemic administration of the CCK_2 agonist, Pentagastrin increases startle amplitudes (Zhou *et al*., 1996). In addition, pentagastrin, via intra-amygdaloidal infusion (Frankland *et al*., 1997), and pentagastrin and CCK-8s, via intracerebroventricular infusion (Frankland *et al*., 1996), also increase startle amplitudes. Intra-amygdaloid administrations of pentagastrin potentiated startle responses dose-dependently. With increasing intra-amygdaloid doses of pentagastrin, the level of responding compared to the baseline (vehicle) group increased exponentially (Frankland *et al*., 1997). This study also demonstrated that these same infusions into the amygdaloid region of pentagastrin had no effect on locomotor activity within the same time range. This suggests that changes in the level of startle responding were not attributable to changes in spontaneous locomotor activity in the open field model (Frankland *et al*., 1997). However this model differs in its' nature to that of the acoustic startle apparatus in which the animal is placed in a chamber in which its' movement is restricted. A more reliable model would perhaps be that which is able to measure spontaneous

locomotor activity, within the same chamber to that in which the animal was tested for the acoustic startle (such as has been studied here). This study also demonstrated that the potentiation of startle, by infusions of pentagastrin, was attenuated by a systemically administered pre-treatment of the CCK_2 receptor specific antagonist L-365260. Systemic administration of the quinoline derived CCK_2 antagonist, LY-288513, attenuates potentiated startle generated by; diazepam withdrawal (Rasmussen *et al*., 1993), and by chronic nicotine withdrawal (Rasmussen *et al*., 1996). Frankland *et al*., (1997) demonstrated that L-365260 only weakly attenuates, without reaching significance, potentiation of startle induced by intra-amygdaloidal administrations of pentagastrin. Frankland *et al*., (1997) also demonstrated that intra-amygdaloidal administration of the CCK_2 antagonist, PD-135158 attenuated the potentiation of acoustic startle, induced by intracerebroventricular infusions of pentagastrin. This infusion of PD-135158 however, exhibited no effects upon the baseline level (Rasmussen *et al*., 1996). Frankland *et al*., (1997) also showed that intracerebroventricular administrations of pentagastrin were able to potentiate startle responses by 70 to 100%. These increases were attenuated by intra-amygdaloidal infusions of another CCK_2 receptor specific antagonist, PD-135158. It was also demonstrated, that neither intra-striatal nor intra-accumbal administration of pentagastrin, at several doses, were able to increase the level of startle responding compared to the vehicle group. It appears in fact that intra-striatal pentagastrin administration, at a dose of 10nM, may decrease the level of acoustic startle compared to vehicle group. Subsequently, the Frankland study (1997) provides strong evidence that the pentagastrin increased startle responding is gated primarily at the amygdala formation. These studies demonstrate that the CCK_2 antagonists tested are able to attenuate the potentiation of startle induced by; (1) cue conditioned fear (to a stimuli previously paired with a noxious unconditioned event), (2) pharmacologically induced fear from intra-amygdaloid and intracerebroventricularly administered pentagastrin, and (3) diazepam withdrawal induced anxiety. Fendt *et al*., (1995) infused CCK-8s into the caudal pontine reticular formation (PnC) and observed an increased number of acoustically activated spikes recorded from single PnC units. In addition this

infusion produced a modest increase (30-40%) in startle responding, although only at the dose of 5-picogram per ml. At higher doses, 2.5 and 10 pg/ml this startle potentiating effect was no longer observed. The giant neurones of the caudal pontine reticular formation are thought to form an obligatory relay circuit in mediation of the acoustic startle responses (Frankland *et al*., 1996).

In clinical studies, the endogenous cholecystokinin tetrapeptide, CCK-4, produced an increase of eye-blink startle amplitude (in response to a series of acoustic stimuli) from baseline values during a continuous intravenous administration in healthy volunteers (Shlik *et al*., 1999).

Aims of this study

This study tested a series of cholecystokinin forms. It was observed previously that intraperitoneal administration of these specific forms; CCK-8s (sulphated octapeptide), CCK-8us (unsulphated octapeptide) and CCK-4 (unsulphated tetrapeptide) at defined doses were able to induce an anxiety-like state (in reversal of the effects of chlordiazepoxide) upon the elevated plus-maze model of anxiety (see chapter 3). Furthermore it was found that combining these forms, at these doses and at half these doses, that this anxiety-like behavioural change was no longer observed. For this reason it was decided to examine whether these phenomena are observable upon the acoustic startle model of anxiety. In addition, in order to determine whether these effects are produced via the CCK_2 receptor subtype, two CCK_2 antagonists were tested for their ability to alter startle responding. These antagonists, the peptoidal CI-988 and the quinoline derived LY-288513 were administered intraperitoneally, either alone or concurrently, with one of the three cholecystokinin peptide forms.

Materials and Methods

Animals

Male rats of the Sprague Dawley strain (Laboratory Animal Centre, National University of Singapore, Singapore), 250-320g were housed in groups of five to seven and maintained with free access to food and water *ad libitum*, in an animal room for one week prior to experimentation. Room lights were on from 07:00 to 19.00 hours. Animals were randomly assigned treatment groups using a simple computer program, random number sequence generator (Random Number Generator v.1.0 by Colin Greengrass, 1998). This simple program (see Appendix C.1 for listing) generated random whole numbers within a range corresponding to an assigned treatment group. In order to maintain similar animal numbers in each group, fulfilment of a criterion of group loading was employed. Thereby, a random number would be generated and an animal in sequence would be assigned to the corresponding group number. When the maximum number of animals approved for each group had been reached, any occurrence of that number in the sequence would be deemed invalid and ignored. The next valid number in the sequence would be taken until all the treatment groups were filled. A 30 minute period, from injection to behavioural testing, was preferred due to an abudance of literature pertaining to this time period for efficacy of cholecystokinin forms.

Drugs and Administration

Cholecystokinin peptide agonists, CCK-4 (Trp-Met-Asp-Phe-NH_2); CCK-8us (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2); (Biotechnology Centre, National University of Singapore), CCK-8s (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; American Peptide Company and Princeton Biomolecules) ; the cholecystokinin peptoidal antagonist CI-988 ([R-(R*,R*)]-4-[[2-[[3-(1H-Indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1.3,7]dec-2-yloxy)carbonyl]-amino]propyl]-amino]-1-phenylethyl] amino]-4-oxobutanoate 1-deoxy-1-(methylamino)-D-glucitol; Research Biochemicals International), and LY-288513 ([trans-N-(4-bromophenyl)-3-

oxo-4, 5-diphenyl-1-pyrazolidinecarboxamide]; kindly donated by Eli Lilly and Company, USA) were dissolved in dimethylsulphoxide (DMSO; Merck) with either chlordiazepoxide hydrochloride (a generous gift from Roche) at 1.5mg/ml or DMSO alone. The drugs or vehicle were administered by intraperitoneal injection in a volume of 1ml per kg body weight. Two separate injections were given to each animal. Animals were used only once. Drugs were administered intraperitonally 30 minutes prior to testing, as has been previously described in literature. Cholecystokinin peptide was stored under desiccation at -70^{o}C. Cholecystokinin peptide solutions were prepared freshly each day approximately two hours prior to testing. Powdered peptide was dissolved in DMSO and specific concentrations were prepared through serial dilution with thorough vortex mixing at each stage.

Auditory Startle Response Apparatus

A computer controlled auditory startle chamber was used (SR-LAB, San Diego Instruments Inc.). The startle apparatus consisted of a transparent Perspex cylindrical animal chamber, 8.7cm in diameter, with sliding Perspex gates at both ends. The animal was placed in this chamber, with both gates closed, during testing. The animal chamber rested on a weighted Perspex frame within a sound proofed isolation cabinet. This cabinet provided inflow-outflow ventilation, a constant background white noise level of 70-decibels, and a constant light level. Both stimulus noise bursts and background white noise was delivered via a speaker mounted approximately 2cm above the animal on the exterior of the animal chamber. Intensity of the startle response was determined using a piezoelectric sensor mounted below the animal chamber. Motion data was recorded via an interface assembly linked to an IBM computer with relevant software (San Diego Instruments, PSR2 Software program). Noise levels, measured in decibels, were determined by means of a sound pressure level meter. This allowed precise adjustment of noise levels. The chamber was thoroughly cleaned with 70% ethanol between sessions and allowed to dry. The test room was maintained with controlled light levels and temperature with white noise delivered at 70dB. Behavioural experiments were performed

between 14:00-18:00hours.

An apparatus-naïve animal was placed in the compartment and underwent an acclimatisation period for 180 seconds in the presence of 70dB of white noise (background). Ideally within this acclimatisation period, peak amplitude readings would have been taken, in order to give a measure of spontaneous locomotor activity, however, the operating program software did not allow for this option in the protocol. Immediately following this period each animal was exposed to twenty-five 100-millisecond bursts of white noise at 120dB with an eight-second period of background noise between each burst. The peak amplitude of each startle reflex movement was recorded. Sequential recording of peak amplitude of movement (means of five responses) exhibits an acoustic startle profile, which is plotted on a chart versus the sequential 120dB noise burst (in groups of five). The protocol pertaining to measurement of spontaneous locomotor activity within the acoustic startle chamber relied on a simple modification of the acoustic startle program, whereby the level of the startle stimulus noise was set to that of background noise (70dB) and data taken periodically.

SR-Lab Software Program

A dedicated computer software program dictated a sequence of twenty-five 100-millisecond bursts of 120dB white noise with an intermittent background noise level of 70dB. There was an eight second interval between 120dB bursts. This is listed in Appendix C.2.

Statistical Analysis

Statistical analysis was performed using statistical software (SPSS V9.0, SPSS Inc. USA). The data were subjected to one-way analysis of variance (ANOVA), followed by Bonferroni post hoc analysis. Probability (p) <0.05 was considered statistically significant. Data are presented as drug group versus startle amplitude means (25 readings in 5 groups of five). Progressive session groups, one to five, are represented by five shaded bars of increasing darkness, trial

block one is lightest in shade, whereas trial block five is darkest.

Results and Data

A series of preliminary validation experiments were conducted.

Figure 2.1: Administration of dimethylsulphoxide (DMSO), which was used as a solvent throughout the experiment, produced no differences in acoustic startle responding when compared to 0.9% Saline.

Figure 2.2: Chlordiazepoxide, at 3.0 and 5.0mg.kg^{-1}, produced a statistically significant decrease in startle amplitudes compared to the DMSO control: chlordiazepoxide; 3.0mg.kg^{-1}, ($F_{2,13}$=14.79, $p<0.05$ (Trial block 1); $F_{2,13}$=71.81, $p<0.001$ (Trial block 2) $F_{2,13}$=42.87; $p<0.001$ (Trial block 3); $F_{2,13}$=69.36, $p<0.001$ (Trial block 4); $F_{2,13}$=23.88, $p<0.01$ (Trial block 5): chlordiazepoxide; 5.0mg.kg^{-1}, $p<0.001$ in trial blocks 1 to 5.

Figure 2.3: A protocol by which locomotor activity within the acoustic startle chamber was determined showed no significant differences between groups compared to DMSO (control); ie. chlordiazepoxide (3.0 mg.kg^{-1}), CCK-4 (0.025 mg.kg^{-1}), CCK-8us (0.01 mg.kg^{-1}), CCK-8s (0.001 mg.kg^{-1}), CI-988 (1.0 mg.kg^{-1}) and LY-288513 (10.0 mg.kg^{-1}).

Figure 2.4: Administration of the cholecystokinin form; CCK-8s (0.001 mg.kg^{-1}) produced a significant increase in startle amplitude when compared to DMSO (control), CCK-8s; 0.001 mg.kg^{-1}, $F_{3,35}$=10.85, $p<0.001$ (Trial block 1); $F_{3,35}$=14.99, $p<0.001$ (Trial block 2); $F_{3,35}$=10.40; $p<0.001$ (Trial block 3); but missed significance on trials 4 and 5. CCK-4 (0.025) narrowly missed significance on trial 2, $p=0.052$. CCK-8us (0.01 mg.kg^{-1}) failed to show any increase in startle amplitude.

Figure 2.5: In comparison between chlordiazepoxide (control) and chlordiazepoxide with cholecystokinin forms, CCK-4 (0.025 mg.kg^{-1}) and CCK-8s (0.001 mg.kg^{-1}) both showed significant increases in startle amplitudes within the first trial period only, CCK-4; 0.025 mg.kg^{-1}, $F_{6,59}$=4.50, $p<0.05$ (Trial block 1), CCK-8s; 0.001 mg.kg^{-1}, $F_{6,59}$=4.5, $p<0.05$ (Trial

block 1). Combined administration of chlordiazepoxide with cholecystokinin forms; CCK-4, CCK-8us and CCK-8s at their peak anxiogenic doses (as determined by elevated plus-maze studies - see chapter three) and at half these maximally effective doses, with each other, produced several affects on startle amplitude. The anxiogenic-like activity of CCK-4 (at 0.025 $mg.kg^{-1}$) was partly attenuated by combination with CCK-8s (0.001 $mg.kg^{-1}$), whereby significant differences with chlordiazepoxide were negated and no significant inhibition was observed between CCK-4 and the CCK-4/ CCK-8s combination. CCK-4 (at 0.025 $mg.kg^{-1}$) in combination with CCK-8us (0.01 $mg.kg^{-1}$) maintained the same significant increase in startle amplitude when compared to the chlordiazepoxide vehicle control, ($F_{6,59}$=4.5, $p<0.01$ (Trial block 1). The anxiogenic-like activity of CCK-8s (at 0.001 $mg.kg^{-1}$) was also attenuated by combination with CCK-8us (0.01 $mg.kg^{-1}$), again significant differences with chlordiazepoxide vehicle group were negated and no significant inhibition was observed between CCK-8s and the CCK-8us/ CCK-8s combination. No anxiogenic-like activity was observed for CCK-4 and CCK-8s at half their anxiogenic doses, as might be expected. Two CCK_2 specific antagonists were tested against the anxiogenic doses of cholecystokinin forms. Prior to this step, a dose response curve was evaluated for both antagonists. It was found that these antagonists produced anxiogenic-like activity with an inverse bell-shaped dose response profile.

Figure 2.6: Administration of CI-988 at doses, 0.002 $mg.kg^{-1}$ and 0.02 $mg.kg^{-1}$ produces an increase in startle amplitude compared to vehicle: CI-988; 0.002 $mg.kg^{-1}$, $F_{5,42}$=10.76, $p<0.01$ (Trial block 1); $F_{5,42}$=7.83, $p<0.05$ (Trial block 2); $F_{5,42}$=5.31, $p<0.05$ (trial block 3); $F_{5,42}$=7.26, $p<0.01$ (trial block 4); $F_{5,42}$=9.24, $p<0.001$ (trial block 5): CI-988; 0.02$mg.kg^{-1}$, $F_{5,42}$=10.76, $p<0.001$ (Trial block 1); $F_{5,42}$=7.83, $p<0.01$ (Trial block 2); $F_{5,42}$=7.26, $p<0.05$ (trial block 4); $F_{5,42}$=9.24, $p<0.01$ (trial block 5).

Figure 2.7: Administration of LY-288513 at doses, 0.001 $mg.kg^{-1}$ and 0.01 $mg.kg^{-1}$ produces an increase in startle amplitude compared to vehicle: LY-288513; 0.001 $mg.kg^{-1}$, $F_{7,82}$=5.71, $p<0.05$ (Trial block 1); $F_{7,82}$=5.43, $p<0.001$ (trial block 3); $F_{7,82}$=5.32, $p<0.05$ (trial block 4);

$F_{7,82}$=9.48, p<0.001 (trial block 5): LY-288513; 0.01mg.kg^{-1}, $F_{7,82}$=5.71, p<0.01 (Trial block 1); $F_{7,82}$=7.47, p<0.001 (Trial block 2); $F_{7,82}$=5.32, p<0.05 (trial block 4); $F_{7,82}$=9.48, p<0.001 (trial block 5).

Figure 2.8: The antagonists, CI-988 (at 1.0mg.kg^{-1}) and LY-288513 (at 10.0mg.kg^{-1}) co-administered with chlordiazepoxide (3.0mg.kg^{-1}) did not modify the anxiolytic-like activity of chlordiazepoxide. The significant decrease in startle amplitude was caused by chlordiazepoxide and not maintained by the antagonist (DMSO versus chlordiazepoxide; 3.0mg.kg^{-1}, $F_{3,28}$=19.63, p<0.001 (Trial block 1); $F_{3,28}$=6.84, p<0.01 (Trial block 2) $F_{3,28}$=4.24; p<0.05 (Trial block 3): DMSO versus chlordiazepoxide + CI-988; 1.0mg.kg^{-1}, $F_{3,28}$=19.63, p<0.001 (Trial block 1); $F_{3,28}$=6.84, p<0.01 (Trial block 2), $F_{3,28}$=4.24; p<0.053 narrowly missing significance (Trial block 3): DMSO versus chlordiazepoxide + LY-288513; 10.0mg.kg^{-1}, $F_{3,28}$=19.63, p<0.001 (Trial block 1) where subsequent trial blocks did not exhibit significant differences.

Figure 2.9: The increase in startle amplitudes produced by CCK-4 (0.025mg.kg^{-1}) was attenuated by CI-988 and LY-288513. CCK-4 (0.025 mg.kg^{-1}) compared to chlordiazepoxide (3.0mg.kg^{-1}) $F_{3,27}$=3.94, p<0.05 (Trial block 1); $F_{3,27}$=3.87, p<0.05 (Trial block 2) $F_{3,28}$=4.39; p<0.05 (Trial block 3). This effect was abolished by CI-988 (1.0mg.kg^{-1}) and by LY-288513 (10.0 mg.kg^{-1}) in trial blocks 1 and 2 where the significant amplitude increase by CCK-4 alone was attenuated. Within trial block 3, LY-288513 inhibited CCK-4 significantly, $F_{3,28}$=4.39; p<0.05.

Figure 2.10: CCK-8s (0.001 mg.kg^{-1}) compared to chlordiazepoxide (3.0 mg.kg^{-1}) control group, produced a significant increase in startle amplitude, $F_{3,28}$=4.39, p<0.01 in trial block 1 and in blocks 2 and 3, $F_{3,28}$=4.39, p<0.05. Co-administration with CI-988 significantly attenuated the increase in amplitude by CCK-8s, $F_{3,29}$=8.39, p<0.05 in trial block 1 compared to CCK-8s alone. LY-288513 attenuated the CCK-8s stimulated amplitude increase to a

significant degree, $F_{3,29}$=8.39, $p<0.01$ compared to CCK-8s in trial block 1 and in block 2, $F_{3,29}$=4.10, $p<0.05$. Partial attenuation of effects was observed for trials blocks 2 and 3 (CI-988) and for 3 (LY-288513) but these missed statistical significance.

Figure 2.11: CCK-8us (0.01 $mg.kg^{-1}$) exhibited no significant activity either alone or co-administered with either antagonist.

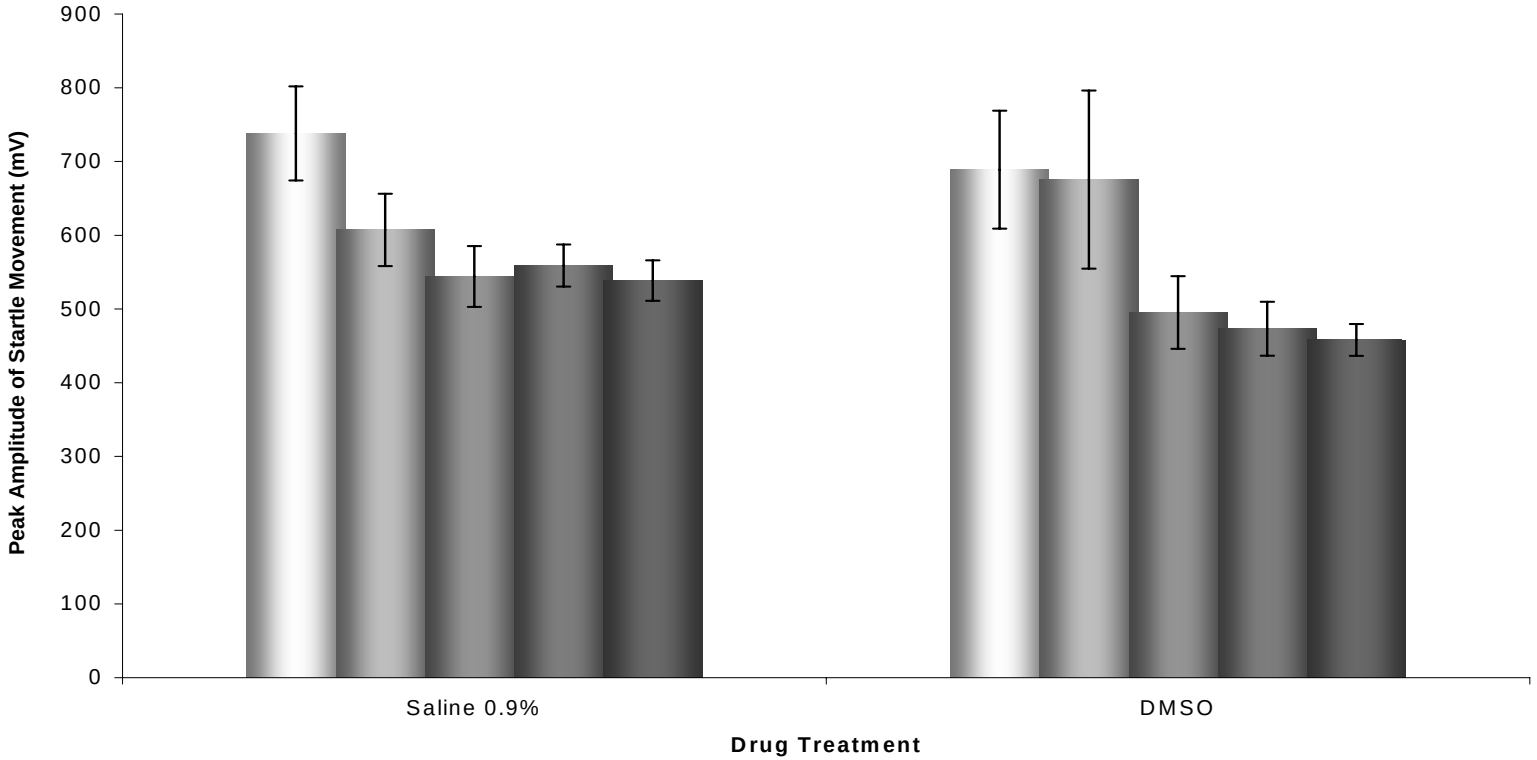


FIG 2.1: Effects of saline (0.9%) and dimethylsulphoxide (DMSO) on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stimuli (results presented as mean with standard error of the mean (SEM)). Consecutive trial blocks are represented from left to right and with darkened shading. Significant differences from the saline group were not observed.

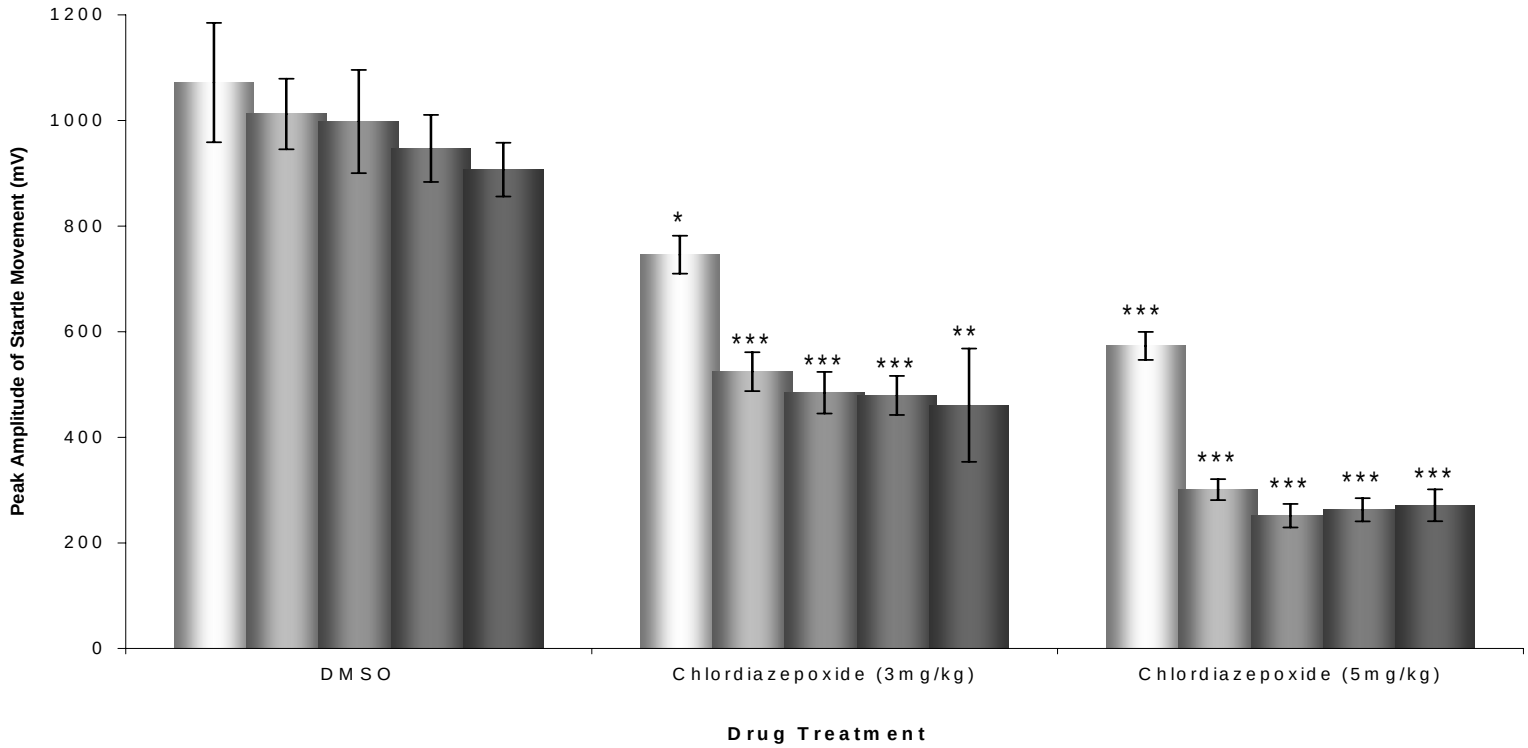


FIG 2.2: Effects of chlordiazepoxide at doses, 3.0 and 5.0 mg.kg^{-1} versus dimethylsulphoxide (DMSO) vehicle, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the DMSO group are shown; *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

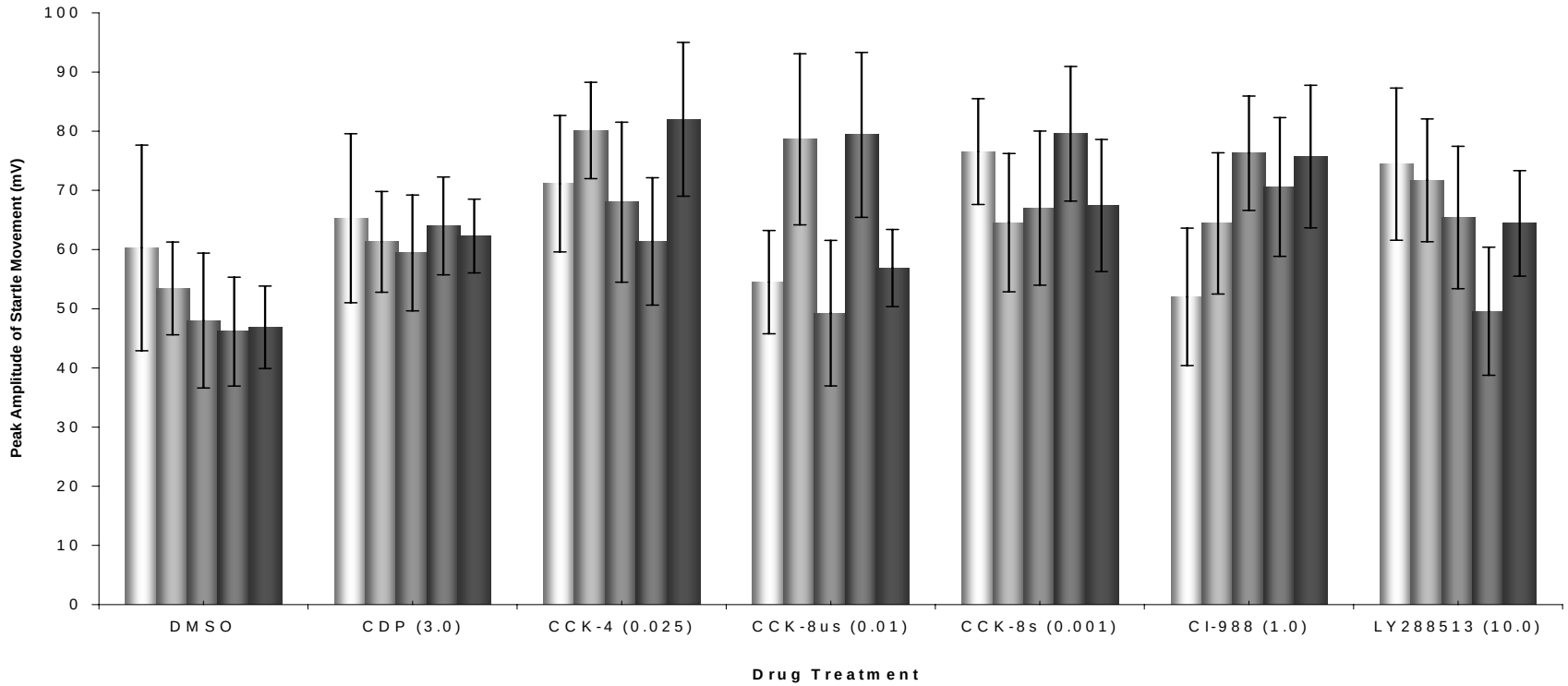


FIG 2.3: Effects of various drugs at specific doses, versus dimethylsulphoxide (DMSO) vehicle on peak amplitude, in rats, in the absence of acoustic startle stimuli, over twenty-five trials. Drugs tested were chlordiazepoxide (3.0mg.kg^{-1}), CCK-4 (0.025mg.kg^{-1}), CCK-8us (0.01mg.kg^{-1}), CCK-8s (0.001mg.kg^{-1}), CI-988 (1.0 mg.kg^{-1}) and LY-288513 (10.0 mg.kg^{-1}). Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual of spontaneous locomotor activity movement amplitudes within the acoustic startle chamber. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the DMSO group were not observed.

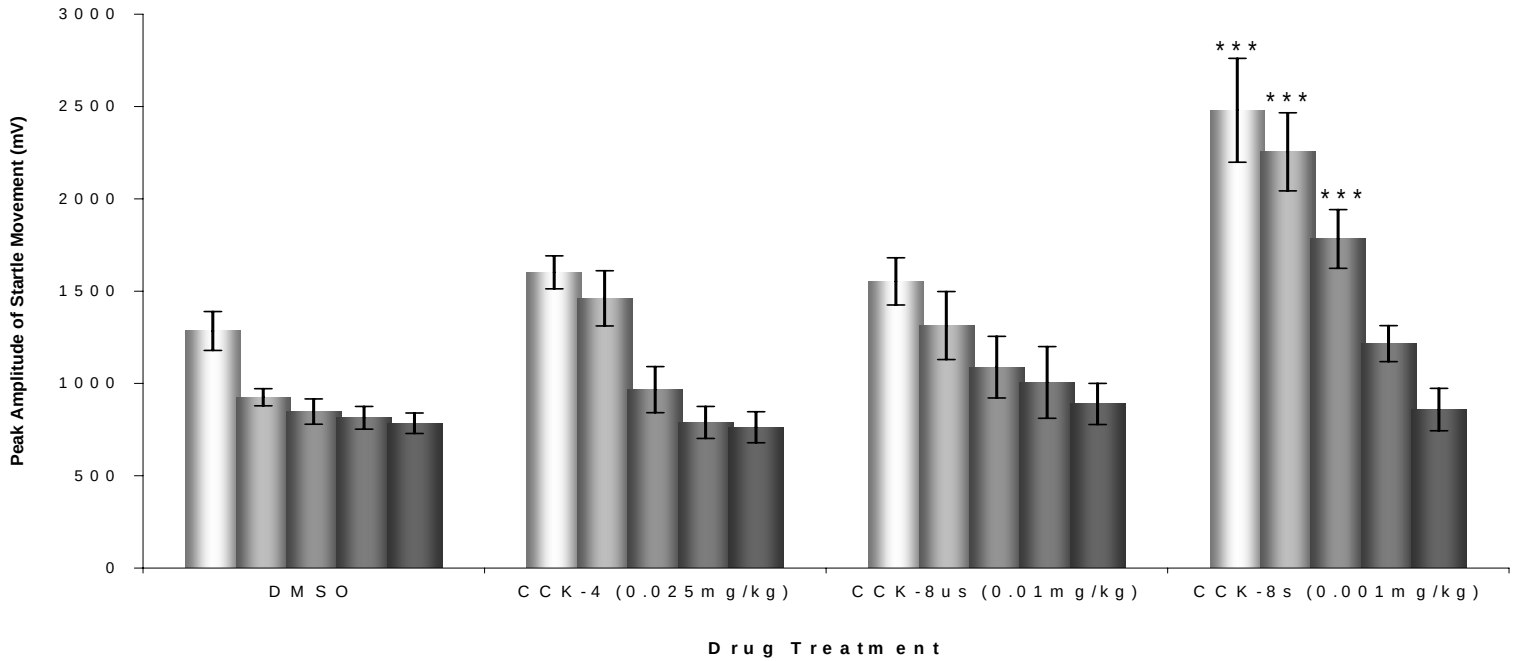


FIG 2.4: Effects of cholecystokinin forms, CCK-4 (0.025 mg.kg^{-1}), CCK-8us (0.01 mg.kg^{-1}), and CCK-8s (0.001 mg.kg^{-1}) versus dimethylsulphoxide (DMSO) vehicle group, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the DMSO group are shown, ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

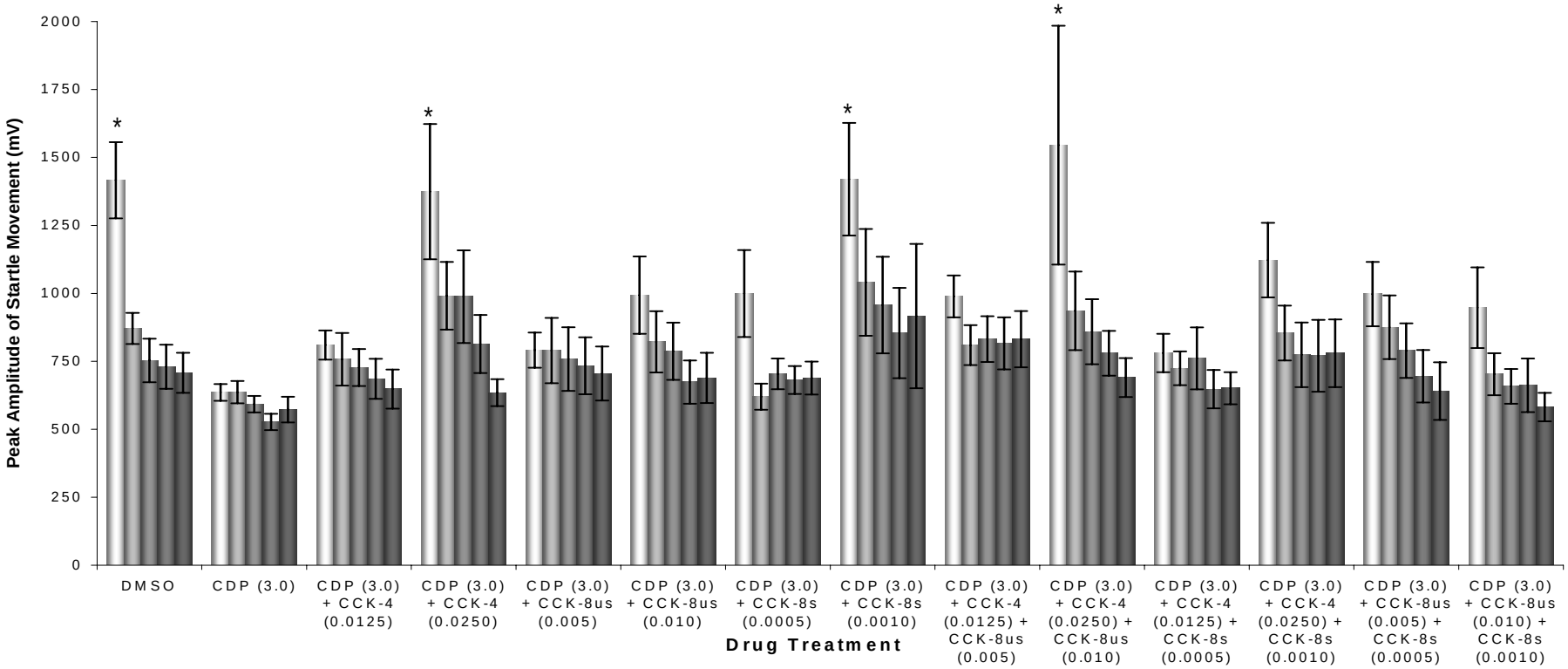


FIG. 2.5: Effects of a range of doses (in mg kg^{-1}) of singularly administered and combined cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination with chlordiazepoxide vehicle, versus the effect of chlordiazepoxide alone, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the chlordiazepoxide group are shown; *$p<0.05$, (Bonferroni post hoc analysis after significant one-way ANOVA).

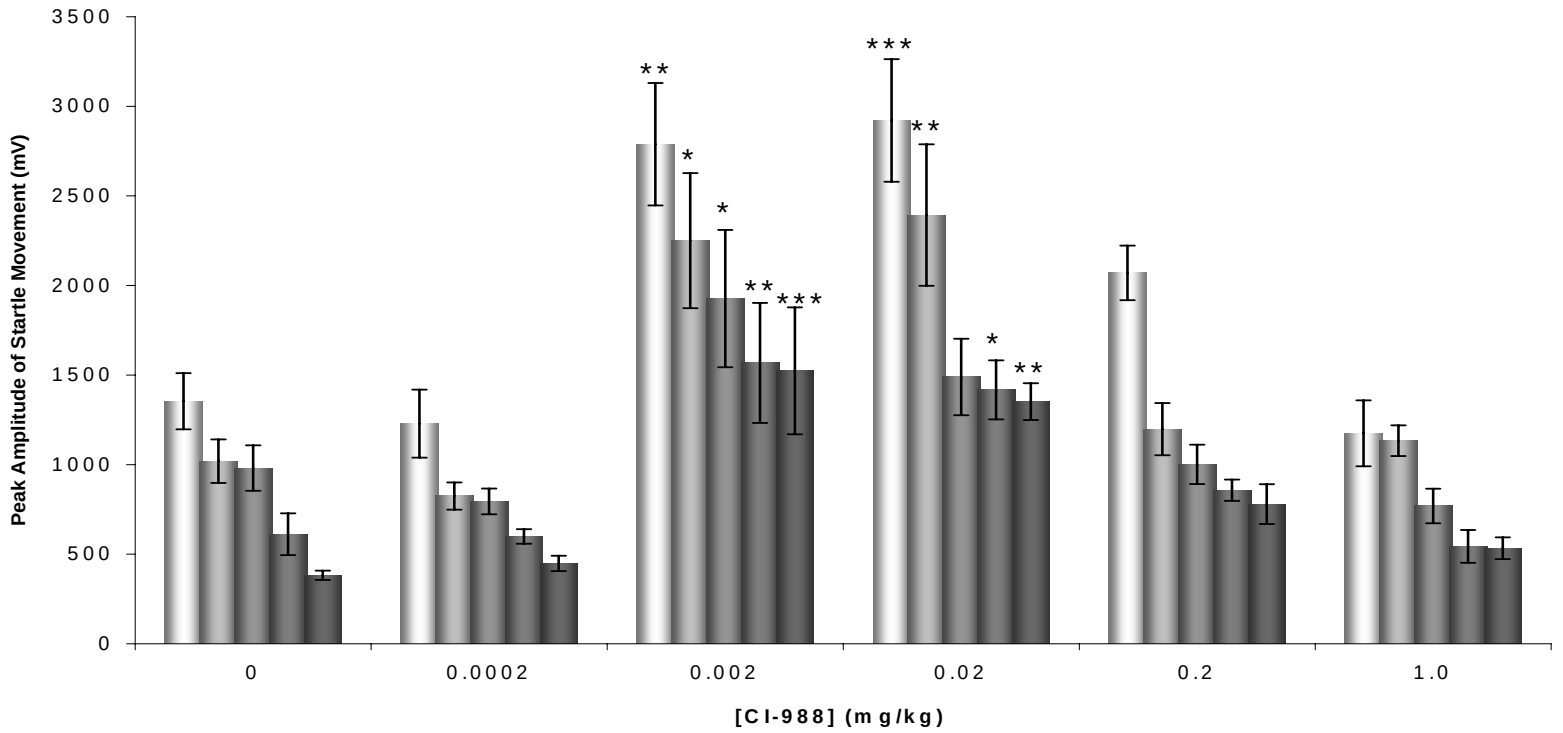


FIG. 2.6: Effects of a range of doses (in mg kg^{-1}) of CCK_2 antagonist, CI-988 with DMSO vehicle, versus the effect of DMSO, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the DMSO group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

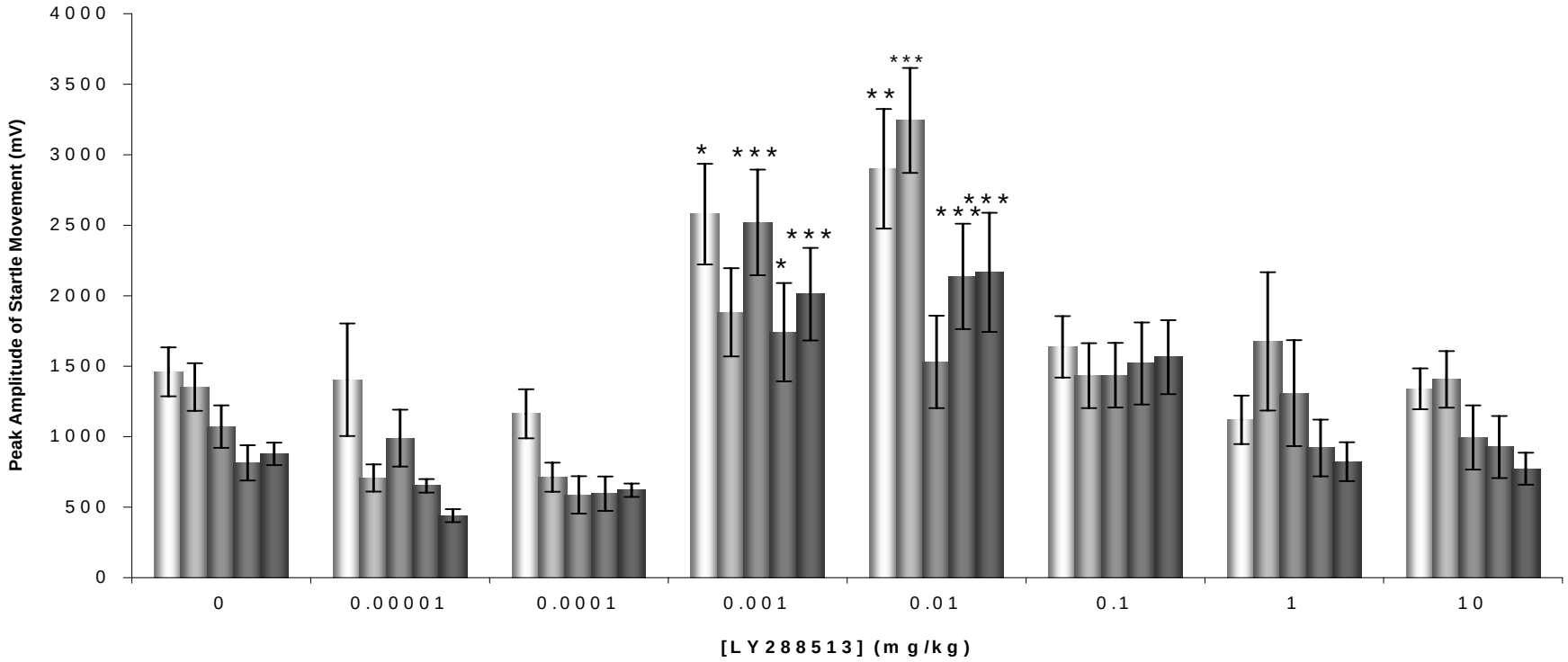


FIG. 2.7: Effects of a range of doses (in mg kg^{-1}) of CCK_2 antagonist, LY-288513 with DMSO vehicle, versus the effect of DMSO, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the DMSO group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

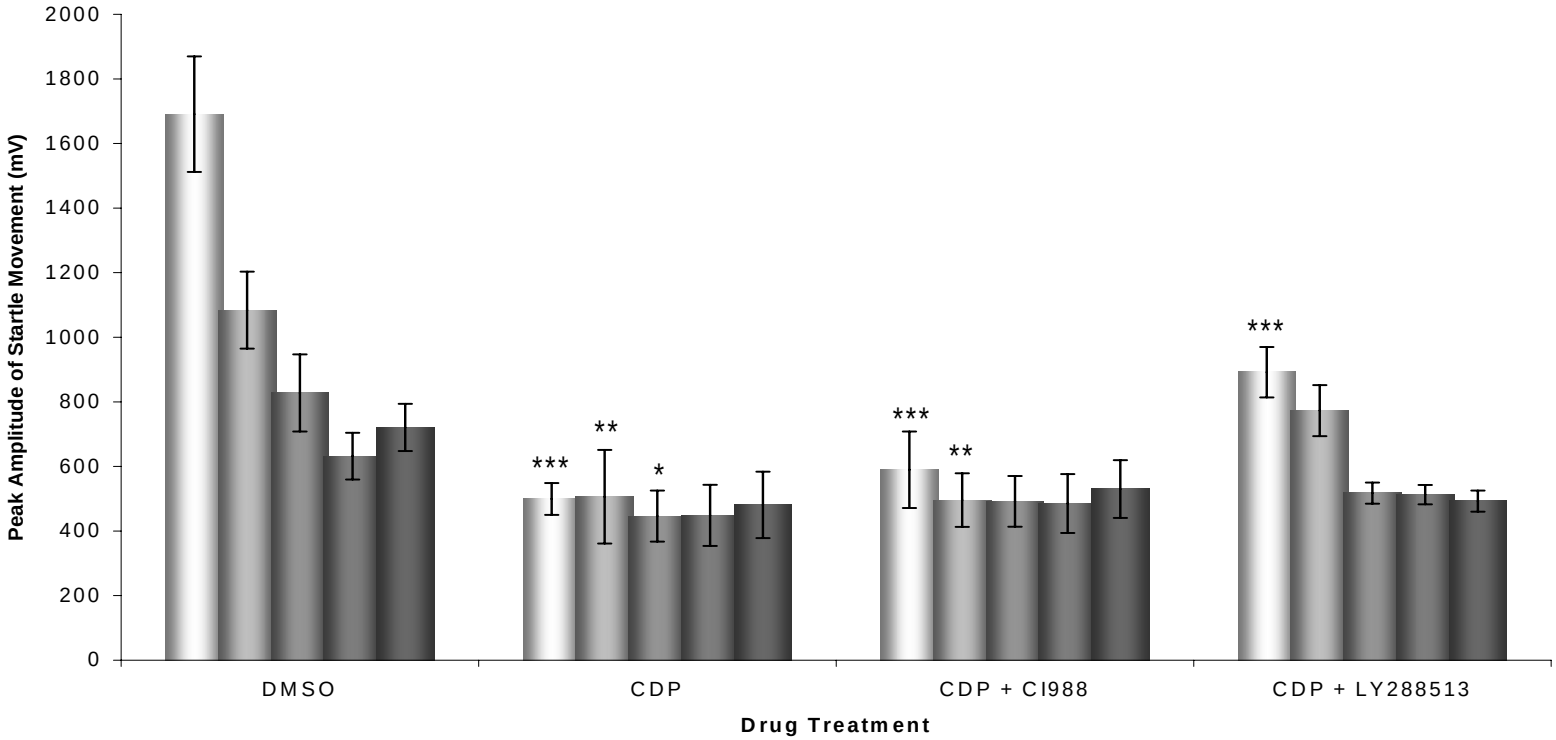


FIG. 2.8: Effects of doses (in mg kg^{-1}) of CCK_2 antagonists, CI-988 (1.0mg.kg^{-1}) and LY-288513 (10mg.kg^{-1}) with chlordiazepoxide (3.0mg.kg^{-1}) vehicle, versus the effect of chlordiazepoxide, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the DMSO group are shown; *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

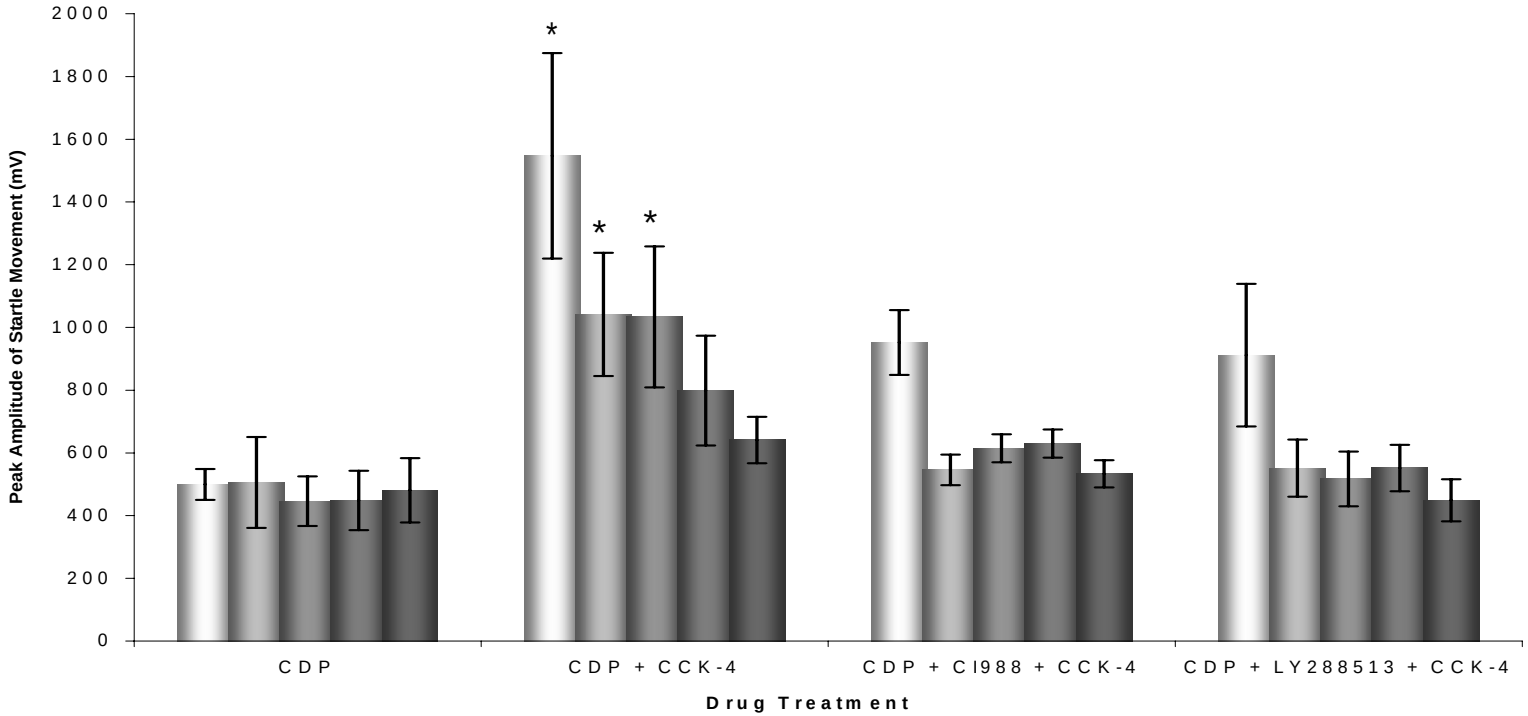


FIG. 2.9: Effects of doses (in mg kg^{-1}) of CCK_2 antagonists, CI-988 (1.0 $mg.kg^{-1}$) and LY-288513 (10.0 $mg.kg^{-1}$) with chlordiazepoxide vehicle on CCK-4 (0.025 $mg.kg^{-1}$) inhibition of chlordiazepoxide activity, versus the effect of chlordiazepoxide, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the chlordiazepoxide group are shown; *$p<0.05$ (Bonferroni post hoc analysis after significant one-way ANOVA).

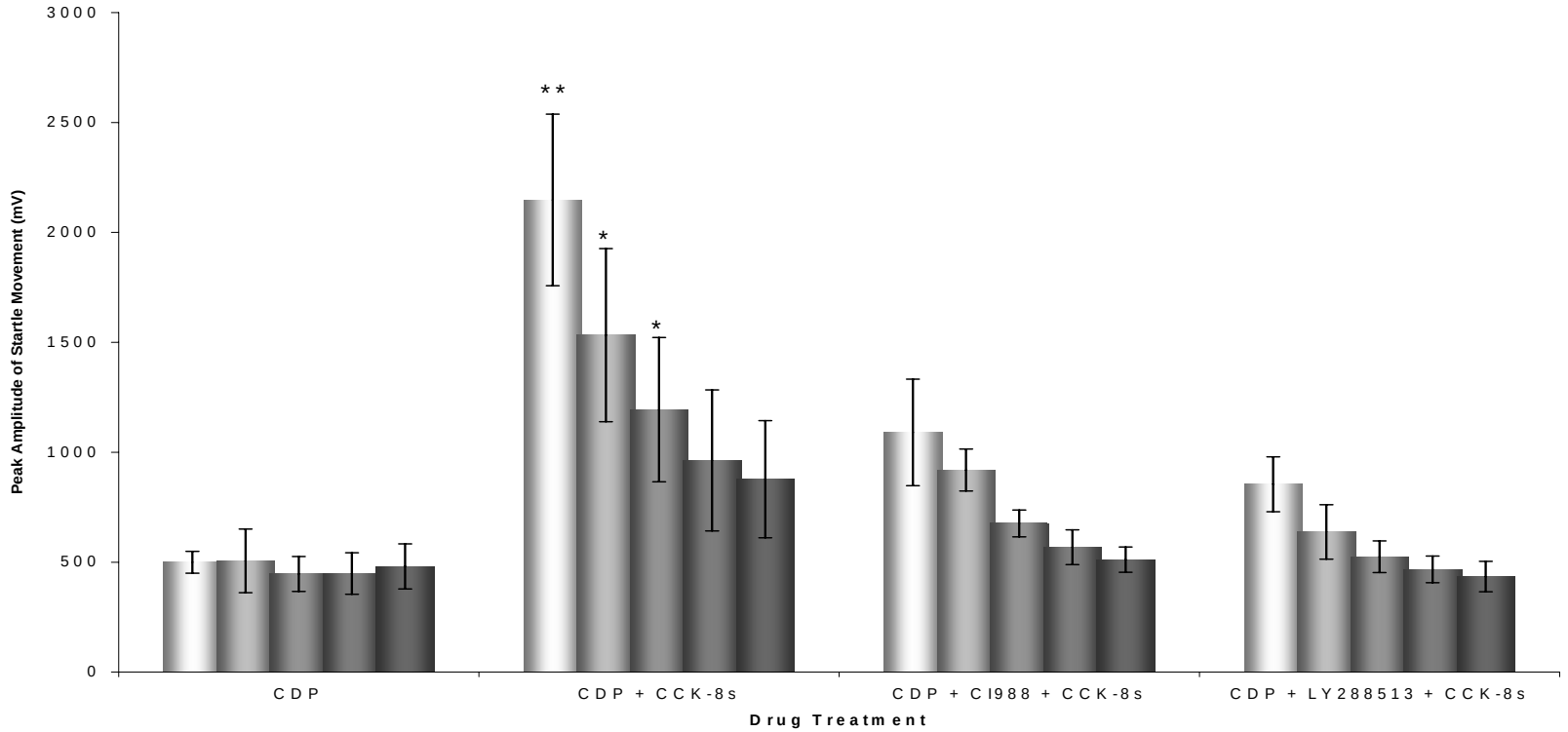


FIG. 2.10: Effects of doses (in mg kg^{-1}) of CCK_2 antagonists, CI-988 (1.0 $mg.kg^{-1}$) and LY-288513 (10.0 $mg.kg^{-1}$) with chlordiazepoxide vehicle on CCK-8s (0.001 $mg.kg^{-1}$) inhibition of chlordiazepoxide activity, versus the effect of chlordiazepoxide, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the chlordiazepoxide group are shown; *$p<0.05$, **$p<0.01$, (Bonferroni post hoc analysis after significant one-way ANOVA).

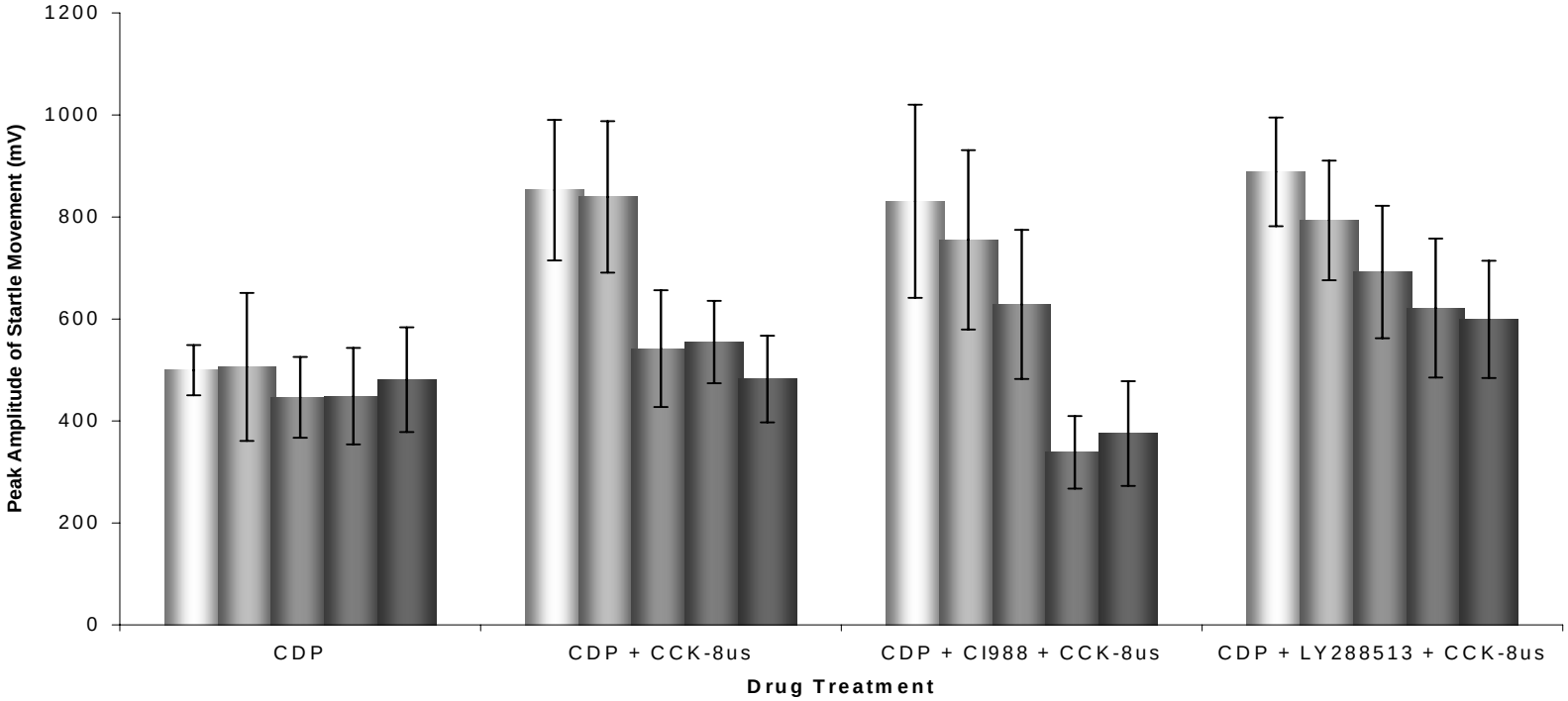


FIG. 2.11: Effects of doses (in mg kg^{-1}) of CCK_2 antagonists, CI-988 (1.0 $mg.kg^{-1}$) and LY-288513 (10.0 $mg.kg^{-1}$) with chlordiazepoxide vehicle on CCK-8us (0.001 $mg.kg^{-1}$) with chlordiazepoxide vehicle, versus the effect of chlordiazepoxide, on acoustic startle peak amplitude, in rats, over twenty-five trials. Five consecutive trial blocks are shown for each treatment (results presented as mean with SEM). These each represent the means of five individual responses to acoustic stimuli. Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the chlordiazepoxide group were not observed (Bonferroni post hoc analysis and one-way ANOVA).

Discussion

The series of preliminary validation experiments showed that, chlordiazepoxide administration, at doses 3.0 and 5.0mg.kg^{-1}, was able to significantly decrease the acoustic startle amplitudes in rats in comparison to the dimethylsulphoxide (DMSO) vehicle, and that the DMSO group did not differ from saline. Given that chlordiazepoxide is known to exhibit muscle relaxant properties, it might be assumed that this effect underlies the decrease in startle amplitudes. Ostensibly this may be partially dismissed given positive chlordiazepoxide effects on other non-motor based measures inherent to the acoustic startle stimulus.

An inhibition of acoustic startle induced ultrasonic vocalisations is observed with rats administered diazepam or flunitrazepam (Kaltwasser 1991). This phenomenon however may also be affected by the sedative and hypothermic properties of benzodiazepines. Hart *et al*., (1998) observed that cardioacceleratory defensive response, associated with anxiety, is enhanced by FG 7142, and attenuated by chlordiazepoxide.

Given this evidence it is probable that chlordiazepoxide is effective in decreasing anxiety levels. However, validation of this model, thus showing that solely the anxiolytic properties of chlordiazepoxide underlie the lowering of startle amplitudes is problematical. There remains a possibility that the muscle relaxant properties of chlordiazepoxide are a factor in this phenomenon, although perhaps not the major element.

In order to validate the proposed anxiogenic component of this model, the use of cholecystokinin itself, both as validator and ligand of study, was preferred over benzodiazepine inverse agonists. Benzodiazepine inverse agonists, such as FG-7142 (N'-methyl-beta-carboline-3-carboxamide) and DMCM (6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate) have shown non-efficacious results, unexpected of their pharmacological nature, in a series of studies. DMCM has been observed to lack any activity in the acoustic startle paradigm (Hijzen *et al*., 1991) or on acoustic startle induced ultrasonic vocalization in the rat

(Olivier *et al*., 1996). FG-7142 has been observed to have no effects on acoustic startle (Rochford *et al*., 1997), nor any effects on acoustic startle induced ultrasonic vocalization in the rat (Kaltwasser, 1991; Olivier *et al*., 1996). Berntson *et al*., (1997) found that FG-7142 attenuates both the somatic and cardiovascular components of the acoustic startle response. Hence, use of benzodiazepine inverse agonists, in validation of this model was prohibited by its' unexpected and somewhat inconsistent nature. Given that CCK-4 and CCK-8, at specific doses, have a well-defined anxiogenic-like profile, and that there seemed to be no alternative anxiogenic agent, the option of validation with these cholecystokinin agents was elected, in addition to a full characterisation of their action. Dimethylsulphoxide (DMSO) was used as a solvent throughout the experiment. This was due to the difficulty in dissolving chlordiazepoxide in saline, coupled to the ease with which cholecystokinin forms were dissolvable in DMSO.

It may be considered noteworthy that chlordiazepoxide, at 3.0 and 5.0mg.kg^{-1}, produced statistically significant decreases in startle amplitudes throughout the trial sequence, whereas the anxiogenic cholecystokinin agents used only produced significant increases within the initial startle period. Given that clinically, chlordiazepoxide at higher doses produces sedation, it was considered necessary to rule out an explanation of sedation underlying this phenomenon, rather than anxiolysis. Therefore, an analysis of the gradient during the habituation phase was carried out. Examination of the line gradients demonstrates a habituation profile with an exponential phase between trial blocks 1 and 2, followed by a plateau phase between trial blocks 3 and 5 (DMSO, exponential phase, -59.52 y/x, chlordiazepoxide, 3.0mg.kg^{-1}, exponential phase -221.6; 5.0mg.kg^{-1}, exponential phase, -272.3). It would be expected that a relatively flat habituation profile, would be observed for sedated animals, whereby this habituation would not occur. This is clearly not the position here with chlordiazepoxide treated animals and the marked exponential phase. In addition, further evidence for the anxiolytic-like property of chlordiazepoxide was provided when measuring

spontaneous activity in the startle chamber, where chlordiazepoxide showed no inhibition of locomotor activity.

Spontaneous locomotor activity within the acoustic startle chamber was measured and showed no significant differences between drug groups tested at their effective doses. This further discounts possibility of effects of sedative action or locomotor stimulation on startle amplitude, over the range of drugs tested.

Comparison between chlordiazepoxide (control) and chlordiazepoxide with cholecystokinin forms yielded significant increases in startle amplitudes within the first trial period for CCK-4 (0.025 $mg.kg^{-1}$) and CCK-8s (0.001 $mg.kg^{-1}$). CCK-8s at this dose produces an anxiogenic-like response in nondrugged animals (versus DMSO vehicle) and drugged animals (co-administered with chlordiazepoxide). Interestingly, CCK-4 appears to counteract the effects of chlordiazepoxide, but is unable to produce an anxiogenic-like action when compared to DMSO vehicle. Given that CCK-4 narrowly missed significance in trial block two when compared with DMSO, it may be derived that ceiling effects were observed in this case. This effect is apparent where an anxiety state of the entire batch of rats used in the experiment was at a suitably high level, as such that any further increase in anxiety would be masked by the confines of the experiment. This account of ceiling effects, nonetheless, is still rather questionable. The maximum startle amplitude measured by this apparatus is around two and a half times that which is produced within the CCK-4 group (4095 mV maximal compared to 1600 mV observed). A fundamental mechanism underlying this phenomenon, although derived solely from conjecture, may be that CCK-4 is able to counteract chlordiazepoxide activity due to some dampening effect upon benzodiazepine receptor activity, and that it may exhibit little or no anxiogenic activity itself within the startle paradigm. A more plausible explanation for these events, however, is that this observation may be apparent whereby the anxiety level produced by CCK-8s may be of a higher intensity compared to that of CCK-4 / CCK-8us. Where, these anxiety states might be observed as vaguely equivalent on the elevated

plus-maze, an acoustic startle paradigm might be less sensitive to anxiety levels. Furthermore, ceiling effects regarding high anxiety levels on the plus-maze would make individual anxiety levels, conferred by each drug species to be equivalent. Subsequently only, the very high level of anxiety induced by a highly efficacious, CCK-8s would observable within the less sensitive, acoustic startle paradigm. I feel that this is the most likely explanation of events. It cannot be assumed that two quite separate anxiety models, measuring very different behaviour, would be identical in their sensitivity to anxiety level.

It may be plausible to propose that cholecystokinin driven anxiogenesis would be gated via the CCK_1, whereby CCK-8s has equivalent affinity for CCK_1 and CCK_2 receptor subtypes, where CCK-4 has specific affinity for only CCK_2 receptor subtypes (Hunter *et al*., 1993). However, the following study showed that the two CCK_2 receptor specific antagonists, CI-988 and LY-288513, at doses 1 and 10 $mg.kg^{-1}$ respectively, were able to attenuate the effects of CCK-4 and CCK-8s. This produced a marked, and statistically significant decrease in startle amplitude when co-administered with CCK-8s (in comparison with CCK-8s alone). Administration of CI-988 and LY-288513 abolished the significant difference in amplitude increase associated with CCK-4, although only reaching significance compared to CCK-4 in trial block three. This data implies that the increases in amplitude are gated strongly at the CCK_2 receptor.

Co-administration of cholecystokinin forms, CCK-4 (at $0.025mg.kg^{-1}$) and CCK-8s ($0.001mg.kg^{-1}$) with chlordiazepoxide, yielded some interesting data. The individual activities of the two forms, at these doses, were negated upon combination. This would be as expected where an inverse bell shaped dose response curve were apparent. Within the elevated plus-maze, such a dose response curve was observed, where subsequently the dose eliciting a maximal anxiogenic-like activity was selected for assessment within the startle paradigm. The combination of the cholecystokinin forms at these doses would be equivalent to any single form at twice the dose, at which it is observed to be relatively inactive in the plus-maze. Interestingly, however, a combination of both forms at half this dose did not yield any data

indicative of anxiogenesis. If both forms exhibited activity on one receptor subtype in order to elicit an anxiety-like effect in this paradigm, then a combination of both forms at half their maximally active doses would be expected to elicit an effect similar to that of either form individually. This is clearly not the case. It may therefore be hypothesized that these forms individually achieve their anxiogenic-like activities via independent mechanisms, most probably through distinct sub-populations of the CCK_2 receptor subtype. Furthermore, it may be proposed that a combination of activities at both sites will nullify any anxiogenic effects of activation at a single site. This equilibrium, underlying the normal non-anxious state, when disturbed would result in an anxious state. This may also be supported with evidence from dose response studies using CCK_2 antagonists. the diphenylpyrazolidinone, LY-288513 and peptoidal based, CI-988, antagonists, exhibited bell shaped dose response curves with regard to anxiogenic-like behaviour on acoustic startle amplitude. These findings are, however not in disagreement with Helton *et al*., (1996), who observed that LY-288513 had no effects on acoustic startle and exhibited anxiolytic properties on the elevated plus-maze. At the highest dose tested ($10mg.kg^{-1}$), it was found that this anxiogenic-like activity was abolished and similarly in Helton's study, doses of 100 to $1000mg.kg^{-1}$ did not elicit this response. The bell shaped dose response curve, with anxiogenic doses of a 100 times smaller magnitude, observed here, may be explained again by the two-site hypothesis for CCK_2 receptors. If these sites had differing affinity for one antagonist, then at a lower dose one such site will be activated and at a higher dose, both sites will be activated. This would support my CCK_2 receptor equilibrium hypothesis, whereby activation of either receptor, causes an imbalance resulting in an anxious state, whereas activation of both receptor sites forces equilibrium, thus a non-anxious state is apparent. This unusual dose response curve, which may be observable for both agonists (although not tested here) and antagonists for the same receptor, would be explained almost flawlessly by the aforementioned hypothesis. Furthermore, effects of cholecystokinin forms in combination showing an absence of activity, is indicative of such a

hypothesis.

Recent biochemical studies have perhaps supported this phenomenon. Pommier *et al*., (1999) demonstrated that CCK_2 receptors are coupled to two secondary messenger cascades, a pertussis toxin-sensitive (phospholipase C pathway leading to the production of inositol phosphate and arachidonic acid), and a pertussis-insensitive G protein pathway (phospholipase A2 pathway leading to the release of arachidonic acid). Previous binding studies have suggested the existence of two or more affinity states for CCK_2 receptors, which could correspond to different coupling states of the receptor to G proteins (Harper *et al*., 1999; Harper *et al*., 1996). According to this model, agonists or antagonists could recognize preferentially either conformation of the activated receptor, leading to variable behaviour in a system containing a single receptor type (Pommier *et al*., 1999).

An area of concern observed within this experiment however is that of inconsistency in amplitude between different sets of experiments using the same treatments. This may be partially explained by several disruptive factors affecting housing conditions which were apparent. These factors were not within the control of the experimenter. These factors may also explain the marked habituation to startle observed with the DMSO group in figure 2.5. This profile appears to mimic that of the putative anxiogenic agents.

With an attempt in equating these findings to the human condition, it may be speculated that activation of one subsystem may confer certain behavioural changes indicative of particular anxiety disorders, whereas activation of the other may produce changes related to a different set of anxiety-like symptoms. This may partly explain the range of different anxiety disorders in the clinical condition, although this is highly speculative.

CHAPTER THREE – ANIMAL MODELS OF UNCONDITIONED FEAR: ELEVATED PLUS-MAZE

Introduction

The elevated plus-maze test of anxiety is derived from the work of Montgomery (1955) on the relation between fear and exploratory drives in rats, the basic premise of which was that environmental novelty evokes both fear and curiosity, thereby creating a typical approach-avoid conflict (Rodgers and Cole 1994). Montgomery (1955), found that rats consistently showed higher levels of exploration of enclosed alleys than in open alleys when faced with a choice of alley type in an elevated Y-maze. These data were interpreted as indicating that open arms engender a higher level of fear than enclosed arms, leading to increased avoidance. The open areas might be associated subjectively with a greater risk of predation whilst the enclosed environments would confer a secure subjective experience.

This observation was further developed by Handley and Mithani (1984) as a potential basis for an animal model of anxiety. Their model was essentially an X-maze elevated 70 cm from ground level with two enclosed and two open arms (both opposite each other). A range of benzodiazepine agonists increased the ratio of open over total arm entries whereas several inverse agonists decreased this ratio significantly, conferring a predictive validity over a ten-minute trial period. From this evidence they proposed that the X-maze might provide a valid model of 'fear-motivated' behaviour in rodents. This study was followed by a more extensive investigation by Pellow *et al*., (1985) which examined behavioural and physiological changes in addition to pharmacological effects. Both studies used the same rat strain and mazes of similar dimensions with procedural variation. Pellow *et al*., used a 5-minute exposure to a hole-board apparatus followed by a 5-minute period on the elevated plus-maze. In addition, Pellow *et al*., instituted an open over total time measure as an index of anxiety. A cross-species sensitivity to the elevated plus-maze as a measure of anxiety was subsequently confirmed by

Lister (1987) using NIH Swiss mice. Lister adapted the maze with an appropriate decrease in dimensions. Using the procedural modification utilised by Pellow *et al*., (1985) with prior hole-board exposure and a 5-minute test, Lister observed a similar pharmacological and behavioural profile to that described for rats. An important variation conferred within the Lister model was in the use of clear Perspex in construction of the closed arm walls. This adaptation allowed equalisation of light levels in all four arms which would eliminate the rodent specific preference for darkened areas over lighted. Hence, this variation provided a more exact model of rodent preference for enclosed over open areas rather than for light level differences.

Avoidance of the open arms of the maze is consistent with the view that these areas evoke a stronger fear reaction than the enclosed arms, thereby providing the model with face (and possible construct) validity (Treit *et al*., 1993a). Pellow *et al*., (1985) observed more fear-related behaviours; defecation, immobility, and freezing on the open arms of the maze, which may provide evidence of face validity. In addition, they observed that inescapable confinement upon the open arms produced a significant two-fold increase in plasma corticosterone level compared to those confined within the closed arms. This may also provide some evidence of the construct validity for the model.

Rodgers and fellow researchers (Lee and Rodgers, 1991; Rodgers *et al*., 1992) observed that exposure of mice to the elevated plus-maze produces a non-opioid form of 'stress analgesia', an effect that is prevented by prior treatment with anxiolytic drugs. This may be an innate response to stress whereby, an animal under assault, will subjectively focus on escape, rather than pain resulting from injury. This phenomenon of plus-maze antinociception has been independently confirmed for both rats (Taukulis and Goggin, 1990), and mice (Conceicao *et al*., 1992). These studies provide further evidence for face and construct validity within this paradigm.

Pharmacological validation of the elevated plus-maze

Benzodiazepine specific receptor ligands:

Anxiolytic-like activity on the plus-maze has been observed following microinfusion of midazolam into several areas of the brain; basolateral amygdala (Green and Vale, 1992), the dorsal periaqueductal grey region (Russo *et al*., 1993) and the septum (Pesold and Treit, 1994; Pesold and Treit, 1992; Pesold and Treit, 1996).

Withdrawal from a benzodiazepine chronic treatment schedule shows an anxiogenic-like profile on the maze (Baldwin and File, 1988; File *et al*., 1987) which can be reversed by the benzodiazepine receptor antagonist, flumazenil (which alone exhibits no activity) (File, 1990).

Non-benzodiazepine GABA receptor specific ligands:

A range of other compounds exert an influence on the GABA receptor/chloride channel complex. Barbiturates, as well as progesterone (Reddy and Kulkarni, 1997) and several pregnane-related steroids; alphaxalone (Britton *et al*., 1991), and pregnanolone (Rodgers and Johnson, 1998), in addition to exerting barbiturate-like effects on the GABA receptor/chloride channel, also produce anxiolytic effects in this test. As might be predicted, consistent anxiolytic-like activity has also been reported for ethanol, which is blocked by benzodiazepine receptor antagonists/inverse agonists such as Ro154513 and FG7142 (Lister, 1988). Withdrawal from chronic ethanol treatment produces an anxiogenic-like behavioural profile on the maze (Baldwin *et al*., 1991; File *et al*., 1991; Rassnick *et al*., 1993) which can be reversed by chlordiazepoxide, flumazenil and baclofen (File *et al*., 1991; File *et al*., 1992), alpha-helical corticotrophin releasing factor (CRF) (Baldwin *et al*., 1991), buspirone (Lal *et al*., 1991) and mianserin (Lal *et al*., 1993). Pentylenetetrazol and picrotoxin, which inhibit the functioning of the GABA receptor/chloride-channel complex, have consistently been reported to produce anxiogenic-like effects (Treit, 1987). Several direct $GABA_A$ and $GABA_B$ agonists (e.g.

muscimol) have also been reported to have anxiolytic effects (Corbett *et al*., 1991), while the $GABA_B$ agonist baclofen, is apparently anxiolytic only in handling-naive rats (Andrews and File, 1993). Essentially, therefore the elevated plus-maze is bi-directionally sensitive to changes in anxiety induced by benzodiazepine receptor ligands. The phenomenon of benzodiazepine-withdrawal anxiogenesis further adds to the pharmacological validity of the test for agents of this class.

Adrenoceptor ligands:

The effects of alpha-adrenoceptor ligands on anxiety in the elevated plus-maze have been highly variable. Several α1- and α2-agonists studied have proven quite equivocal regarding their effects on the maze. The relatively non-selective α2-antagonist, yohimbine, however has consistently produced anxiogenic-like effects in the elevated plus-maze while effects of more selective α2-antagonists are much less convincing. ß-adrenoceptor antagonists have also been inconsistent in effect (for review see Rodgers and Cole, 1994). However, it is observed that several of these "beta-blockers" exhibit additional affinity for serotonergic receptors. A study by Njung'e *et al*., (1993), alluded to this conclusion where of a range, of non-specific and receptor-selective ß-ligands tested, only those antagonists with additional high affinity for $5HT_{1A}$ receptors (i.e. pindolol and alprenolol), exert anxiolytic effects in the elevated plus-maze. The remaining antagonists and a series of agonists were inactive. This evidence appears to suggest that, given the reputed relationship of α2-adrenergic function and panic disorder, and ß-adrenoceptors in generalised anxiety (Nutt, 1991), predictive (pharmacological) validation for these conditions is not satisfied within the plus-maze.

Serotonergic receptor ligands:

In the plus-maze, PCPA-induced 5-HT depletion has generally been reported to reduce anxiety (Critchley *et al*., 1992; Moser, 1989; Treit *et al*., 1993b). In converse, systemic administration of the 5-HT precursor, L-5-HTP, is reported to exert anxiogenic-like effects in the maze

(Soderpalm *et al*., 1989). Handley and McBlane (1993) observed that 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT), a putative $5HT_{1A}$ agonist, produces a contradictory behavioural profile at the same dose with anxiolytic effects or anxiogenic-like effects depending upon the level of maze lighting (high and low illumination respectively). Similar highly variable results have been obtained with buspirone when administered systemically (Cole and Rodgers, 1994). Andrews and File (1993) have attributed this pattern of inconsistency to anxiogenic-like effects of buspirone produced only in handling habituated rats and zaclopride only in those handling naïve. $5HT_2$ and $5HT_3$ receptor antagonists have also yielded similarly inconsistent results in the plus-maze. Serotonergic reuptake inhibitors are also highly inconsistent in their activity on the maze. Pharmacological intervention with serotonergic agents appears to depend strongly upon endogenous anxiety levels and varies between genetic strain, housing conditions, handling experience and the specific conditions under which the test is conducted (Rodgers and Cole, 1994).

Cholecystokinin receptor ligands:

Kharro *et al*., (1989) were the first to report that systemic administration of the cholecystokinin (CCK) agonists, caerulein (non-selective) and pentagastrin (CCK_2 selective) decreased the exploratory activity of mice in the elevated plus-maze. Harro *et al*., (1990) went on to demonstrate that this anxiogenic-like action of caerulein was attenuated by acute pre-treatment with the cholecystokinin antagonist proglumide at high doses, but not with diazepam. Furthermore, proglumide was observed to increase exploratory activity of rats in the plus-maze. Mannisto *et al*., (1994) observed that the "anxiogenic" effect of caerulein was antagonised by the CCK_2 specific antagonist, L-365260, but was increased by the cholecystokinin non-selective antagonist devazepide (at high doses). L-365260 and devazepide at these doses, alone were unable to modify behaviour. It is of note however that co-administration of caerulein with devazepide also inhibited behavioural indices within the open field test, thus indicating an inhibitory effect upon locomotor activity. Caerulein alone

exhibited no modification of activity in this open field test.

Several studies have demonstrated that peripheral administration of the cholecystokinin tetrapeptide, CCK-4 decreased exploratory activity in an elevated plus-maze, (Harro and Vasar, 1991a; Hadjiivanova *et al*., 1995). This action was attenuated with cholecystokinin receptor antagonists; proglumide, lorglumide, L-365260 and devazepide (Harro and Vasar 1991a). Rex *et al*., (1994) confirmed that a peptidase resistant form, BOC-CCK-4 also modified behavioural indices within the plus-maze, indicative of anxiety.

While Rex *et al*., (1994) found CCK-8s to be without effect within the dose range tested, Vasar *et al*., (1994) observed that CCK-8s, at 2.5-10 $\mu g.kg^{-1}$; via the subcutaneous (s.c.) route, decreased the exploratory activity of mice in an elevated plus-maze. Interestingly though, L-365260, (1-100 $\mu g.kg^{-1}$; i.p.), proglumide, (0.1-1 $\mu g.kg^{-1}$, i.p.), and devazepide, at a high dose, (100 $\mu g.kg^{-1}$) actually potentiated the anti-exploratory effect of CCK-8s (2.5 $\mu g.kg^{-1}$).

Johnson and Rodgers (1996) in contrast observed that CCK-4 (12.5-100 $\mu g.kg^{-1}$) failed to produce any significant behavioural effects in the murine plus-maze, while CCK-8s induced signs of behavioural inhibition at 100 $\mu g.kg^{-1}$ without altering anxiety-related indices.

Structural modification of the CCK-8 peptide has yielded several highly selective CCK_2 agonists exhibiting good bioavailability and degradative resistance. Following intravenous (i.v.) injection of one such analogue BC-264, in tritiated form, the ligand was found in its intact form in mouse brain tissue. Interestingly, BC-264 systemically administered increased the anxiety-like responses of the "anxious" rat and decreased these responses in "non anxious" animals in the elevated plus-maze (Dauge *et al*., 1992)). In a study by Derrien *et al*., (1994) while the nonselective cholecystokinin agonist BDNL and selective CCK_2 agonist BC-197 induced anxiogenic-like effects in the plus-maze, BC-264, however, had no observed activity. The effects of BDNL were not modified by L-365260, but were suppressed by CI-988 and by high doses of L-364718 (a selective CCK_1 antagonist). BC-197 induced effects were also

blocked by CI-988. BC-264 produced anxiogenic-like dose dependent effects when administered with L-365260 (200 $\mu g.kg^{-1}$) and opposing effects with CI-988 (2 $\mu g.kg^{-1}$). Binding competition with [^{3}H]pBC-264 using guinea pig, mouse, and rat brain membranes adhered significantly to a two-site model than to a one-site model with competition from BC-197 but not with BC-264 (Derrien *et al*., 1994). This, examined further in chapter six, indicates that where BC-197 exhibits differing affinities for two distinct receptor sites, BC-264 exhibits a very similar affinity for these sites (which is not revealed by saturation analysis). Structural modification of CCK-4 yielded another highly selective CCK_2 receptor ligand, JMV-320 which also decreased exploratory activity in the elevated plus-maze but not in the open field test, indicating an anxiety-like behaviour within the plus-maze (Hadjiivanova *et al*., 1995).

Systemic Administration of Cholecystokinin Antagonists:

A study first utilising newly synthesised peptoidal based CCK_2 specific antagonists, CI-988 and PD-135158 demonstrated that they produced potent anxiolytic effects in the mouse black/white box test, the rat elevated plus-maze test and the rat social interaction test after systemic administration. In addition these antagonists were marked by an absence of sedation and long-term abuse potential. Furthermore, both attenuated withdrawal anxiety from a variety of abused drugs (Hughes *et al*., 1990; Costall *et al*., 1991). CCK_2 selective antagonists PD-135666 (CCK_2 selectivity 260:1) and PD-141479 (30:1) and the unselective CCK_1 and CCK_2 receptor antagonist PD-142898 (1.1:1) also produced anxiolytic effects (Singh *et al*., 1995).

Administration of synthetic antagonists, PD-135158 (cholecystokinin non-specific) or CI-988 ($1mg.kg^{-1}$ s.c.) attenuated heightened anxiety-like behaviour in the murine plus-maze resulting from chronic ethanol administration. CI-988 and L-365260 also produced anxiolytic-like effects alone (Wilson and Little, 1998).

The CCK_2 specific antagonist, L-365260 also produced an increase of indices within the plus-maze, indicative of anxiolysis. Both the non-CCK_2 antagonists, 3S-(-) enantiomer of L-365260

and devazepide failed to modify exploratory behaviour, suggesting that CCK_2 receptor activation underlies this behaviour (Rataud *et al*., 1991)

A pyrazolidinone related CCK_2 antagonist LY-288513 (3, 10 $mg.kg^{-1}$, IP; 10, 30 $mg.kg^{-1}$, PO) produced an anxiolytic-like action in mice with a magnitude of effect similar to that of diazepam, except without affecting muscle tone, neuromuscular coordination, or sensorimotor reactivity (Helton *et al*., 1996). Novel quinazolinone and arylurea derived CCK_2 antagonists are also reported as orally active in the plus-maze (Padia *et al*., 1998).

Revel *et al*., (1998) demonstrated that CR-2945, an antranilic acid derived cholecystokinin antagonist showing a very high selectivity for CCK_2 receptors (9000:1) over CCK_1, also showed significant dose-dependent anxiolytic-like effects in four rodent tests of anxiety including the mouse black/white box, rat elevated plus- and zero- maze, and punished licking. Within this study commonly used benzodiazepine derived CCK_2 antagonists PD-135158 and L-365260 were inactive in the elevated plus-maze after oral administration. The magnitude of the activity of CR-2945 was comparable to that of diazepam, but without sedation and ataxia. Furthermore, a chronic treatment with CR-2945 (seven days) did not induce tolerance or withdrawal anxiety in rats.

In contrast to these positive findings, Johnson and Rodgers (1996) observed that cholecystokinin receptor antagonists; devazepide, L-365260 and PD-135158 within the range 1.0 $\mu g.kg^{-1}$-1.0$mg.kg^{-1}$, were without significant effect on the murine plus-maze. Dawson *et al*., (1995) also noted a lack of effect of CCK_2 antagonists L-740093 (0.1-1.0$mg.kg^{-1}$), L-365260 (0.00001-10.0$mg.kg^{-1}$), and CI-988 (0.01-1.0$mg.kg^{-1}$) within the plus-maze in rats. Griebel *et al*., (1997) again confirmed a lack of activity of CCK_2 antagonists PD-135158 (0.001-1.0$mg.kg^{-1}$) and LY-288513 (1-3$mg.kg^{-1}$) within the plus-maze.

Central Administration of Cholecystokinin Antagonists:

Holy and Wisniewski (1998) observed that intracerebroventricularly administered CCK-8 and CCK-4 induced an anxiogenic-like effect in the plus-maze. Intracerebroventricular administrations of caerulein or pentagastrin increased dose dependently the level of anxiety on the plus-maze. CI-988 dose dependently antagonised an anxiogenic-like response to pentagastrin but not that induced by pentylenetetrazol when administered systemically (Singh *et al*., 1991). Intracerebroventricularly infused CCK-antisense oligodeoxynucleotide into rats (over 3 days) was observed to significantly decrease anxiety-like behaviour in rats within the plus-maze (Cohen *et al*., 1998).

Intra-amygdaloidal administration with 20ng of Boc-CCK-4 and 1ng of CCK-8s failed to influence anxiety-related behaviour within the plus-maze according to Huston *et al*., (1998). However, Belcheva *et al*., (1994) observed that bilateral microinjections of CCK-8 into the amygdala confirmed the anxiogenic-like effect of CCK-8 on the plus-maze.

Meanwhile, Dauge *et al*., (1989b) observed that administration of CCK-8s into the posteromedial nucleus accumbens produced a decrease in exploration within the elevated plus-maze which was reversed by systemic administration of the selective CCK_1 antagonist L-364718. This was not observed after administration of CCK-8us in this accumbal region. Administration into the anterior nucleus accumbens however also had no effect. Ladurelle *et al*., (1995) went on to show that with intra-accumbal administration, an anxiogenic-like effect of CCK-8s was observed in the elevated plus-maze, but only in rats previously submitted to a novel environment (four-hole box).

Benzodiazepine Interactions:

With regard to an association between the benzodiazepine and cholecystokinin systems there are several important pieces of evidence connecting them. It has been demonstrated previously

that acute administration of benzodiazepine anxiolytic agents at low doses antagonise CCK-8 induced activation of hippocampal pyramidal neurones (Bradwejn and De Montigny, 1985) and that activation of the GABA receptor-chloride channel complex appears to inhibit the release of cholecystokinin from the rat cerebral cortex (Yaksh *et al*., 1987)

Despite some conflicting results, it is generally concluded that with in-vitro receptor binding studies of brain homogenates the number of benzodiazepine binding sites and the affinity of these does not change with long-term benzodiazepine agonist treatment (Heninger and Gallager 1988). However, Harro *et al*., (1990b), observed that an up-regulation of sulphated [^{3}H]CCK-8 receptor binding sites is evident after chronic diazepam administration for 2 weeks. A single acute anxiolytic dose of diazepam however did not produce any change in binding of either sulphated [^{3}H]CCK-8 or [^{3}H]flunitrazepam.

The benzodiazepine inverse agonist, FG-7142 with acute administration increased cholecystokinin mRNA levels in specific regions of the amygdala and the hippocampus. The benzodiazepine antagonist, flumazenil, which was inactive when given alone, significantly antagonised the anxiogenic-like activity of CCK-8us and the anxiolytic-like effects of devazepide (high dose) and L-365260 (Chopin and Briley 1993). This indicates a definite role of the GABA receptor in gating the anxiety-like effects of cholecystokinin.

Harro *et al*., (1990a) observed that rats separated into subgroups with high and low exploratory activity on the elevated plus-maze differed in binding to benzodiazepine and cholecystokinin receptors. It was established that the anxious (low exploration) group exhibited a significantly decreased number of benzodiazepine receptors in frontal cortex and hippocampus and a decrease of CCK-8 specific receptors in the hippocampus. Non-anxious animals had a significantly lower number of CCK-8 specific receptors in frontal cortex. Caerulein and pentagastrin administered intraperitoneally at plus-maze "anxiogenic" doses decreased the number of benzodiazepine binding sites in rat frontal cortex. Acute treatment of rats with FG-

7142, an anxiogenic-like benzodiazepine receptor inverse agonist, did not influence upregulation of CCK-8 specific receptor binding in the frontal cortex (Harro *et al*., 1990a) but did increase cholecystokinin mRNA levels in the basolateral amygdala and the CA3 pyramidal cell layer of the hippocampus (Brett and Pratt, 1995). This demonstrates the high region specificity underlying anxiety-like behaviour. Koks *et al*., (1997) showed that under this same dichotomy, "anxious" rats showed a significantly lower affinity (K_D) of $5HT_{2A}$ receptors in the frontal cortex compared to control and "non anxious" rats. No differences in density (B_{MAX}) were found. An increase in cholecystokinin receptor density in the hippocampus was also observed in the "anxious" rats. Harro *et al*., (1990b) observed that rats undergoing diazepam withdrawal, exhibited an increase in anxiety-like behaviour on the plus-maze, yet showed no changes in [^{3}H]flunitrazepam binding. However the apparent number of sulphated [^{3}H]CCK-8 binding sites was significantly increased in the primary olfactory cortex, frontal cortex and hippocampus. Acute diazepam treatment had no influence on binding.

Methodological Disparities:

Detailed assessment of plus-maze literature indicates that inconsistencies observed in pharmacological studies cannot be ascribed to species differences despite major strain differences in basal levels of plus-maze anxiety (Costall *et al*., 1989; Mangiafico *et al*., 1989; Pare, 1992; Pellow *et al*., 1985) and mice (Miyamoto *et al*., 1992; Rodgers and Cole, 1993a; Rodgers and Cole, 1993b; Trullas and Skolnick, 1993). Anxiety indices are also reported to increase with age (Frussa-Filho *et al*., 1991).

An interesting recent study may have provided additional indicators for the marked differences between findings of different groups with apparently similar methodology. Koks *et al*., (2000) observed that caerulein (5 $\mu g.kg^{-1}$, s.c.) produced the most robust action in animals brought immediately from the experimental room prior to the experiment, and kept in isolation after caerulein administration. Caerulein failed to modify exploratory activity in rats both familiar

with the experimental room, and kept in home-cages after injections. The action of caerulein in these "stressed" rats was attenuated by L-365260 (100 $\mu g.kg^{-1}$, i.p.). Furthermore marked seasonal fluctuations were observed to occur in the exploratory activity of rats, with activity greatly decreased in July compared to November. Moreover rats displaying the inhibited "summer" activity had an increased density of cholecystokinin and 5-HT_2 receptors in the frontal cortex and increased density of cholecystokinin receptors in the hippocampus. Furthermore, it has also been observed that rats tested ante meridiem (08-12.00) exhibit greater exploration than those tested post meridiem (14-17.00) (Griebel *et al*., 1993).

Vasar *et al*., (1993a) observed that isolation of male rats significantly reduced their exploratory activity in the elevated plus-maze compared with those group housed. Caerulein at a high dose significantly decreased the exploratory behaviour of rats housed in groups, but not in the isolated rats. This was perhaps due in part to floor effects produced by an isolation-induced decrease in indices within the vehicle group. This was further examined in the following study. Interestingly, low doses of caerulein increased exploratory indices of isolated rats in the plus-maze. Concurrently, social isolation of the rats increased the number of [^{3}H]pCCK-8 binding sites in the frontal cortex, but not in the other forebrain structures (the mesolimbic area, striatum and hippocampus), without any changes in the density of benzodiazepine receptors observed. In other studies it has, quite conversely, been observed that housing isolation either exhibits no effect (Frussa-Filho *et al*., 1991) or is anxiogenic (Janowska *et al*., 1991). Furthermore, isolated housing for periods of greater than seven days is observed to decrease plus-maze anxiety of mice (Hilakivi *et al*., 1989; Rodgers and Cole, 1993b). In addition, behavioural profiles are not predictive of dominant or subordinate social status in male mice (Hilakivi-Clarke and Lister, 1992).

While the effects of prior handling have not been studied extensively, this procedure has been reported to either increase (Andrews and File, 1993) or have no effect upon (Brett and Pratt, 1990) basal levels of open arm exploration in rats. Irrespective of effects on basal anxiety

however, handling has been found to significantly alter responses to specific drugs, including diazepam, cocaine, zacopride and buspirone (Andrews and File, 1993; Brett and Pratt, 1990; File and Andrews, 1991; Rogerio and Takahashi, 1992). Hole board exposure prior to plus-maze testing also appears to reduce basal anxiety levels in both rats (Pellow *et al*., 1985) and mice (Lister, 1987; Rodgers and Cole, 1993a). Similar effects of prior novelty exposure have also been reported in rats (DaCunha *et al*., 1992). A range of stressors have been observed as anxiogenic on the plus-maze, including saline injection (Adamec *et al*., 1991), immobilization (Albonetti and Farabollini, 1992; Handley and McBlane, 1993), electric shock (Steenbergen *et al*., 1990), social defeat (Heinrichs *et al*., 1992; Rodgers and Cole, 1993b), cat odour (Zangrossi and File, 1992a; Zangrossi and File, 1992b) and cat exposure (Adamec and Shallow, 1993).

The influence of the level of illumination on plus-maze performance is equivocal, with groups either reporting no effect (Falter *et al*., 1992; Handley and McBlane, 1993; Pellow *et al*., 1985) or reductions in open arm exploration (Benjamin *et al*., 1990; Lee and Rodgers, 1990; Morato and Castrechini, 1989). Studies examining the effects of repeated exposure to the plus-maze are examined later in this thesis.

Lister (1987) observed that measures of time spent and entries onto the open arms were heavily dependent upon anxiety level while total arm entries appeared to be measures of both locomotor activity and anxiety. Concurrent assessment of additional behavioural measures may thus be necessary in order to assist interpretation. However, many of these pre-testing regimes modify behaviour within the highly sensitive plus-maze, while post-trial assessment within these models is again influenced by the anxiogenic nature of plus-maze exposure. Therefore, the hope that, concurrent testing might aid interpretation of plus-maze activities is perhaps an erroneous expectation.

The effects of stressors upon plus-maze behaviour show, not only decreased open arm activity, but also display depressed levels of general activity and rearing in the maze (Rodgers and Cole, 1993a). Prior stress also increases risk assessment measures. During assessment of a range of anxiolytic compounds, Cole and Rodgers (1994), observed that risk assessment measures are generally more sensitive to drug action than open arm time and entries.

Materials and Methods

Animals:

Male rats of the Sprague Dawley strain (Laboratory Animal Centre, National University of Singapore, Singapore), 250-320g were housed in either groups of five to seven or singularly, and maintained with free access to food and water *ad libitum*, in an animal room for one week (or ten days in the case of isolation experiments) prior to experimentation. Room lights were on from 07:00 to 19:00 hours. Animals were brought into the brightly lit, test room immediately prior to plus-maze testing and handled only at that time for weighing and injection procedures. Operator handling was near identical throughout the sample. Animals were randomly assigned treatment groups using a simple computer program random number sequence generator (Random Number Generator for Q-Basic by Colin Greengrass, 1998). This program (see Appendix C for listing) generated random whole numbers within a range corresponding to an assigned treatment group.

Isolation Protocol

Isolation was carried out in opaque cages with animals randomly assigned from the initial batch. Testing was carried out under a regime in which each isolated sample was isolated for precisely 10 days. Initially upon delivery of 24 rats, 20 were group housed (five cages of 4 per cage), and four were housed in isolation. On the second day, four animals inhabiting one group housing cage were separated for isolation housing, with a remaining 16 group housed. On the third day, another group-housed sample was isolated leaving 12 rats group housed and 12 isolated. On day ten, the first batch of isolated animals were tested on the plus-maze. Upon subsequent days those animals that had been isolated sequentially were tested in that order, thus conferring isolation for a fixed period. Cages were identical in material and dimensions for both group-housed and isolated animals. Food and water was provided *ad libitum*.

Drugs and Administration

Cholecystokinin peptide agonists: CCK-4 (Trp-Met-Asp-Phe-NH_2) and CCK-8us (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2; Biotechnology Centre, National University of Singapore); CCK-8s (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; American Peptide Company and Princeton Biomolecules) ; the cholecystokinin peptoidal antagonist CI-988 ([R-(R*,R*)]-4-[[2-[[3-(1H-Indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1.3,7]dec-2-yloxy)carbonyl]-amino]propyl]-amino]-1-phenylethyl]amino]-4-oxo-butanoate-1-deoxy-1-(methylamino)-D-glucitol; Research Biochemicals Inter-national (RBI)), the pyrazolidine based CCK_2 antagonist, LY-288513 (trans-N-(4-bromophenyl)-3-oxo-4,5-diphenyl-1-pyrazolidine-carboxamide; generous gift from Eli Lilly and company) were dissolved in dimethylsulphoxide (DMSO; Merck) with either chlordiazepoxide hydrochloride (7-Chloro-2-(methylamino)-5-phenyl-^{3}H-1,4-benzodiazepine 4-oxide; A generous gift from Roche) at 1.5mg/ml or in DMSO alone. In validation studies FG-7142 (N-methyl-D-carboline-3-carboxamide; RBI) was also utilised under the same protocol. The drugs or vehicle were administered by intraperitoneal injection in a volume of 1ml per kg body weight. Two separate injections were given to each animal. Cholecystokinin peptide was stored under desiccation at -70^{o}C. Cholecystokinin peptide solutions were prepared freshly each day approximately two hours prior to testing. Powdered peptide was dissolved in DMSO and specific concentrations prepared through serial dilution, with thorough vortex mixing at each stage.

Behavioural Procedures

Elevated Plus-maze: Experiments were similarly conducted to those described previously (Pellow *et al*., 1985; Lister, 1987) with minor modifications. The plus-maze consisted of two open arms (50 x 10cm) with a 1cm high surrounding wall and two enclosed arms (50 x 10cm) with a 50cm high surrounding wall. The arms extended from a central platform (10 x 10cm) and were arranged such that the two open arms were opposite each other as were the closed

arms. The floor of the open arms, the central platform, and the closed arms was made of black Perspex. The surrounding walls of the arms were made of clear Perspex. The maze was elevated 50cm from the floor on a wooden platform. The test room was maintained with controlled light levels and temperature with white noise delivered at 70dB. The maze platforms and walls were thoroughly cleaned with 70% ethanol between sessions and allowed to dry. At the beginning of the experiments, naïve rats were placed on the central platform facing one of the open arms. The total number of visits to the open arms, the total number of visits to the closed arms, the cumulative time spent in the open arms and the cumulative time spent in the closed arms were subsequently measured for a 5 minute period using manual operation of a counter/timer unit (see appendix B.1). An arm visit was recorded when a rat had all four paws on one arm. These sessions were videotaped. Behavioural experiments were performed between 14:00-17:00hours. Animals were used only once. Drugs were administered intraperitonally 30 minutes prior to testing.

Locomotor Activity: Open field motor activity was assessed for some animals immediately following plus-maze testing. The apparatus consisted of a clear Plexiglas chamber of 33cm width by 42cm length and walls 20cm in height. The top of the chamber was covered during testing with a steel lid with air holes. The chamber was placed on a steel surface. The test room was maintained with controlled light levels and temperature with white noise delivered at 70dB. The chamber was thoroughly cleaned with 70% ethanol between sessions and allowed to dry. Locomotor activity was measured using an Opto-varimex mini activity monitoring system (Columbus Instruments, USA). An apparatus naïve animal was placed in the chamber and allowed a two-minute acclimation period within the chamber before testing. The total number and the ambulatory beam interruptions were subsequently measured for 5 minutes. Ambulatory locomotor activity is a measure by which repetitive interruptions of the same beam are ignored. Approximation of values for grooming/rearing activity is achieved by subtraction of total from ambulatory counts, which represents activity without ambulation.

Statistics

Statistical analysis was performed using statistical software (SPSS V9.0, SPSS inc. USA). The data were subjected to one-way analysis of variance (ANOVA), and Bonferroni post hoc analysis. Probability ($p < 0.05$) was considered statistically significant. Data are presented as drug group means with standard error of the mean (SEM).

Results and Data

Figure 3.1.1: Administration of chlordiazepoxide, in saline at 3.0 $mg.kg^{-1}$ produced a statistically significant increase in time spent in the open arms compared to a saline control, within the elevated plus-maze; chlordiazepoxide; $3.0mg.kg^{-1}$, ($F_{3,32}$=49.66, $p<0.001$) at the expense of significantly lower time spent in the closed arms ($F_{3,32}$=49.66, $p<0.001$). The established anxiogenic agent FG-7142 ($5.0mg.kg^{-1}$) in saline, was unable to significantly alter time spent in the open arms. FG-7142 did however produce a significant decrease in time upon the central platform ($F_{3, 32}$=6.63, $p<0.01$) and a significant increase in time spent within the closed arm ($F_{3,32}$=38.35).

Figure 3.1.2: Correspondingly, numbers of open arm entries were also significantly increased ($F_{3,32}$=29.56, $p<0.001$). Furthermore, an increase in the total number of entries into both arms was observed ($F_{3,32}$=13.64, $p<0.01$). Despite an inability to alter the number of individual closed or open arm entry counts, the number of total arm entries was significantly decreased in FG-7142 treated rats ($F_{3,32}$=13.64, $p<0.01$).

Figure 3.2.1: Chlordiazepoxide ($3.0mg.kg^{-1}$) in DMSO increased time spent in open arms ($F_{3,35}$=28.46, $p<0.001$) offset by a decrease in closed arm time ($F_{3,35}$=24.20, $p<0.01$). Despite FG-7142 failing to significantly alter time spent in the open arms, time upon the central platform was significantly decreased ($F_{3, 35}$=5.58, $p<0.01$) and time spent within the closed arm was increased ($F_{3,35}$=24.2, $p<0.01$). FG-7142 ($5.0mg.kg^{-1}$) in addition prevented the increase in open arm time observed with chlordiazepoxide ($3.0mg.kg^{-1}$) with co-administration (statistical significance compared to chlordiazepoxide alone ($F_{3,35}$=28.46, $p<0.001$).

Figure 3.2.2: Open arm entries were also significantly elevated $F_{3, 35}$=28.17, $p<0.001$) in the Chlordiazepoxide ($3.0mg.kg^{-1}$) in DMSO group. FG-7142 also produced a significant decrease in open arm ($F_{3,35}$=28.17, $p<0.05$); closed arm ($F_{3,35}$=9.80, $p<0.001$); and total entries ($F_{3,35}$=18.97, $p<0.001$) similarly to that observed with FG-7142 in saline. FG-7142 ($5.0mg.kg^{-}$

^{1}) in combination with chlordiazepoxide (3.0mg.kg^{-1}) significantly decreased open arm entries (when compared to chlordiazepoxide alone ($F_{3,35}$=28.17, p<0.001).

Figure 3.2.3: With subsequent testing of spontaneous open field locomotor activity, it was observed that both chlordiazepoxide and FG-7142 inhibited activity significantly: for ambulatory activity, chlordiazepoxide ($F_{3,35}$=5.28, p<0.05), FG-7142 ($F_{3,35}$=5.28, p<0.05); for non-ambulatory activity, FG-7142 ($F_{3,35}$=3.69, p<0.05); for total locomotor activity, chlordiazepoxide ($F_{3,35}$=5.68, p<0.05), FG-7142 ($F_{3,35}$=5.68, p<0.05). A combination of chlordiazepoxide (3.0mg.kg^{-1}) and FG-7142 (5.0mg.kg^{-1}) did not alter locomotor activity.

Cholecystokinin Peptide Forms

The effects of cholecystokinin forms upon reversal of increased open arm time and entries induced by chlordiazepoxide (3.0mg.kg^{-1}) were examined.

Figure 3.3.1: Dose response data for the cholecystokinin tetrapeptide, CCK-4, in combination with chlordiazepoxide (3.0mg.kg^{-1}) showed that only a dose of 0.025mg.kg^{-1} was able to significantly decrease percentage time spent in the open arms ($F_{5,43}$=7.54, p<0.001) compared to chlordiazepoxide alone. A resultant increase in closed arm time was observed in counterbalance ($F_{5,43}$=4.31, p<0.01). No other significant differences were observed although there appears to be a trend towards an inverse bell shaped dose response curve with regard to time spent on open arms and vice versa for time spent on the closed arms.

Figure 3.3.2: Examination of open arm entry data yielded an interesting correlation whereby number of open arm entries appeared to exhibit two separate dose response troughs. CCK-4 decreased the number of open arm entries only at the dose 0.025mg.kg^{-1} ($F_{5, 43}$=3.41, p<0.05), corresponding to that of open arm time. The total number of arm entries however was significantly decreased at both 0.025mg.kg^{-1} ($F_{5,43}$=3.89, p<0.05) and 0.075mg.kg^{-1} ($F_{5,43}$=3.89, p<0.05), without any significant changes at the intermediate dose 0.05mg.kg^{-1}.

Figure 3.4.1: An inverse bell shaped dose response curve was observed for data of the effects of CCK-8us on (%) open arm time within a dose range of 0.005 to 0.1mg.kg^{-1}. CCK-8us, only at 0.01mg.kg^{-1}, significantly reversed the effects of chlordiazepoxide (3.0mg.kg^{-1}) upon (%) open arm time spent ($F_{4,36}$=6.69, p<0.001) while closed arm time was increased at this dose ($F_{4,36}$=4.83, p<0.01).

Figure 3.4.2: CCK-8us demonstrated a significant decrease in open arm entries only at a dose of 0.01mg.kg^{-1} ($F_{4,36}$=4.23, p<0.01).

Figure 3.5.1: Examination of a CCK-8s dose response relationship yielded an inverse bell shaped dose response curve. Within a dose range of 0.0003 to 0.01mg.kg^{-1}, CCK-8s reversed the effects of chlordiazepoxide only at doses 0.001mg.kg^{-1} ($F_{6,47}$=5.58, p<0.05) and 0.002mg.kg^{-1} ($F_{6,47}$=5.58, p<0.01) with significant decreases in open arm time. In compensation, at these doses closed arm time was increased; 0.001mg.kg^{-1} ($F_{6,47}$=8.19, p<0.05) and 0.002mg.kg^{-1} ($F_{6,47}$=8.19, p<0.001).

Figure 3.5.2: Data for effect of CCK-8s on number of arm entries showed a similar distribution to that of CCK-4. At a dose of 0.0005mg.kg^{-1} the number of open arm entries ($F_{6,47}$=6.86, p<0.05), closed arm entries ($F_{6,47}$=5.94, p<0.01), and the resultant total number ($F_{6,47}$=8.31, p<0.001) were significantly inhibited. This also occurred at the dose 0.002mg.kg^{-1}, with significant inhibition of number of open arm entries ($F_{6,47}$=6.86, p<0.05), closed arm entries ($F_{6,47}$=5.94, p<0.05), and the resultant total number ($F_{6,47}$=8.31, p<0.01). Yet again the intermediate dose of 0.001 did not show a significant decrease in number, although did narrowly miss significance for open arm entries ($F_{5,43}$=3.89, p=0.064).

Figure 3.6.1: A combination of cholecystokinin forms at both half and maximally effective doses, with regard to attenuation of chlordiazepoxide effects upon time spent in the open arms, yielded quite unexpected data. In comparison with the chlordiazepoxide group, data for cholecystokinin forms alone were in conformity with earlier dose response studies. A dose of

0.0125mg.kg^{-1} failed to evoke a response, while a higher dose of 0.025mg.kg^{-1} significantly inhibited the activity of chlordiazepoxide in increasing open arm time ($F_{15,114}$=15.81, p<0.01). CCK-8us also acting in conformity with the earlier dose response study showed no significant effects at 0.005mg.kg^{-1} and a significant inhibition of chlordiazepoxide function at 0.01mg.kg^{-1} ($F_{15,114}$=15.81, p<0.001). CCK-8s tested at three doses produced significant inhibition at doses 0.001mg.kg^{-1} ($F_{15,114}$=15.81, p<0.001) and 0.002mg.kg^{-1} ($F_{15,114}$=15.81, p<0.001) and not at 0.0005mg.kg^{-1}. Interestingly the maximal dose of CCK-8us (0.01mg.kg^{-1}) also induced a significant increase in time spent on the central platform ($F_{15,114}$=3.05, p<0.05). Interestingly, a combination of CCK-4 and CCK-8 (both forms) at either half or at their maximally effective doses failed to elicit inhibition of chlordiazepoxide activity. A combination of CCK-8us and CCK-8s at these doses maintained the same inhibition of chlordiazepoxide activity, compared to chlordiazepoxide alone, chlordiazepoxide (3.0) with CCK-8us (0.005) and CCK-8s (0.0005; $F_{15,114}$=15.81, p<0.001); chlordiazepoxide (3.0) with CCK-8us (0.005) and CCK-8s (0.001; $F_{15,114}$=15.81, p<0.01); chlordiazepoxide (3.0) with CCK-8us (0.010) and CCK-8s (0.002; $F_{15,114}$=15.81, p<0.001). With the exception of CCK-8us at 0.01mg.kg^{-1}, most decreases in open arm time were compensated by increases in closed arm time or were absorbed by both closed arm and central platform time increases.

Figure 3.6.2: Numbers of arm entries were decreased in the CCK-8s group only at the dose 0.002mg.kg^{-1} ($F_{15,114}$=6.83, p<0.05) and in the CCK-8us (0.01) / CCK-8s (0.002) combined group ($F_{15,114}$=6.83, p<0.001). In the combined group this significant decrease in arm entries was accompanied by a significant decrease in total arm entries ($F_{15,114}$=2.83, p<0.05).

Figure 3.6.3: An identical treatment protocol, tested within an open field locomotor activity model, failed to demonstrate any significant differences between combination groups. Furthermore, a broad standard error was observed within most groups. Trends observed may imply an inhibition of locomotor activity in maximal dose groups for CCK-8us and CCK-8s.

Figure 3.7.1: A dose response study of the peptoidal CCK_2 antagonist, CI-988 (0.002 to 1.0mg.kg^{-1}) did not yield time data with significant differences.

Figure 3.7.2: A dose response study of the peptoidal CCK_2 antagonist, CI-988 (0.002 to 1.0mg.kg^{-1}) did not yield entry data with significant differences.

Figure 3.7.3: A dose response study of the peptoidal CCK_2 antagonist, CI-988 (0.002 to 1.0mg.kg^{-1}) co-administered with chlordiazepoxide (3.0mg.kg^{-1}) yielded data analogous to that of the aforementioned cholecystokinin agonists, in that an inverse bell shaped dose response curve was observed for open arm time data. A dose of 0.02mg.kg^{-1} produced a significant decrease in chlordiazepoxide induced open arm time ($F_{9,85}$=9.37, $p<0.05$).

Figure 3.7.4: This phenomenon was also observed in a significant decrease in number of open arm entries ($F_{9,85}$=4.77, $p<0.001$) and in total arm entries ($F_{9,85}$=3.72, $p<0.01$). Interestingly once again this yielded an interesting correlation whereby the total number of entries appeared to exhibit two separate dose response troughs. At the highest dose tested the total number of entries was again significantly inhibited ($F_{9,85}$=3.72, $p<0.01$) with the intermediate dose of 0.2mg.kg^{-1} without effect. Higher doses were tested due to CI-988 small sample size. Dose response studies of CI-988 with DMSO (vehicle) in the absence of chlordiazepoxide showed no significant effects on any indices.

Figure 3.8.1: Dose response studies of the pyrazolidinone related CCK_2 antagonist, LY-288513, in DMSO (vehicle) demonstrated a broadly dose dependent increase in time spent within the open arms (figure 3.8.1.). LY-288513 at doses, 0.001mg.kg^{-1} ($F_{8,63}$=9.87, $p<0.05$); 0.1mg.kg^{-1} ($F_{8,63}$=9.87, $p<0.01$); 1.0mg.kg^{-1} ($F_{8,63}$=9.87, $p<0.01$); and 10mg.kg^{-1} ($F_{8,63}$=9.87, $p<0.001$) significantly increased time spent within open arms with a steady increase in effect with increasing dose. Time spent within the central platform was unaffected while time spent upon the closed arms was only significantly decreased at the highest dose tested, 10mg/.kg^{-1}

($F_{8,63}$=5.44, p<0.001).

Figure 3.8.2: A profile describing LY-288513, in DMSO (vehicle) effects upon arm entries differed only slightly from that of measures of time spent. Total and closed arm entries were unaffected across the dose range while doses, 0.1mg.kg^{-1} ($F_{8,63}$=6.17, p<0.01) and 10mg.kg^{-1} ($F_{8,63}$=6.17, p<0.001) significantly increased open arm entries. The intermediate, 1mg.kg^{-1} dose was seemingly unaffected.

Studies of cholecystokinin peptide antagonism by CI-988 and LY-288513 showed a general attenuation of effects. A co-administration of chlordiazepoxide and each cholecystokinin form showed near identical data to that of previous studies with a significant decrease in time spent within the open arm; CCK-4 at 0.0025mg.kg^{-1} ($F_{8,83}$=23.36, p<0.001); CCK-8us ($F_{8,83}$=23.36, p<0.001); and CCK-8s ($F_{8,83}$=23.36, p<0.001) compared to chlordiazepoxide alone. As previously shown a combination of chlordiazepoxide and CI-988 (1.0mg.kg^{-1}) shows no differences from chlordiazepoxide alone.

Figure 3.9.1: Addition of CI-988 to both the CCK-4/chlordiazepoxide and CCK-8s/chlordiazepoxide combination, restores chlordiazepoxide activity. Perhaps a relatively extraneous observation is that CI-988 co-administered with CCK-8us/chlordiazepoxide not significantly attenuating CCK-8us decreased open arm time, compared to chlordiazepoxide alone. With this comparison, CI-988 with CCK-8us/chlordiazepoxide maintains a significant difference compared to an unusually high open arm time measure in the chlordiazepoxide group (mean 61.37). However, at least partial attenuation of the CCK-8us effect is observed where, compared to the CCK-8us/chlordiazepoxide group, CI-988 with CCK-8us/chlordiazepoxide shows significantly increased open arm time ($F_{8,83}$=23.36, p<0.05).

Figure 3.9.2: Measures for number of entries follows a similar pattern to that of time spent within each sector, with both CCK-4 ($F_{8,83}$=11.59, p=0.065) and CCK-8us ($F_{8,83}$=11.59, p=0.057) narrowly missing significance differences from chlordiazepoxide in open arm

entries, and CCK-8s achieving significant inhibition ($F_{8,83}$=11.59, p=0.01). Similarly to time spent measures, a combination of CI-988 with either, CCK-4/chlordiazepoxide and CCK-8s/chlordiazepoxide causes a loss of significant effect of cholecystokinin forms. In addition the number of open arm entries for the CCK-4/chlordiazepoxide with CI-988 group is significantly greater than that of CCK-4/chlordiazepoxide ($F_{8,83}$=11.59, p<0.01) and likewise for CCK-8s/chlordiazepoxide with CI-988 compared to CCK-8s/chlordiazepoxide ($F_{8,83}$=11.59, p<0.001).

Figure 3.10.1: Antagonism of cholecystokinin effects by LY-288513 showed significant attenuation with co-administration. Firstly, significant differences from the chlordiazepoxide treatment group were observed for CCK-4 at 0.025mg.kg^{-1} ($F_{8,63}$=6.84, p<0.05); CCK-8us ($F_{8,63}$=6.84, p<0.01); and CCK-8s ($F_{8,63}$=6.84, p<0.001). This effect was largely at the expense of time spent in the closed arms. LY-288513 in combination with chlordiazepoxide at 1.0mg.kg^{-1} showed no significant differences from chlordiazepoxide alone. The effects of cholecystokinin forms on open arm time were attenuated by LY-288513 (1.0mg.kg^{-1}); with LY-288513/CCK-4/chlordiazepoxide significantly greater than CCK-4/chlordiazepoxide ($F_{8,63}$=6.84, p<0.05); and with LY-288513/CCK-8us/chlordiazepoxide significantly greater than CCK-8us/chlordiazepoxide ($F_{8,63}$=6.84, p<0.01). Differences between LY-288513/CCK-8s/chlordiazepoxide and CCK-8s/chlordiazepoxide missed significance but demonstrated a trend towards attenuation ($F_{8,63}$=6.84, p=0.077). LY-288513 at 10.0mg.kg^{-1} co-administered with chlordiazepoxide also showed no significant changes in time spent within each sector on the maze.

Figure 3.10.2: Measures for number of arm entries showed no clear distribution, including a lack of effect in CCK-4/chlordiazepoxide and CCK-8us/chlordiazepoxide groups. CCK-8s/chlordiazepoxide treated rats did however show a significant decrease in number of open arm entries ($F_{8,63}$=41.59 p<0.05). LY-288513 at 10mg.kg^{-1} in combination with chlordiazepoxide, resulted in marked inhibition in number of entries on the plus-maze; open

arm entries ($F_{8,63}$=3.87, p<0.01); closed arm entries ($F_{8,63}$=3.33, p=0.079) and total arm entries ($F_{8,63}$=2.68, p<0.05).

Figure 3.11.1: Antagonism of the effects of FG-7142 by CI-988 was studied with no differences between FG-7412 and FG-7142/CI-988 groups observed. Data was found to be indistinguishable between groups. Both FG-7412 and FG-7142/CI-988 groups showed significant inhibition of time spent within the open arms ($F_{2,18}$=7.03, p<0.05 for both) and on the central platforms ($F_{2,18}$=12.57, p<0.01 FG-7412; p<0.001 FG-7412/CI-988) and a significant increase in time spent in the closed arms ($F_{2,18}$=32.48, p<0.001 for both).

Figure 3.11.2: In addition, measures for number of entries were also significantly inhibited in both groups compared to the vehicle group, but no differences were observed between FG-7142 and FG-7142/CI-988 groups.

Figure 3.12.1: Further study of locomotor activity within the open field for these rats, subsequent to plus-maze testing, showed that in both groups activity is significantly inhibited. Ambulatory activity is significantly inhibited by FG-7142 ($F_{2,18}$=10.73, p<0.05) and in total activity ($F_{2,18}$=12.02, p<0.05) whereas FG-7142/CI-988 inhibits activity in all three measures: ambulatory ($F_{2,18}$=10.73, p<0.001); non-ambulatory ($F_{2,18}$=10.06, p<0.001); and total activity ($F_{2,18}$=12.02, p<0.001).

Social Isolation

The effects of social isolation on plus-maze behaviour and psychopharmacology was examined.

Figure 3.13.1: Group housed, chlordiazepoxide treated animals (3.0mg.kg^{-1}) exhibited a significant increase in time spent in the open arm ($F_{5,42}$=6.65, p<0.001) offset with a similar increase in closed arm time ($F_{5,42}$=4.35, p<0.01). This phenomenon was not observed however in socially isolated rats. A similar finding was observed with LY-288513 (10.0mg.kg^{-1}) in that

group housed rats demonstrated a significant increase in time spent on the open arms ($F_{5,42}$=6.65, p<0.01) with socially isolated animals showing no significant differences from the vehicle group housed animals.

Figure 3.13.2: A similar relationship is observed for measures of arm entries with group-housed chlordiazepoxide treated animals exhibiting significantly increased numbers of open arm entries ($F_{5,42}$=10.39, p<0.001). Socially isolated chlordiazepoxide treated animals did not show any differences from the vehicle group housed animals. No significant differences were observed in either LY-288513 groups with regard to numbers of arm entries.

Figure 3.14.1: No significant differences were observed between chlordiazepoxide treated rats with either group housing or social isolation protocols. All remaining treatment groups showed significant decreases in time spent in the open arms; group housed CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); socially isolated CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); group housed CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); socially isolated CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); group housed CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); socially isolated CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001) compared to group housed chlordiazepoxide treated rats. This decrease in open arm time was offset by an increase in time spent in the closed arms in all groups; group housed CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); socially isolated CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); group housed CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); socially isolated CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); group housed CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); socially isolated CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001) compared to group housed chlordiazepoxide treated rats. In comparison with the chlordiazepoxide treated socially isolated group, the remaining isolated groups measures were significantly inhibited; socially isolated CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); socially isolated CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001); and socially isolated CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=23.08, p<0.001). This decrease in open arm time compared to chlordiazepoxide treated

socially isolated animals, was counterbalanced by the increase in time spent in the closed arms in these groups; socially isolated CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); socially isolated CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001); socially isolated CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=14.19, p<0.001).

Figure 3.14.2: Analysis of numbers of arm entries yielded similar data with the exception that the CCK-8us treated, group housed, rats that failed to achieve a significant decrease in number of open arm entries. Other groups all achieved a corresponding significant decrease in open arm entries; group housed CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001); socially isolated CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001); socially isolated CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001); group housed CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001); socially isolated CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001) compared to group housed chlordiazepoxide treated rats. In comparison with socially isolated chlordiazepoxide treated rats, socially isolated CCK-4 at 0.025mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001); socially isolated CCK-8us at 0.01mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001); and socially isolated CCK-8s at 0.002mg.kg^{-1} ($F_{7,56}$=12.96, p<0.001) significantly inhibited numbers of open arm entries. Within these treatment groups no differences in number of closed arm entries nor in total number of entries were observed.

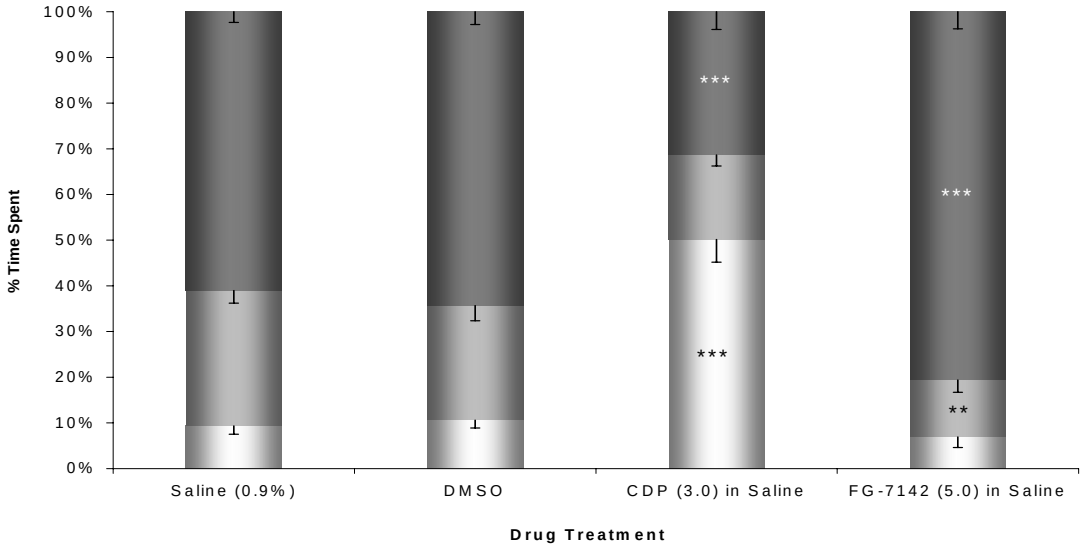


FIG. 3.1.1: Effects in rats of drugs with known anxiolytic (chlordiazepoxide) and anxiogenic (FG-7142) properties, and dimethylsulphoxide (DMSO) versus a saline vehicle on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM); light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the saline (vehicle) group are shown; **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

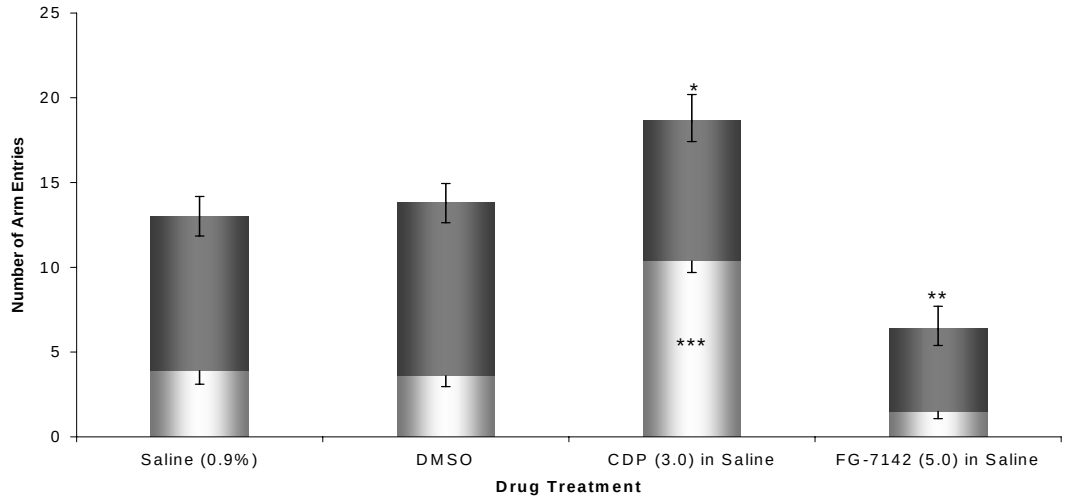


FIG. 3.1.2: Effects in rats of drugs with known anxiolytic (chlordiazepoxide) and anxiogenic (FG-7142) properties, and dimethylsulphoxide (DMSO) versus a saline vehicle on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM); light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the saline (vehicle) group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

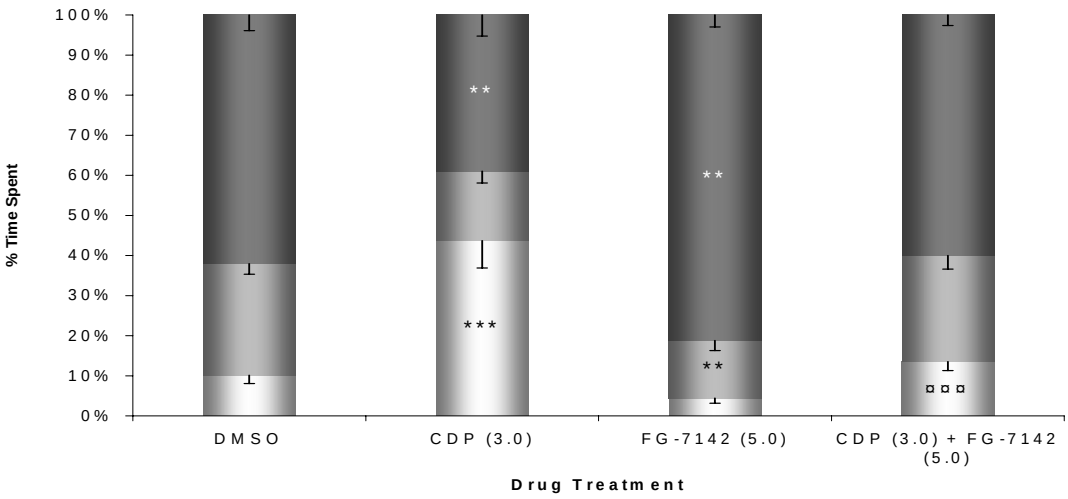


FIG. 3.2.1: Effects in rats of drugs with known anxiolytic (chlordiazepoxide) and anxiogenic (FG-7142) properties and a combination of both, versus a dimethylsulphoxide (DMSO) vehicle on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the DMSO (vehicle) group; **p<0.01, ***p<0.001, and from the chlordiazepoxide group are shown; ¤¤¤p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

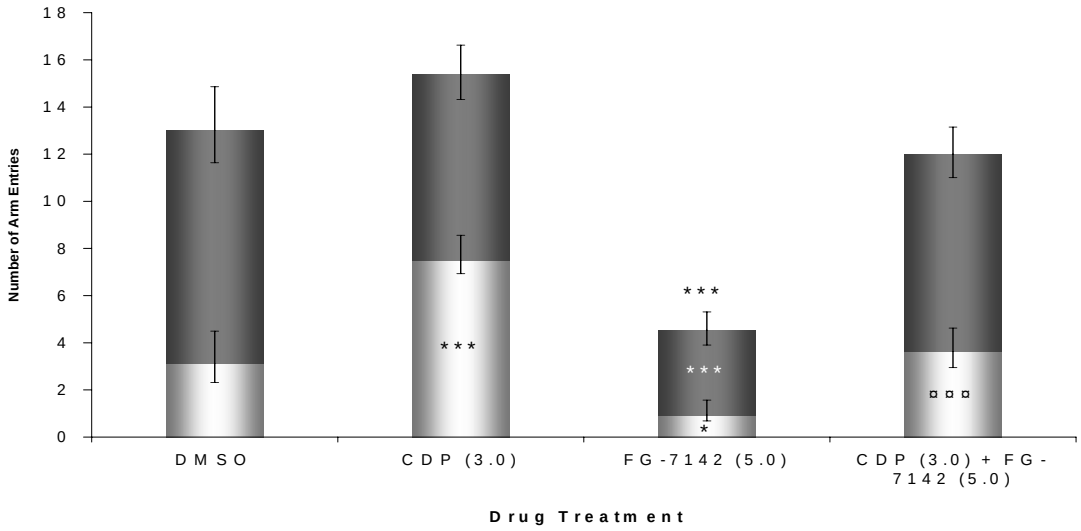


FIG. 3.2.2: Effects in rats of drugs with known anxiolytic (chlordiazepoxide) and anxiogenic (FG-7142) properties and a combination of both, versus a dimethylsulphoxide (DMSO) vehicle on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the DMSO (vehicle) group (*p<0.05, ***p<0.001) and from the chlordiazepoxide group; ¤¤¤p<0.001 are shown for open and closed arm entries within each bar and for total entries atop each bar (Bonferroni post hoc analysis after significant one-way ANOVA).

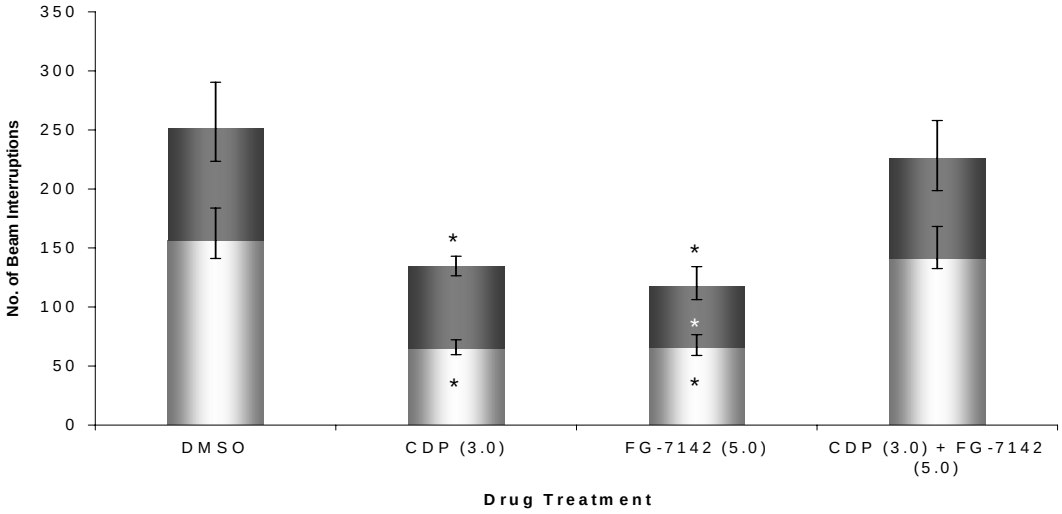


FIG. 3.2.3: Effects in rats of drugs with known anxiolytic (chlordiazepoxide) and anxiogenic (FG-7142) properties and a combination of both, versus a dimethylsulphoxide (DMSO) vehicle on locomotor activity within an open field chamber. Data presented are total, ambulatory and non-ambulatory beam interruptions (results presented as mean with SEM). Light grey bars show ambulatory locomotor activity, dark grey bars show non-ambulatory activity, totalling light grey and grey bars show number of total beam interruptions. Significant differences from the DMSO (vehicle) group are shown for beam interruption counts within each bar and for total entries atop the combined bar; *$p<0.05$, (Bonferroni post hoc analysis after significant one-way ANOVA).

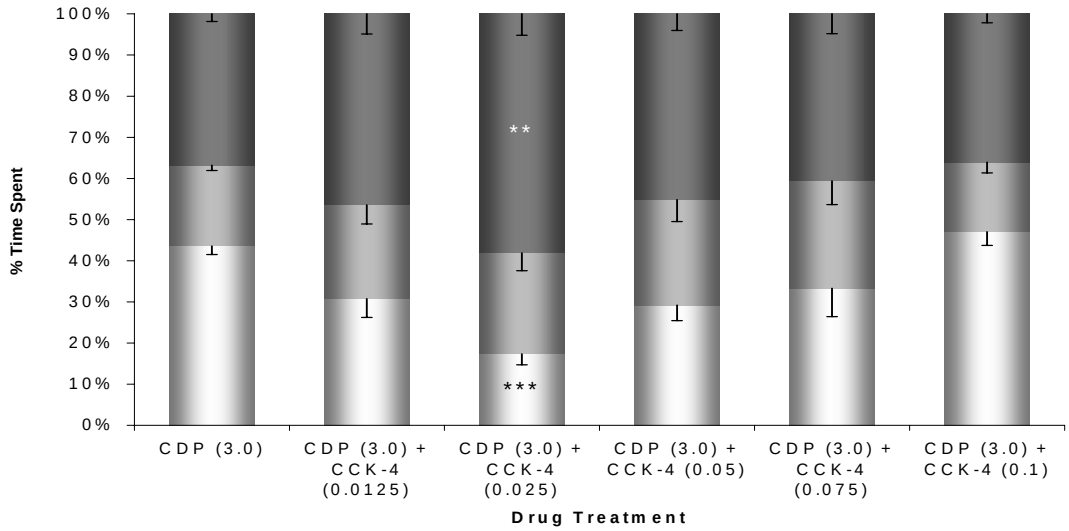


FIG. 3.3.1: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin isoform; CCK-4 in combination with chlordiazepoxide versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

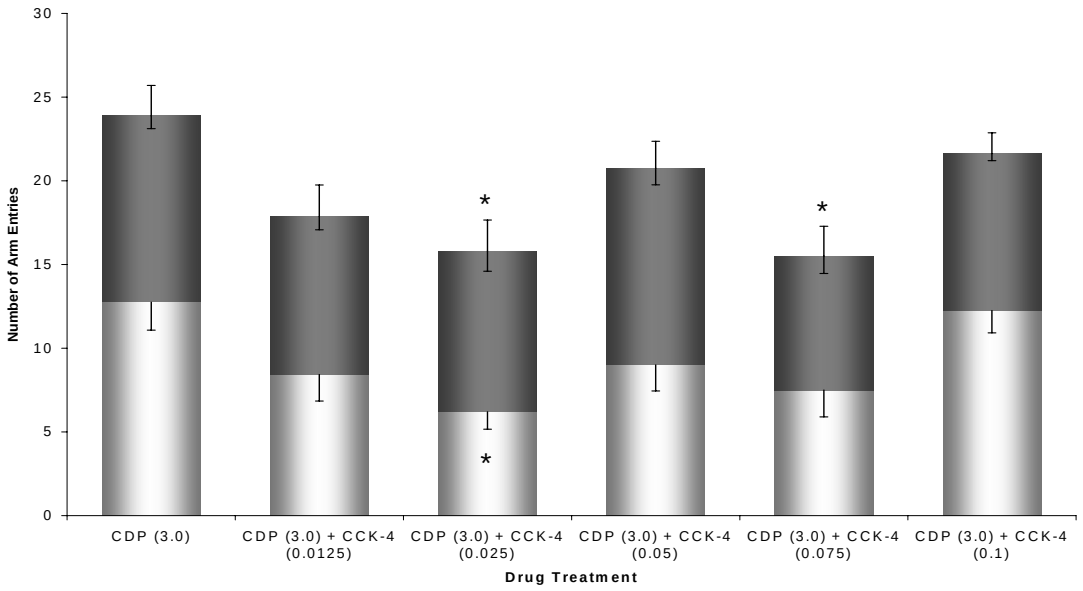


FIG. 3.3.2: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin isoform; CCK-4 in combination with chlordiazepoxide versus the effect of chlordiazepoxide alone on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05 (Bonferroni post hoc analysis after significant one-way ANOVA).

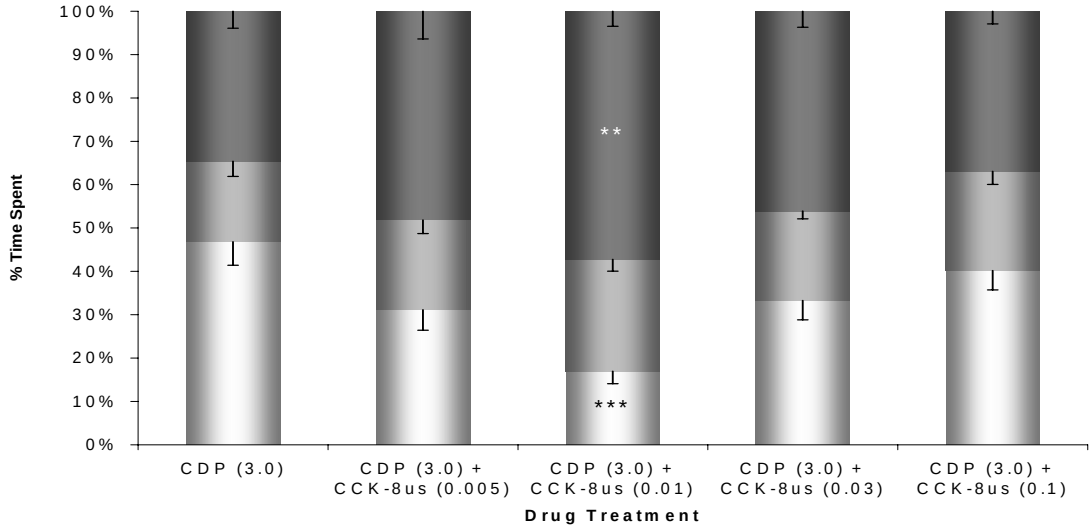


FIG. 3.4.1: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin isoform; CCK-8us in combination with chlordiazepoxide versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

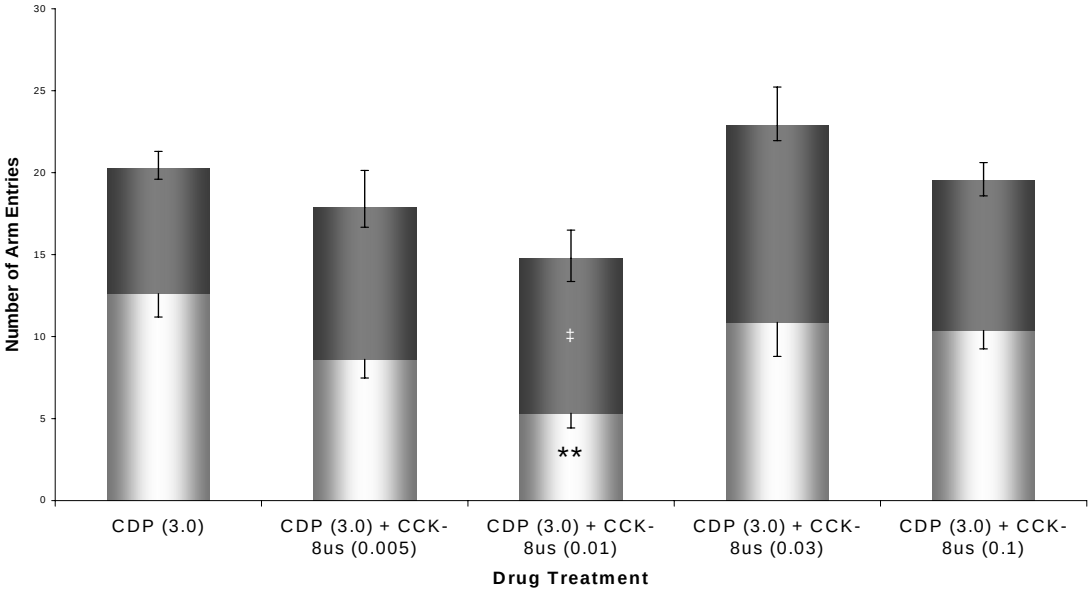


FIG. 3.4.2: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin isoform; CCK-8us in combination with chlordiazepoxide versus the effect of chlordiazepoxide alone on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; **p<0.01, ‡ signifies narrowly missed statistical significance, (Bonferroni post hoc analysis after significant one-way ANOVA).

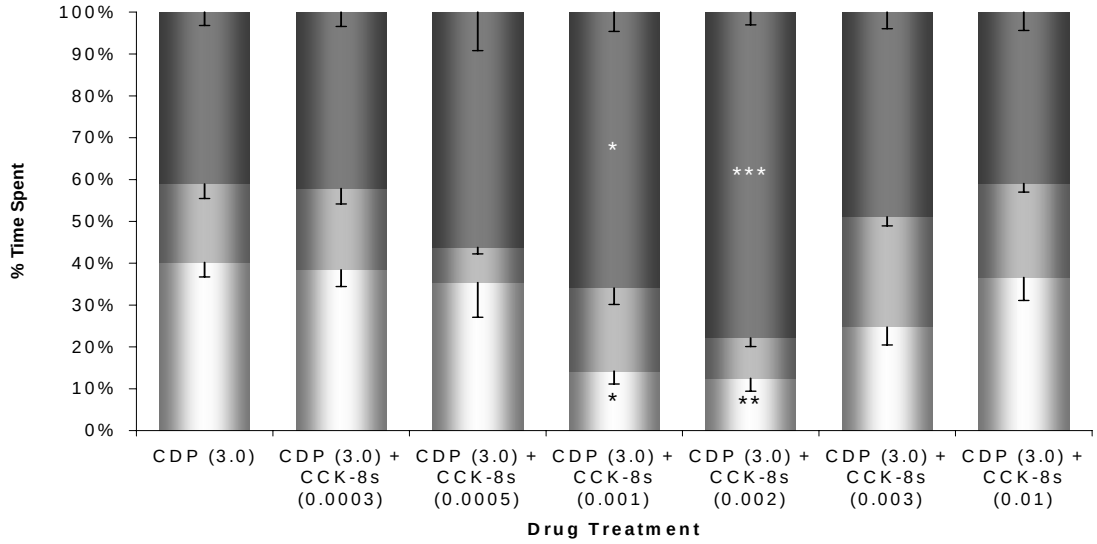


FIG. 3.5.1: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin isoform; CCK-8s in combination with chlordiazepoxide versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

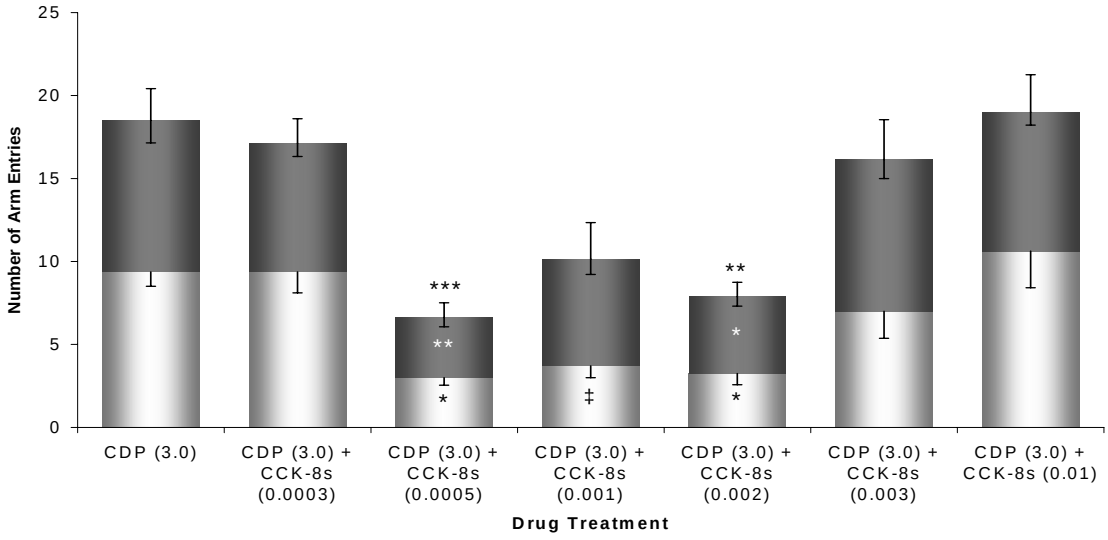


FIG. 3.5.2: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin isoform; CCK-8s in combination with chlordiazepoxide versus the effect of chlordiazepoxide alone on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05, **p<0.01, ***p<0.001, ‡ signifies narrowly missed statistical significance, (Bonferroni post hoc analysis after significant one-way ANOVA).

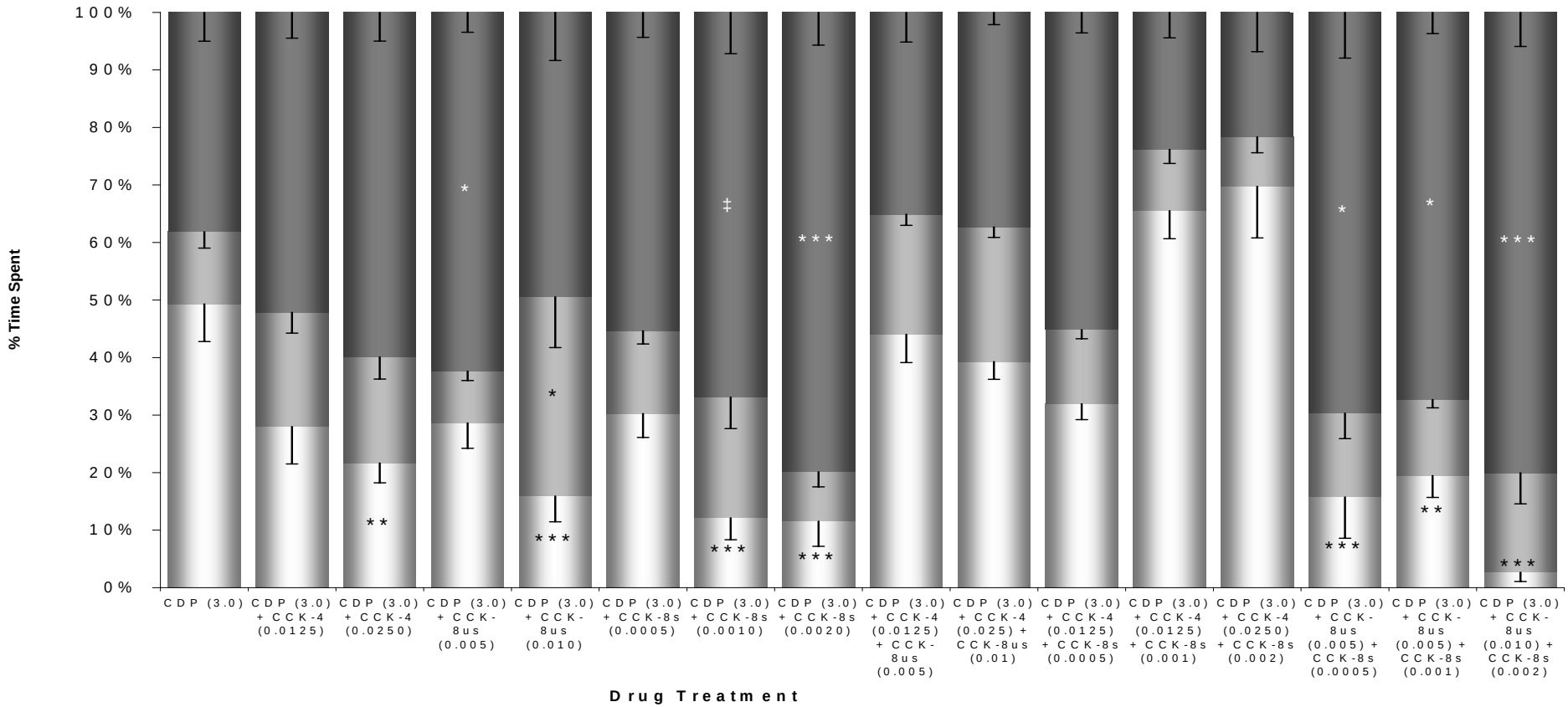


FIG. 3.6.1: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective and half maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; *p<0.05, **p<0.01, ***p<0.001, ‡ signifies narrowly missed statistical significance, (Bonferroni post hoc analysis after significant one-way ANOVA).

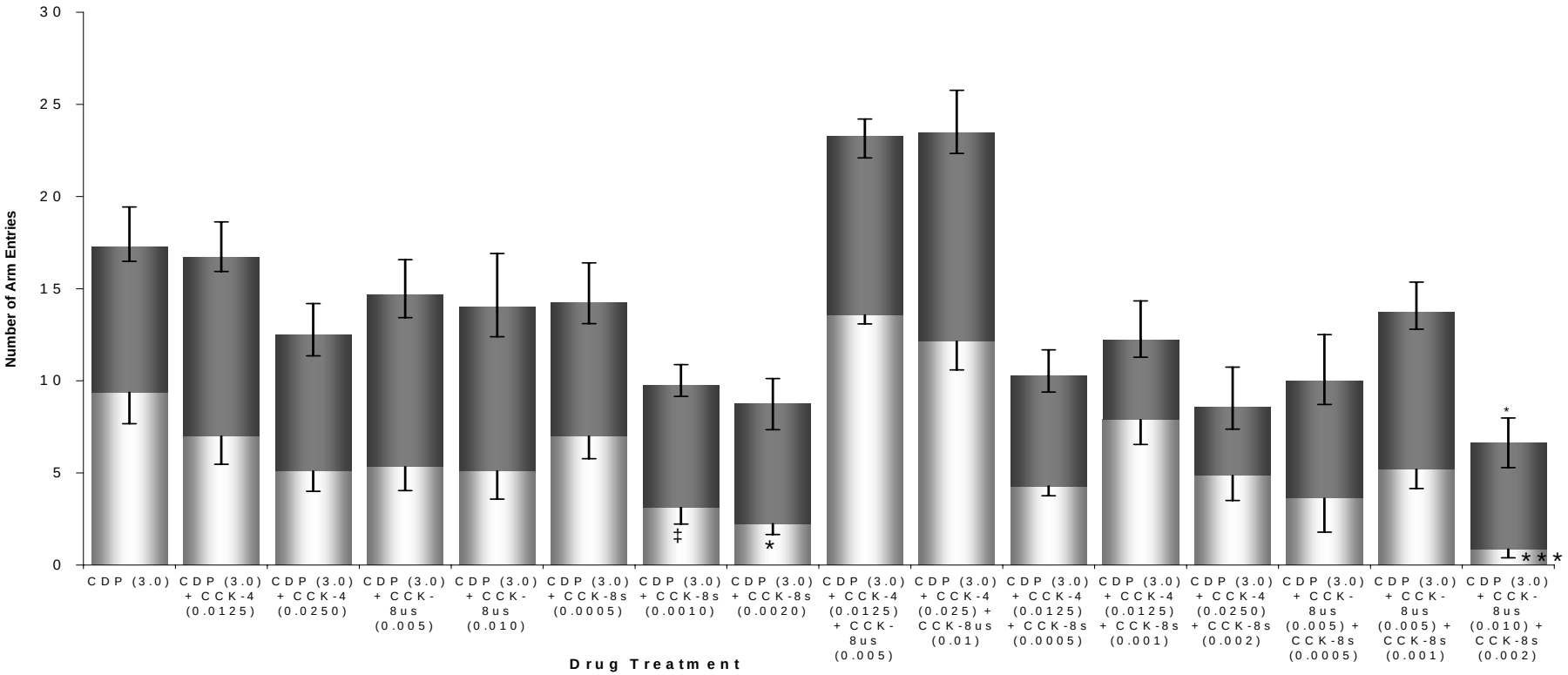


FIG. 3.6.2: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective and half maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05, **p<0.01, ***p<0.001; ‡ signifies narrowly missed statistical significance, (Bonferroni post hoc analysis after significant one-way ANOVA). The symbol ‡ represents close adherence to p=0.05.

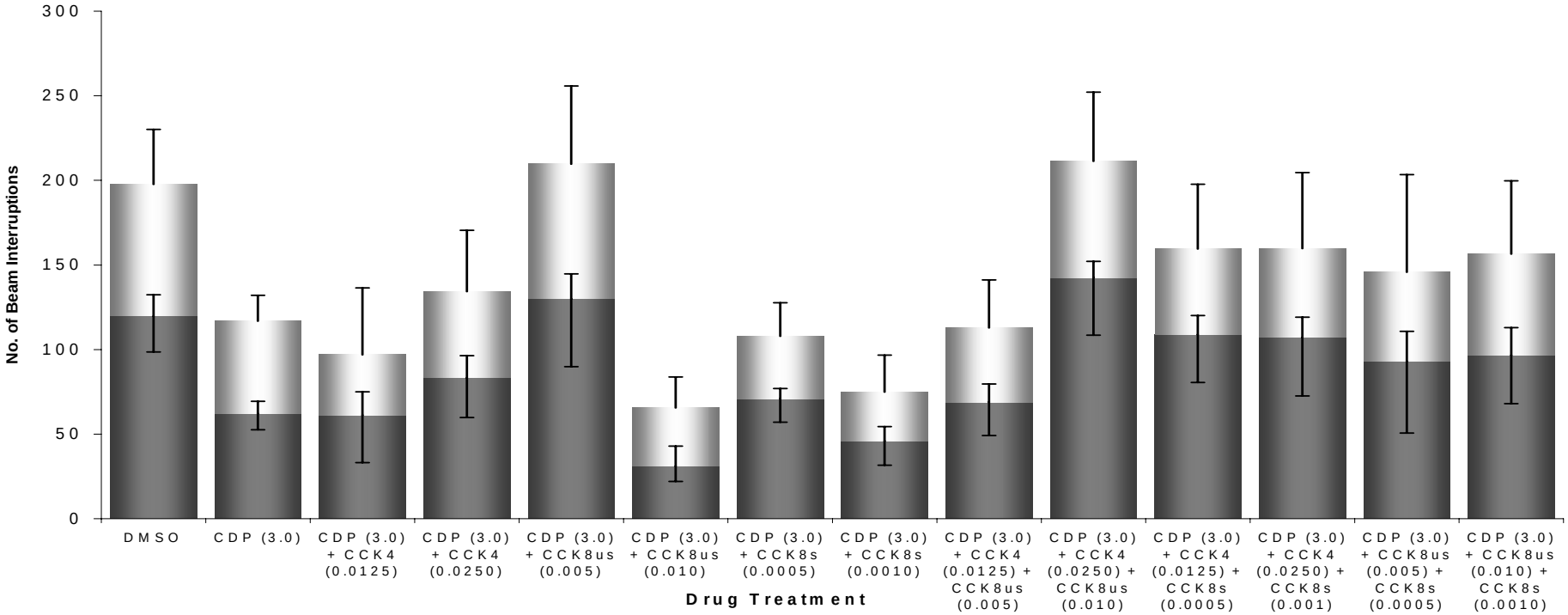


FIG. 3.6.3: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective and half maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone on locomotor activity within an open field chamber. Data presented are total, ambulatory and non-ambulatory beam interruptions (results presented as mean with SEM). Light grey bars show ambulatory locomotor activity, dark grey bars show non-ambulatory activity, totalling light grey and grey bars shows number of total number of beam interruptions. Significant differences from the DMSO group were not observed (Bonferroni post hoc analysis after significant one-way ANOVA).

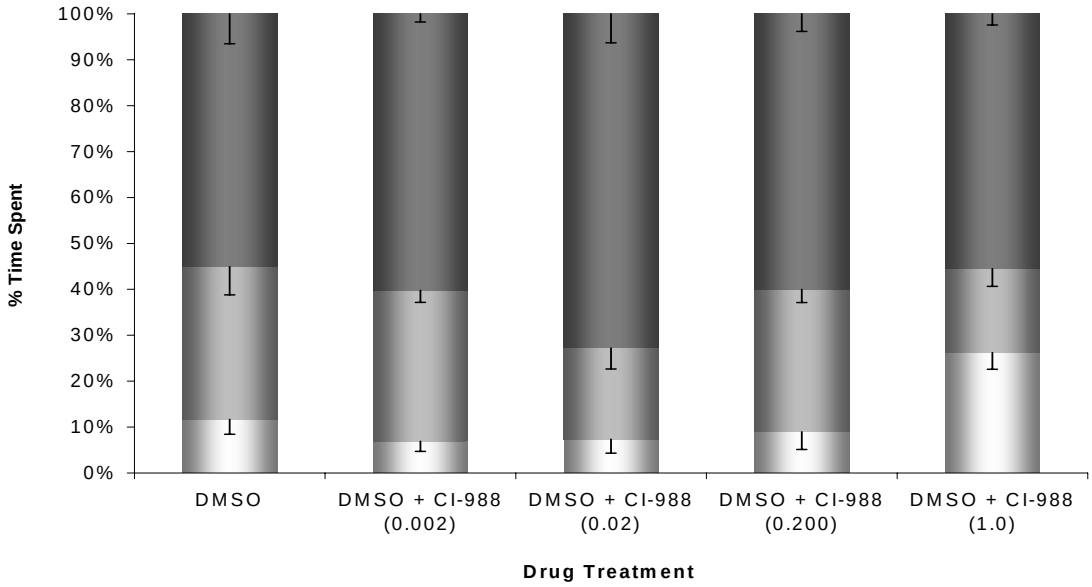


FIG. 3.7.1: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist CI-988 versus the effect of DMSO (vehicle) on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the DMSO group were not observed (Bonferroni post hoc analysis after significant one-way ANOVA).

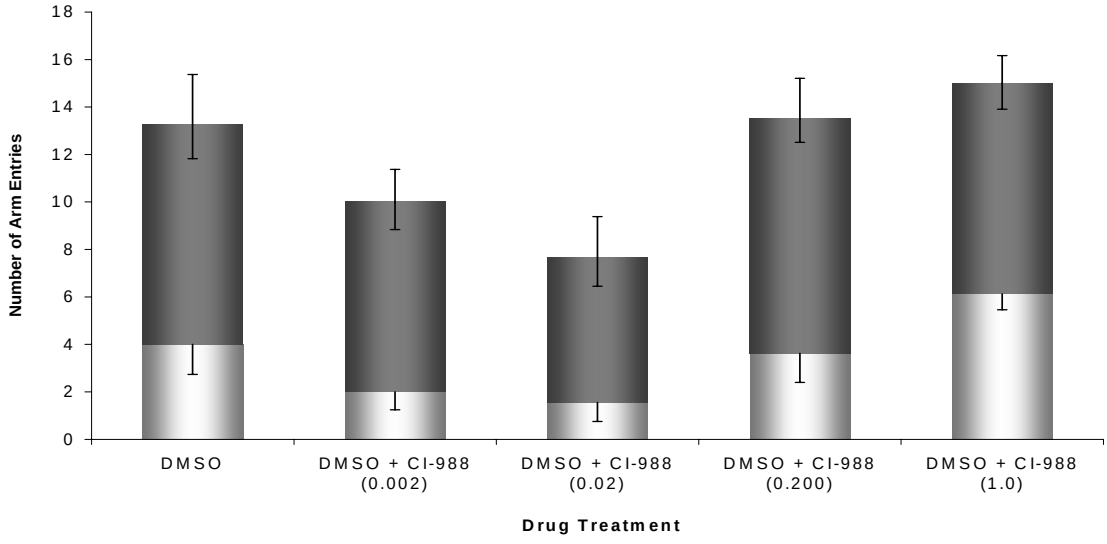


FIG. 3.7.2: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist CI-988 versus the effect of DMSO (vehicle) on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the DMSO group were not observed (Bonferroni post hoc analysis after significant one-way ANOVA).

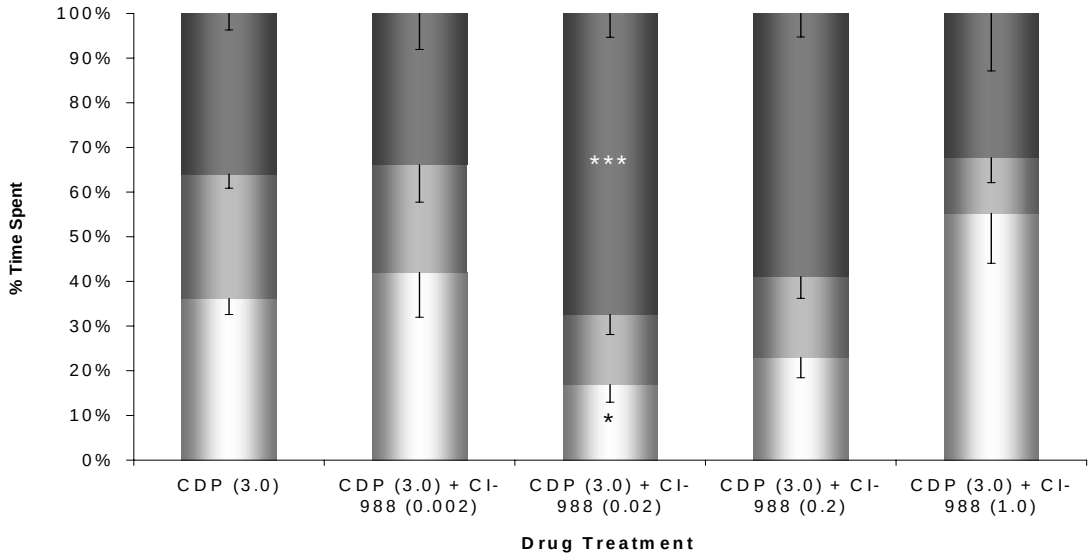


FIG. 3.7.3: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin-2 (CCK_2) receptor antagonist CI-988 in combination with chlordiazepoxide (3.0mg.kg^{-1}) versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; *p<0.05, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

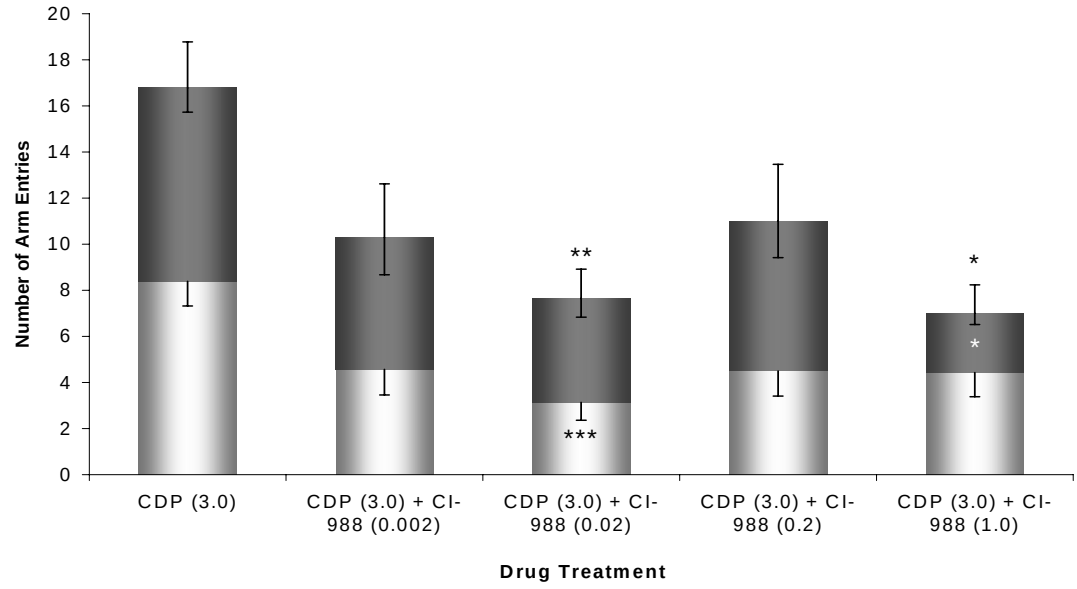


FIG. 3.7.4. Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist CI-988 in combination with chlordiazepoxide (3.0mg.kg^{-1}) versus the effect of chlordiazepoxide on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

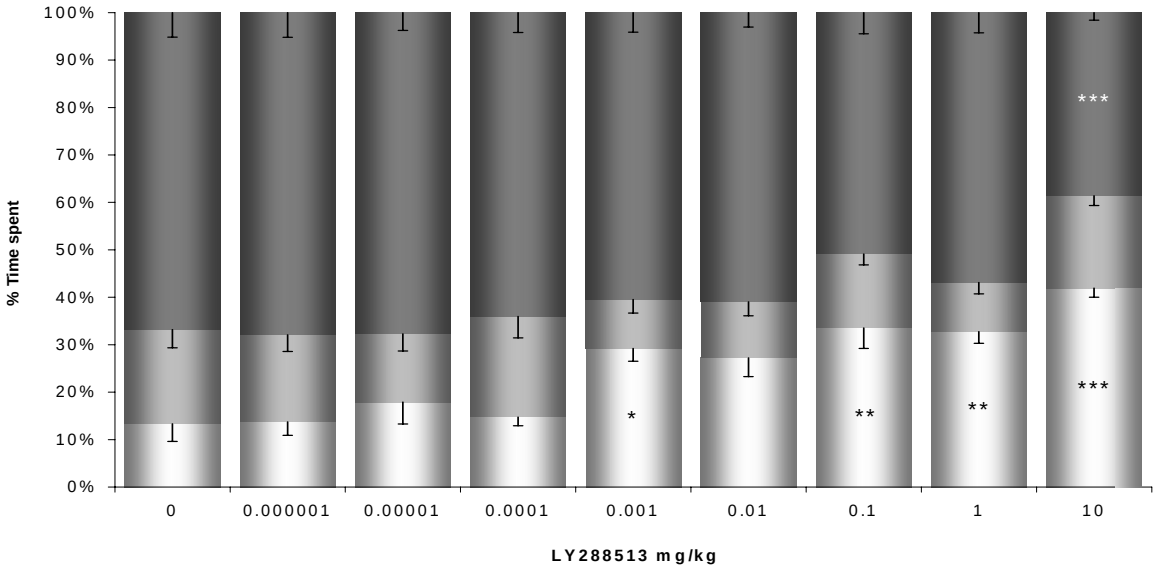


FIG. 3.8.1: Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist LY-288513 in versus the effect of DMSO (vehicle) on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the DMSO group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

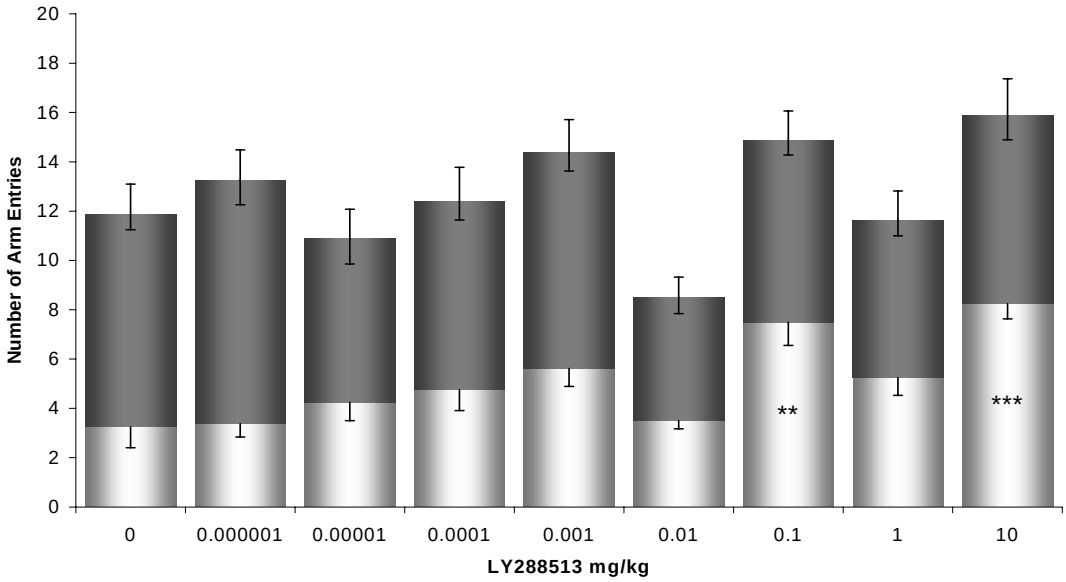


FIG. 3.8.2. Effects in rats of a range of doses (in mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist LY-288513 in versus the effect of DMSO (vehicle) on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the DMSO group are shown for open and closed arm entries within each bar and for total entries atop each bar; **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

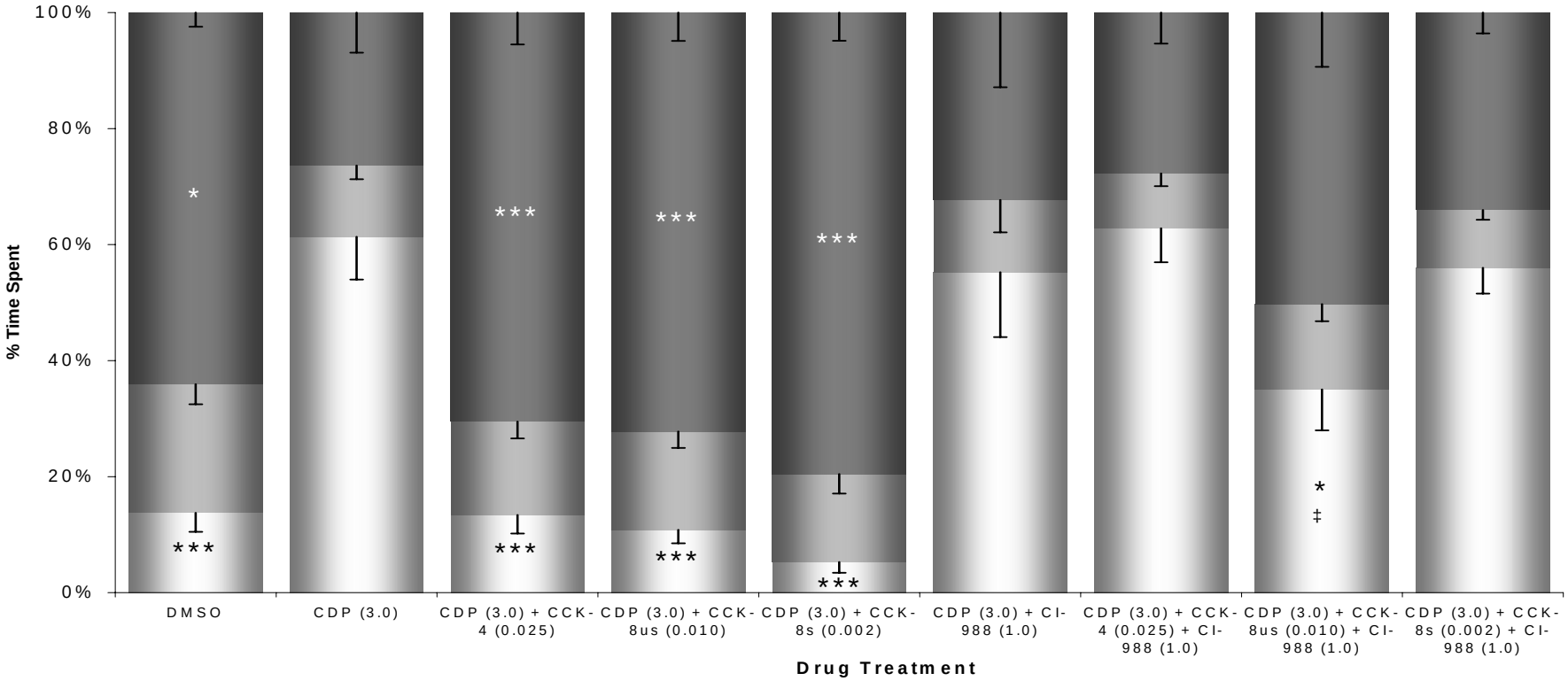


FIG. 3.9.1: Effects in rats of a dose (1.0 mg kg^{-1}) of cholecystokinin-2 receptor (CCK_2) antagonist; CI-988 versus the effect of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; *$p<0.05$, **$p<0.01$, ***$p<0.001$ and from the chlordiazepoxide/CCK-8us group ‡$p<0.05$ (Bonferroni post hoc analysis after significant one-way ANOVA).

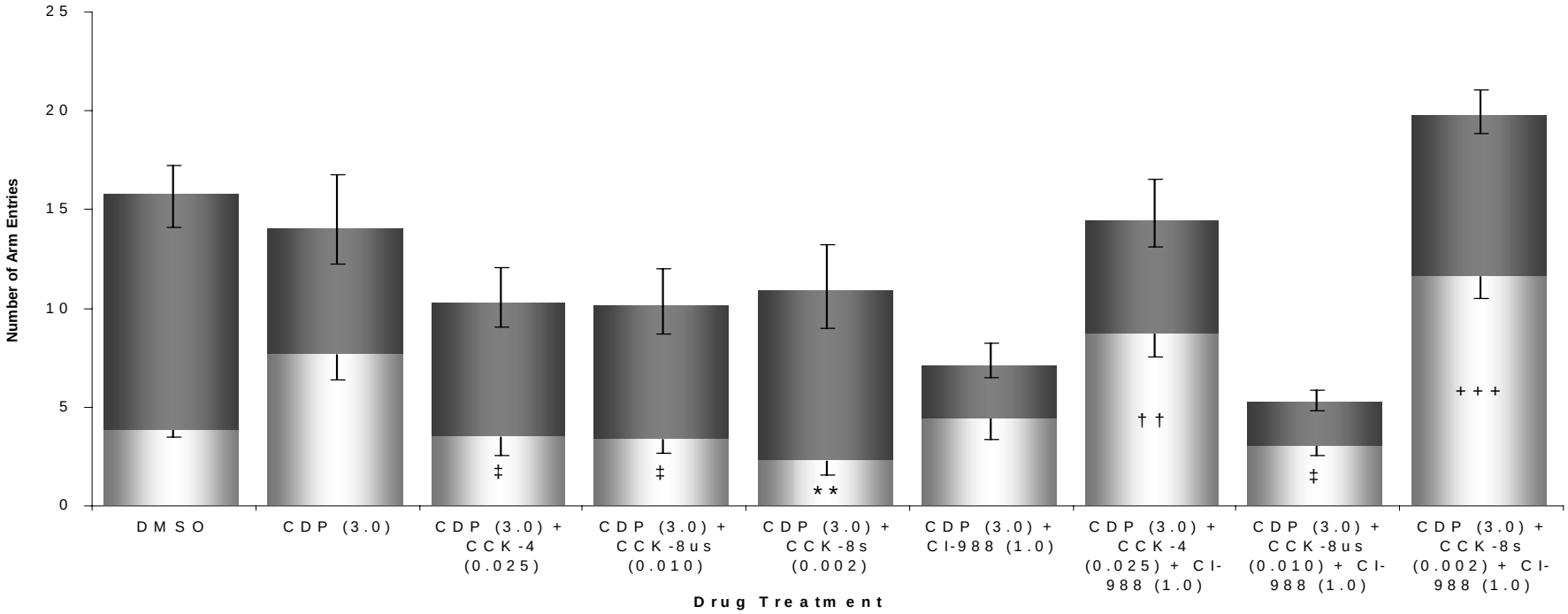


FIG. 3.9.2: Effects in rats of a dose (1.0 mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist CI-988 in versus the effect of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective doses, with chlordiazepoxide; versus the effect of chlordiazepoxide alone on entries onto the open and closed arms of the elevated plus-maze; presented collectively as total entries (results presented as mean with SEM). Grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05, **p<0.01, ***p<0.001; significant differences from the CCK-4/chlordiazepoxide group; †p<0.05, ††p<0.01, †††p<0.001; significant differences from the CCK-8us/chlordiazepoxide group; ‡ p<0.05; and significant differences from the CCK-8s/chlordiazepoxide group;

+p<0.05, ++p<0.01, +++p<0.001 are also shown (Bonferroni post hoc analysis after significant one-way ANOVA).

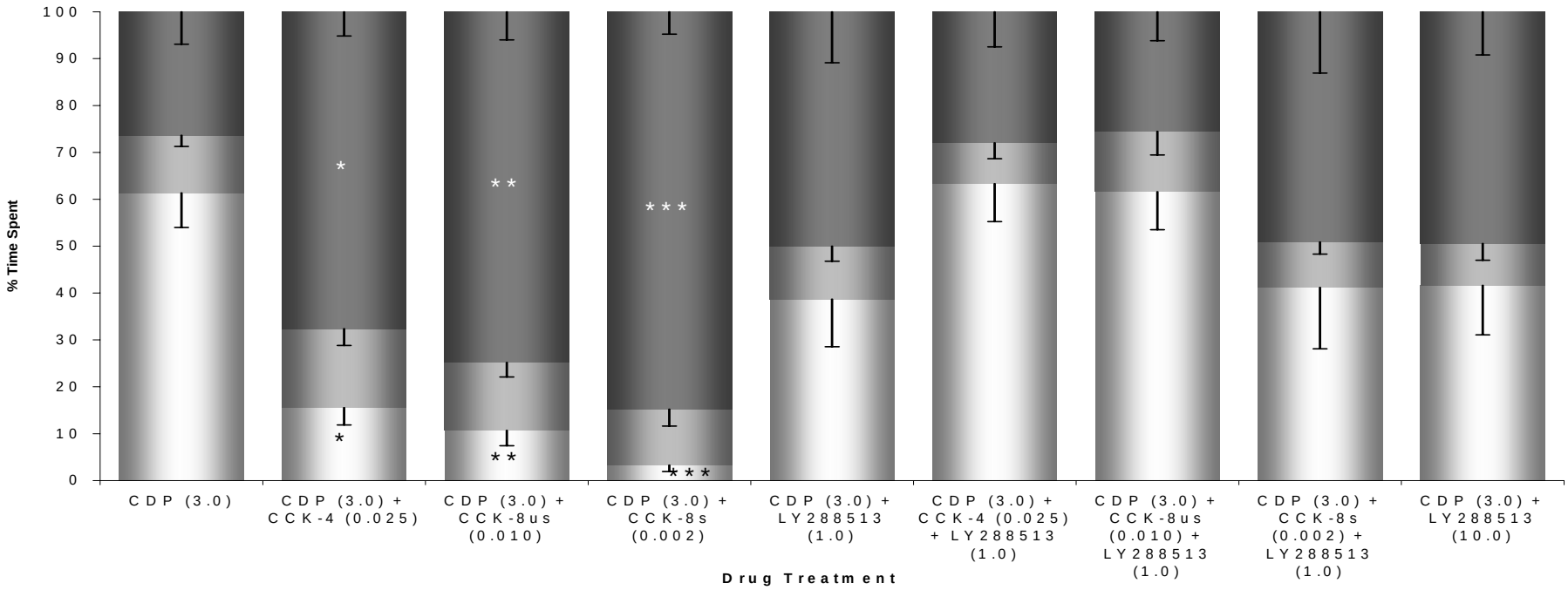


FIG. 3.10.1: Effects in rats of a dose (10 mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist LY-288513 in versus the effect of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

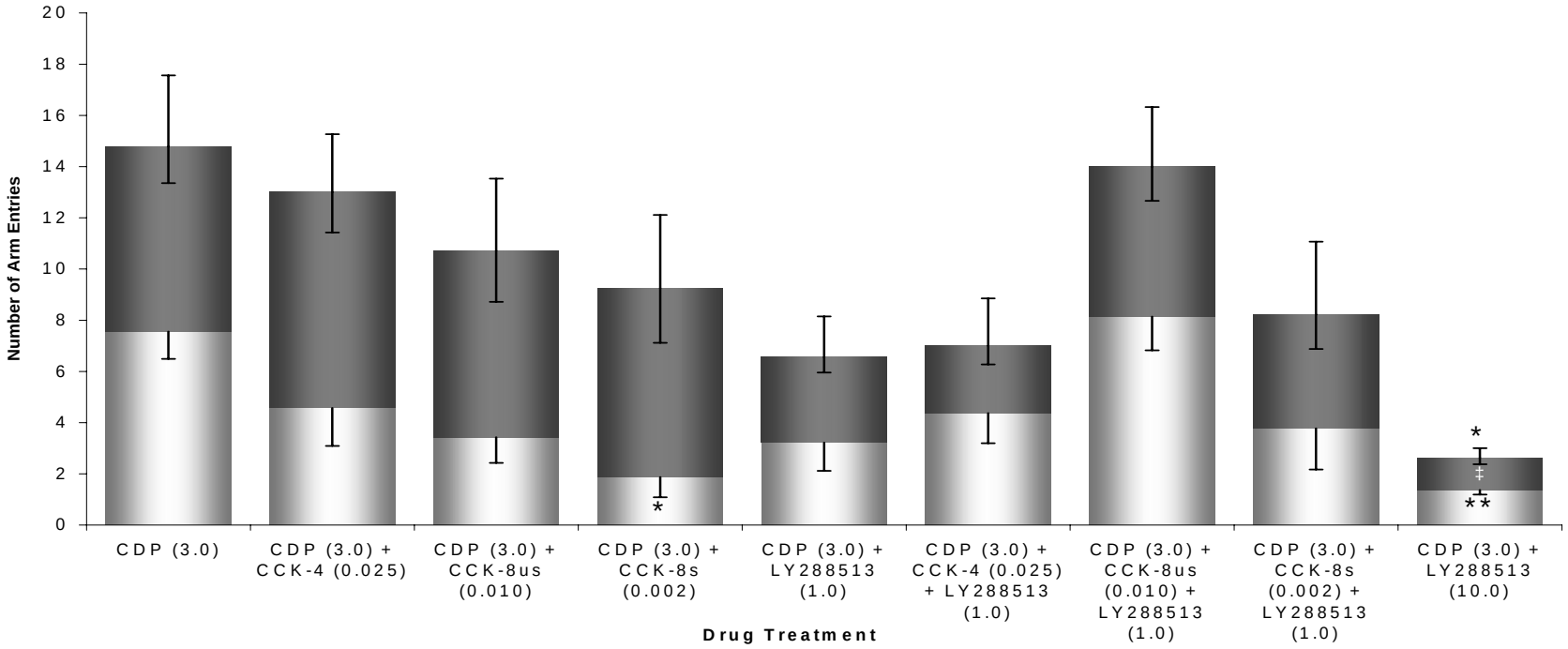


FIG. 3.10.2: Effects in rats of a dose (10 mg kg^{-1}) of cholecystokinin-2 (CCK_2); antagonist LY-288513 versus the effect of cholecystokinin forms; CCK-4, CCK-8us and CCK-8s in combination at maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and dark grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *$p<0.05$, **$p<0.01$, ***$p<0.001$; significant differences from the chlordiazepoxide (3.0) / LY288513 (1.0) group; ‡ $p<0.05$; (Bonferroni post hoc analysis after significant one-way ANOVA).

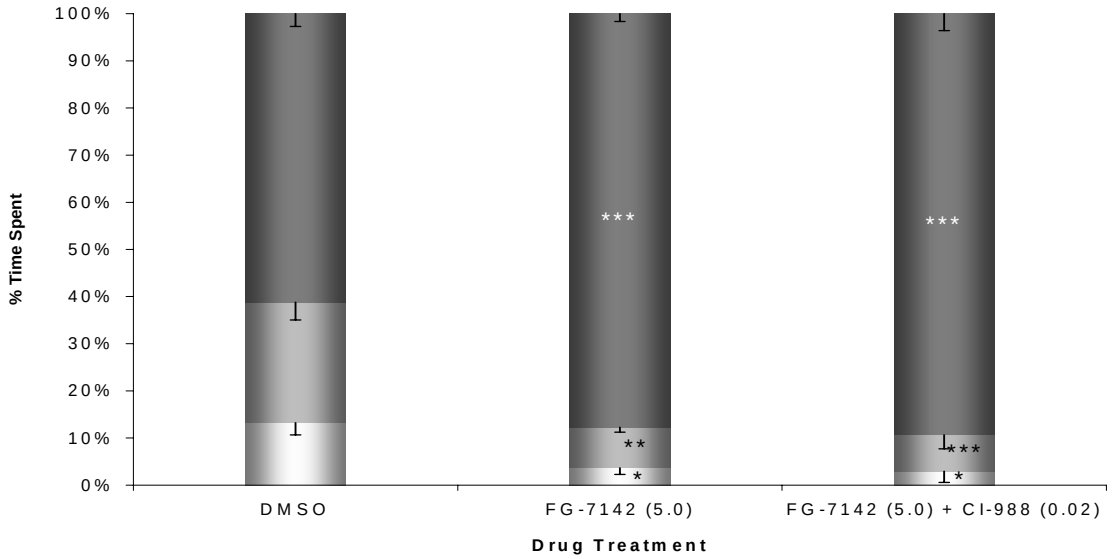


FIG. 3.11.1: Effects in rats of the cholecystokinin-2 (CCK_2); antagonist CI-988 (0.02 mg kg^{-1}) in combination with FG-7142 (5.0 $mg.kg^{-1}$) versus DMSO (vehicle) on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the DMSO (vehicle) group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

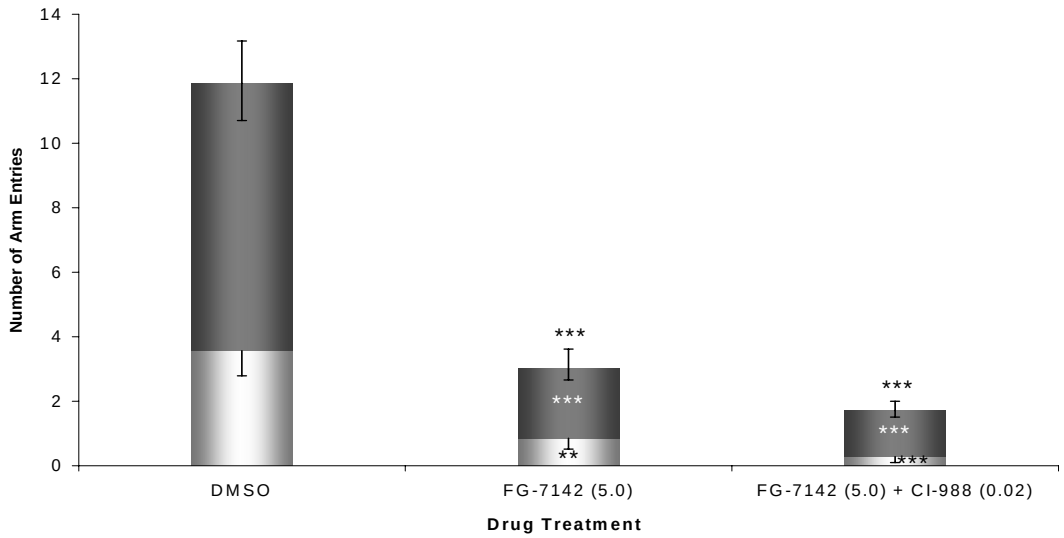


FIG. 3.11.2: Effects in rats of the cholecystokinin-2 (CCK_2); antagonist CI-988 (0.02 mg kg^{-1}) in combination with FG-7142 (5.0 $mg.kg^{-1}$) versus DMSO (vehicle) on entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the DMSO (vehicle) group are shown for open and closed arm entries within each bar and for total entries atop each bar; **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

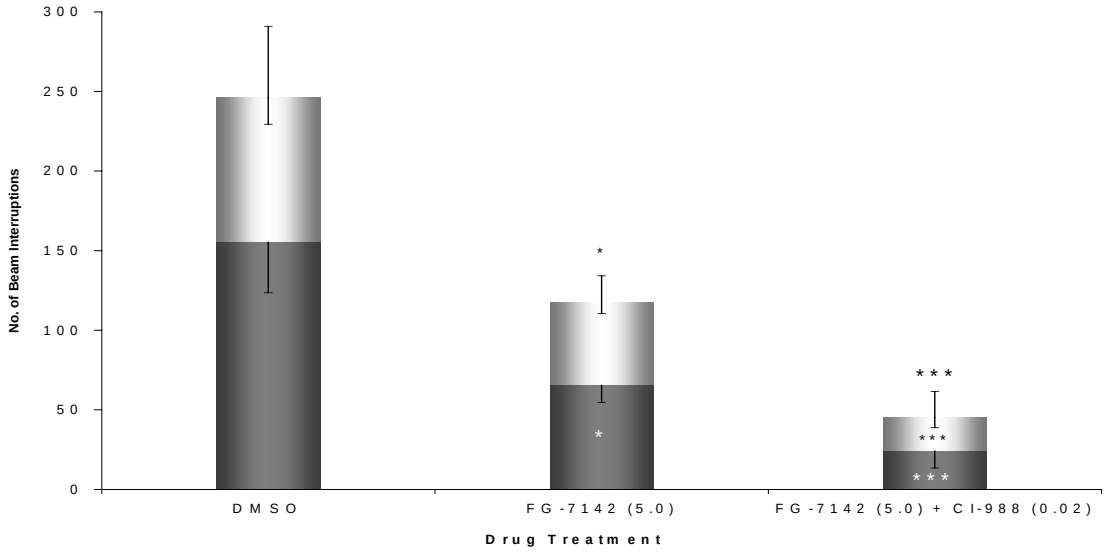


FIG. 3.12.1: Effects in rats of the cholecystokinin-2 (CCK_2); antagonist CI-988 (0.02 mg kg^{-1}) in combination with FG-7142 (5.0 $mg.kg^{-1}$) vs. DMSO (vehicle) on locomotor activity within an open field chamber. Data presented are total, ambulatory and non-ambulatory beam interruptions (results presented as mean with SEM). Light grey bars show ambulatory locomotor activity, dark grey bars show non-ambulatory activity, totalling light grey and dark grey bars show number of total entries. Significant differences from the DMSO group are shown for beam interruption counts within each bar and for total counts atop the combined bar; *p<0.05, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA.

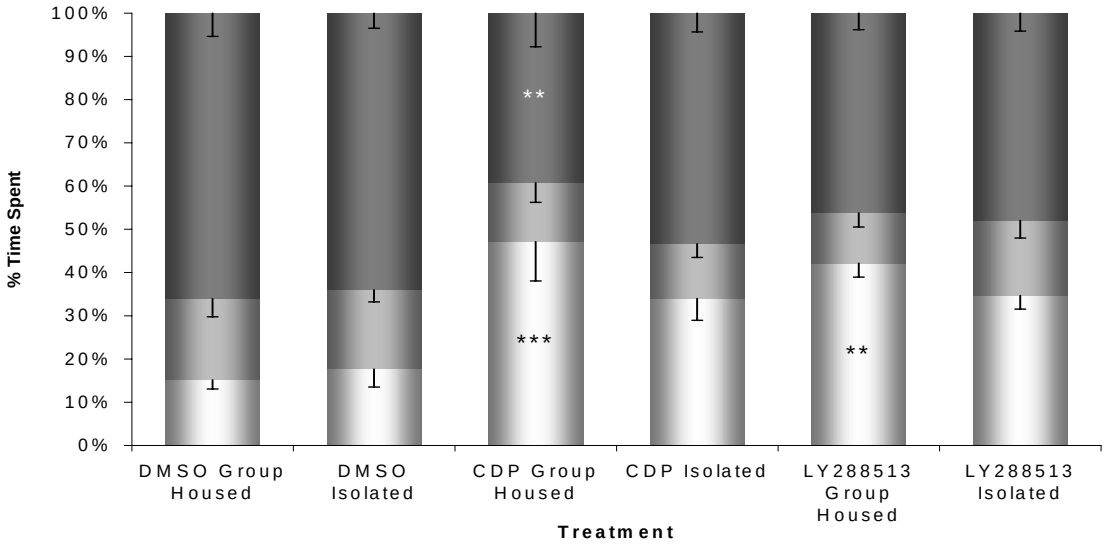


FIG. 3.13.1:. Effects in rats of chlordiazepoxide (3.0$mg.kg^{-1}$) and the cholecystokinin-2 (CCK_2); antagonist LY-288513 (10.0 mg kg^{-1}) versus DMSO (vehicle) between group housed or isolated rats upon time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the DMSO (group housed) group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

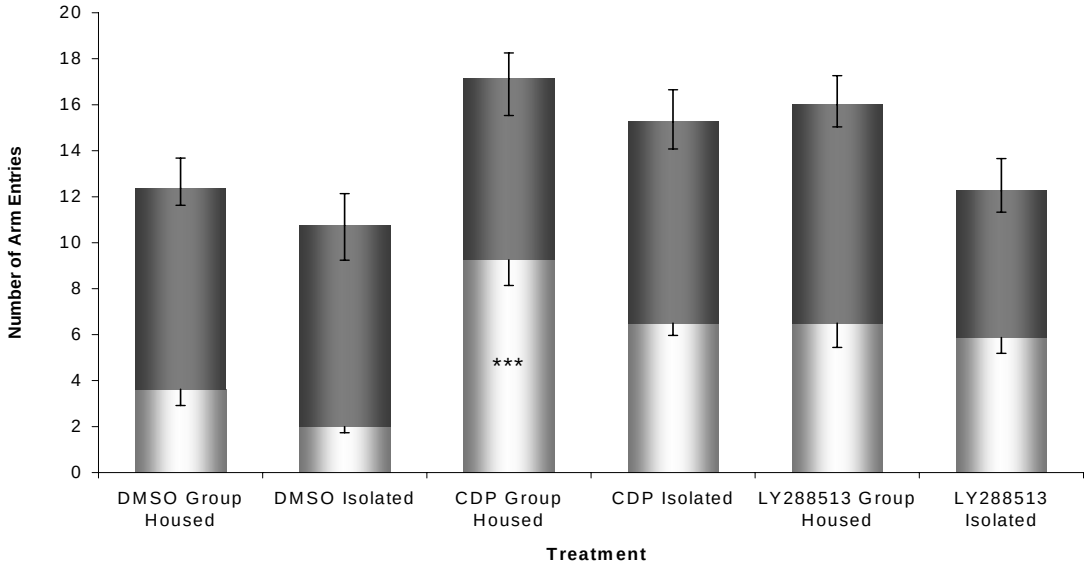


FIG. 3.13.2: Effects in rats of chlordiazepoxide (3.0mg.kg^{-1}) and LY-288513 (10.0 mg kg^{-1}) versus DMSO (vehicle) between group housed or isolated rats upon entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and dark grey bars show number of total entries. Significant differences from the DMSO (group housed) group are shown for open and closed arm entries within each bar and for total entries atop each bar; ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

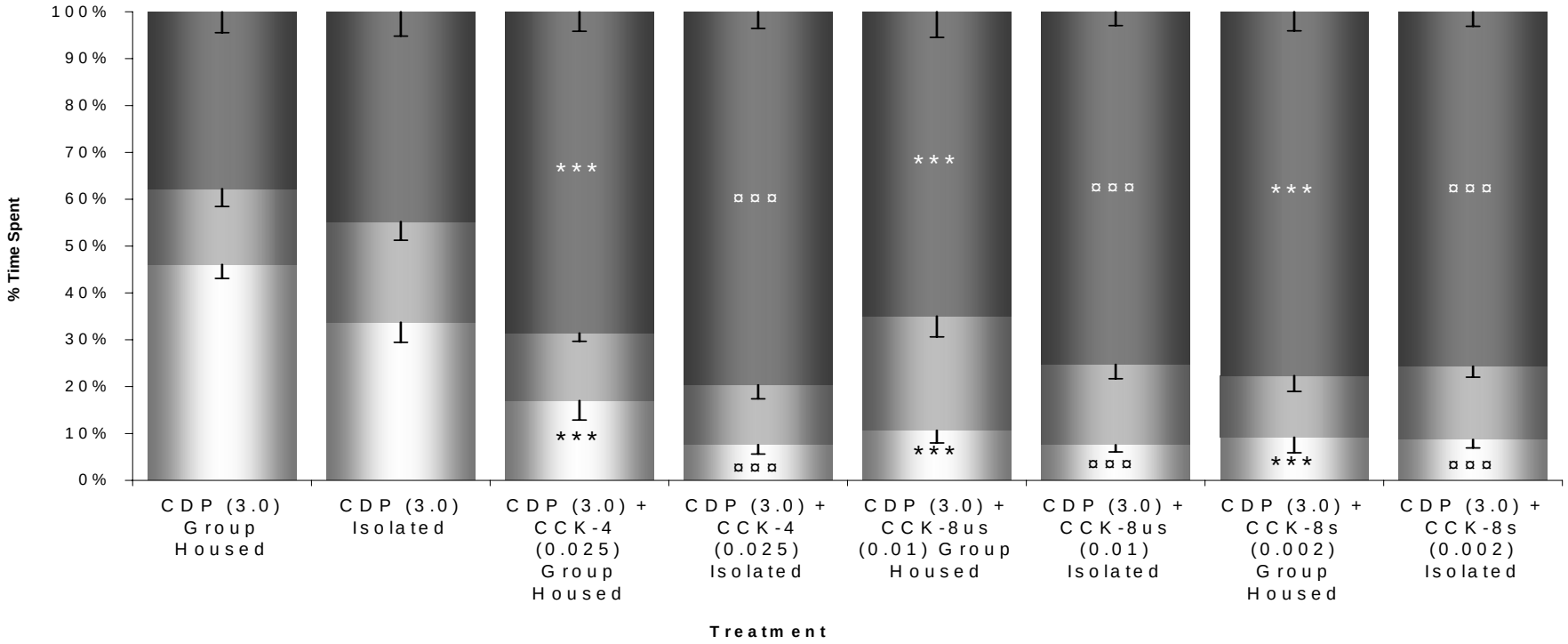


FIG. 3.14.1: Effects in rats of a cholecystokinin forms; CCK-4 (0.025mg.kg^{-1}), CCK-8us (0.01mg.kg^{-1}) and CCK-8s (0.002mg.kg^{-1}) in at maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone, in either group housed or isolated rats, upon entries onto alone on time spent in specific areas of the maze (as a percentage of the total trial time) on the elevated plus-maze (results presented as mean with SEM). Light grey bars show % time spent on open arms, grey bars show % time spent on central platform, dark grey bars show % time spent on closed arms. Significant differences from the chlordiazepoxide (group housed) group are shown; *p<0.05, **p<0.01, ***p<0.001; Significant differences from the chlordiazepoxide (isolated) group are shown; ¤¤¤p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

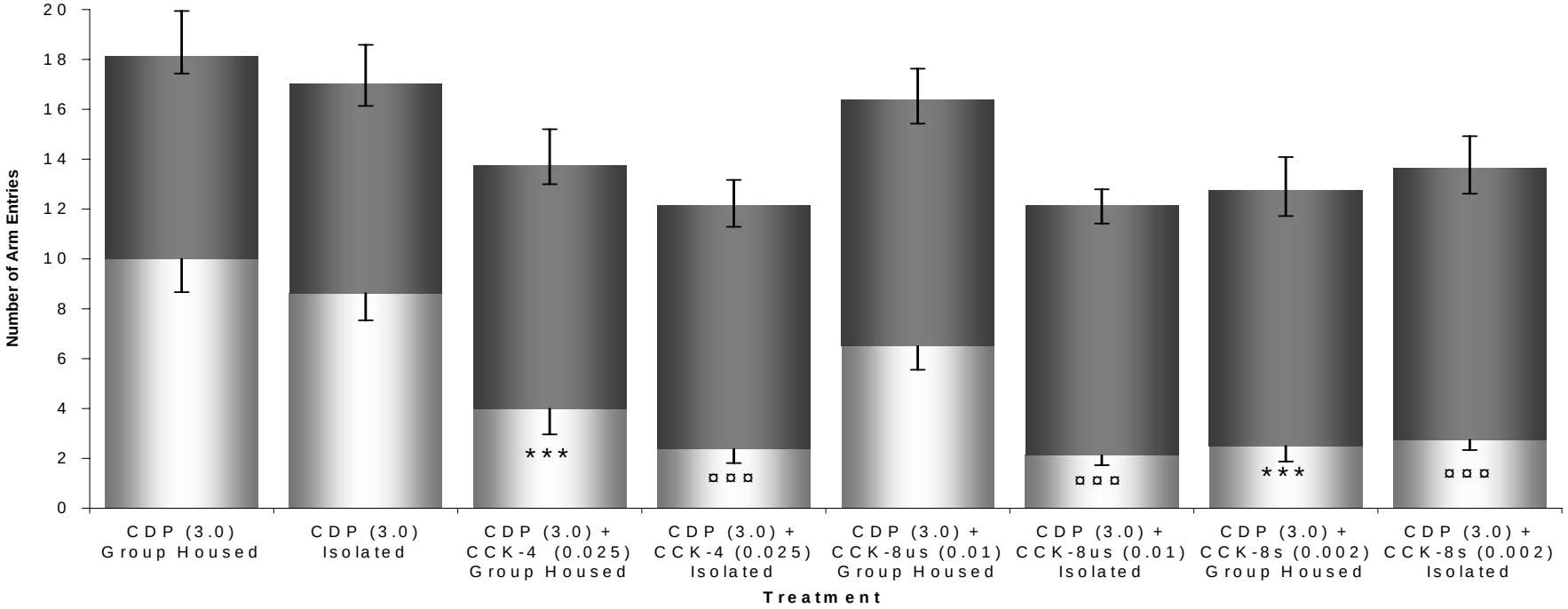


FIG. 3.14.2: Effects in rats of a cholecystokinin forms; CCK-4 (0.025mg.kg^{-1}), CCK-8us (0.01mg.kg^{-1}) and CCK-8s (0.002mg.kg^{-1}) in at maximal effective doses, with chlordiazepoxide versus the effect of chlordiazepoxide alone, in either group housed or isolated rats, upon entries onto the open and closed arms of the elevated plus-maze presented collectively as total entries (results presented as mean with SEM). Light grey bars show number of entries onto open arms, dark grey bars number of entries onto closed arms, combined light grey and grey bars show number of total entries. Significant differences from the chlordiazepoxide group are shown for open and closed arm entries within each bar and for total entries atop each bar; *p<0.05, **p<0.01, ***p<0.001; Significant differences from the chlordiazepoxide (isolated) group are shown; ¤¤¤p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

Discussion

The solvent, dimethylsulphoxide (DMSO) was preferred for service as the vehicle throughout these studies due to solubility issues surrounding cholecystokinin peptides in saline. A general uniformity of data between groups using saline and DMSO vehicles, concludes that DMSO almost certainly is an appropriate vehicle for use within these experiments. The finding that benzodiazepine agonists such as chlordiazepoxide, produce increases in time spent in the open arms within the plus-maze is a long established phenomenon (Pellow *et al*., 1985; Lister, 1987; Trullas *et al*., 1989) and is confirmed in this study. This increase is typically offset by a decrease in time spent in the closed arms. It is significant that time spent on the central platform is not significantly affected here. Time on the central platform may be seen as a decisive period in which an animal evaluates the relative merits of entering each arm. If this time period is altered it may be inferred that the drug under test is exerting an effect on cognitive processes. Therefore, an increased central platform period, without any change in total number of entries, may indicate some impairment in decision-making processes. However, compounded with a downward variation in total entry number this may indicate inhibited locomotor activity. Rats treated with a benzodiazepine inverse receptor agonist FG-7142, produced a statistically significant decrease in central platform time which compounded with a significant decrease in total arm entries, may remain obscure with regard to interpretation using analysis of indices alone. An observed phenomenon within this group however implies that a high level of anxiety underlies this behaviour. Animals were observed to move quickly into the closed arms shortly after a brief "orientation" period in which exploration or central platform based assessment of the open arm occurred. This may imply that FG-7142 activates a hypervigilant state that causes a rapid transition from the central platform to the closed arms. This state of hypervigilance would support face validity criteria given that this is a fundamental symptom within clinical anxiety (Dubovsky 1990).

Corresponding increased numbers of open arm entries were also observed for chlordiazepoxide compared to the vehicle group. This further supports the reputed anxiolytic effects of benzodiazepine agonists implying not only greater time spent within open arms but also accompanying increases in open arm entries. Interestingly, chlordiazepoxide produced a significant increase in total arm entries. This increase implies either some form of locomotor activation or an inhibition of fear within the maze as a whole unit. The latter would be apparent whereby a novel environment such as the plus-maze would incorporate anxiety-promoting elements in both arms. The antithesis of this phenomenon would not extend however to putative anxiogenic agents such as FG-7142. This compound while producing significant inhibition of arm entries and of time on the central platform, affects behaviour such that animals spend a high percentage of time in a single closed arm. A high level of anxiety would be proposed to exert similar effects.

FG7142 however did not produce a significant decrease in time spent in the open arms compared to DMSO vehicle but displayed marked significance in increasing closed arm time. This lack of significant activity of FG-7142 on open arm time was possibly due to floor effects within this model. Unlike previous data (using the saline vehicle) FG-7142 in DMSO produced a significant decrease in open arm, closed arm and total entries. This augmentation of effects using the DMSO vehicle may be due to greater solubility, thus drugs are able to exert greater efficacy.

As would be expected, FG-7142, a benzodiazepine receptor inverse agonist, prevented the increase in open arm indices observed with chlordiazepoxide, indicative of pharmacological opposition. Interestingly, both compounds inhibited spontaneous open field locomotor activity (figure 3.2.3.). It has previously been observed that chlordiazepoxide increases locomotor activity in the open field (Gentsch *et al*., 1987), which seems to be in contradiction with these findings. This phenomenon leads to a possible conclusion that chlordiazepoxide produces some sedation at the tested dose. FG-7142 perhaps not surprisingly inhibited locomotor

activity. It might be expected of a putative anxiogenic agent, that it would decrease exploration within a novel environment. This might also be explained by either increased activation (chlordiazepoxide) or decreased activation (FG-7142) of benzodiazepine receptors inhibiting locomotor activity via either a hypo-attentive state (chlordiazepoxide) producing sedation or via a hyper-attentive state (FG-7142) promoting freezing behaviour.

A combination of chlordiazepoxide (3.0mg.kg^{-1}) and FG-7142 (5.0mg.kg^{-1}) despite both individually inhibiting locomotor activity, produced no difference from that of the vehicle group. A combination of both agents would undoubtedly negate the activity of both individually given their antagonistic pharmacology, and thus should effectively normalise behaviour. It may be noteworthy that measurement of locomotor activity within the first two minutes was not carried out. The exploratory phase of chlordiazepoxide treated rats may have occurred within this initial period. Therefore, within the measured period, the animal will have exhausted an incentive to explore. Interestingly, animals under study appeared to exhibit relatively high levels of endogenous anxiety when compared with those in several other studies. Consequently, cholecystokinin forms were tested in chlordiazepoxide treated animals rather than with nondrugged animals, in order to effectively raise the level of the baseline response. This measure was taken to avoid floor effects. However as is shown in this study dose dependent effects are an important factor.

All three forms of cholecystokinin reversed the effect of chlordiazepoxide with an inverse bell-shaped dose response curve for both time spent and entries into the open arms. This profile has been observed for the synthetic agonist BC-197 (Derrien *et al*., 1994). CCK-4, CCK-8us and CCK-8s exerted significant effects at doses, 0.025mg kg^{-1}, 0.005-0.010 mg kg^{-1}, and 0.0005-0.002 mg kg^{-1} respectively. At higher doses a reversal of chlordiazepoxide activity was no longer observed. This data supports the negative findings of Rex *et al*., (1997), Rex and Fink (1998), and Chopin and Briley (1993) where they failed to observe activity on the plus-maze for CCK-8s within the higher dose range 0.01 - 0.1 mg.kg^{-1}. Where these specific doses of

cholecystokinin forms counteract the activity of chlordiazepoxide, it may be assumed that this is an anxiogenic-like effect on the elevated plus-maze given their reported anxiogenic-like activity alone (Rex *et al*., 1994; Vasar *et al*., 1994; Koks *et al*., 1999; Bickerdike *et al*., 1995; Chopin and Briley, 1993).

Neuropeptide receptor antagonists can be divided into two main classifications, either peptide or non-peptide. Non-peptide cholecystokinin antagonists were found to be chemically very different to the endogenous peptide cholecystokinin forms (Hill *et al*., 1992). Until relatively recently, the mechanism by which these antagonists exert their effects remained an enigma. However, radioligand binding studies combined with mutational analysis of cholecystokinin receptors has revealed the existence of distinct binding domains for peptide agonists and non-peptide antagonists on a single receptor. These techniques have shown that most peptide agonists interact at sites within the extracellular region of the receptor, particularly at the NH_2 terminal strand. Non-peptide antagonists, however, interact at sites within the transmembrane region (Beinborn *et al*., 1993). In a study by Schwartz *et al*., (1995), it was proposed that cholecystokinin receptors, like that of other neuropeptide receptors, follow an allosteric model. This model purports that specific ligands exhibit effects that shift a receptor between either an active or inactive conformational state. Thus, it was proposed that non-peptide antagonists exert their effects by shifting the equilibrium away from the active state. The mechanism by which this activation / inactivation is conferred is probably via a coupling / de-coupling, respectively of the G protein. It may still be possible that these "decoupled" receptors exhibit efficacy but via another mechanism (other than that conferred by the G-protein) (Weatherford *et al*., 1993; Blaker *et al*., 2000).

This mechanism appears to explain the inverted bell-shaped dose-response curve phenomenon exhibited by endogenous cholecystokinin forms. As is demonstrated, these agonists produce an anxiogenic-like response on the elevated plus-maze over a narrow dose range. A possible hypothesis derived from this model is that cholecystokinin peptide agonists are capable of

binding both active sites on the single receptor, albeit with differing affinities. At the anxiogenic dose, cholecystokinin may bind to the peptide specific site and elicit the response. At a higher dose however, cholecystokinin may bind both the peptide specific and the purported non-peptide specific site, the latter site with lesser affinity. The activation at the non-peptide, so-called antagonist site, may shift the receptor to a G protein uncoupled conformation, and thus deactivating the receptor, at least with regard to anxiety-like efficacy.

Brain CCK_2 receptors have also been demonstrated, with radioligand binding studies, to exist in three different affinity states with regard to CCK-8 forms. Two of these states, exhibiting a high or low affinity to CCK-8 were detected. A third state of very low affinity was detected using [^{3}H]-L365,260 (radiolabelled non-peptide antagonist). The third state represents a much larger percentage of the receptor population than the higher affinity states (Huang *et al*., 1994). This study suggests that non-peptide antagonists bind with a greater affinity to the G protein uncoupled conformations (low or very low affinity) and peptide agonists bind with a greater affinity to the high affinity form. Peptide agonists may also bind to the G protein uncoupled receptor conformation but with a much lower affinity.

It may be speculated that cholecystokinin binds with high affinity on the G protein coupled conformation receptor population and elicits a response at a specific dose. At a higher dose however, it may bind both the high and the low affinity receptors, where the low affinity receptors exhibit no activity and thus shift an active receptor population to that of an inactive population. The increased "activation" of the G protein uncoupled conformation may also have an inhibitory effect on the anxiogenic response gated by the active population. Furthermore, it may also be proposed that the purported cholecystokinin non-peptide antagonists are actually acting as agonists at these low affinity receptor sites.

An interesting finding in this study was that CCK-8s significantly decreases locomotor activity (a measure of total number of entries) on the elevated plus-maze. This phenomenon was not

apparent in the CCK-8us and CCK-4 groups. A plausible inference from this observation is that CCK_1 receptor activation produces this decrease in locomotor activity on the elevated plus-maze. CCK-8s exhibits a 500 to 1000 fold greater affinity at the CCK_1 receptor compared to CCK-8us and CCK-4 (Silvente-Poirot *et al*., 1993). This phenomenon is again not manifest at higher doses, which again may be due to a self-limiting CCK_1 receptor mechanism analogous to that described earlier for the CCK_2 receptor. Undoubtedly this decrease in locomotor activity on the maze is the major underlying factor for CCK-8s mediated decreases in open arm entries. However it is unlikely that measures for time spent on the open arms are altered by this factor. It would appear that CCK-8s exhibits efficacy on the elevated plus-maze, which affects both anxiety-like behaviour and locomotor activity, whereas CCK-4 and CCK-8us only exhibit efficacy on anxiety-like behaviour. Therefore, this phenomenon may be dependent upon an equilibration between CCK_1 and CCK_2 receptors, classically purported to underlie anxiety behaviour. This hypothesis may bring into question previous studies that have taken the measure of arm entries, and stated categorically that that is a measure of anxiety-like behaviour, whilst disregarding locomotor activity as a factor.

Examination of open arm entry data, for CCK-4 and CCK-8s groups, yielded an interesting correlation whereby number of open arm entries appeared to exhibit two separate dose response troughs. CCK-4 decreased the total number of arm entries at both $0.025mg.kg^{-1}$ and $0.075mg.kg^{-1}$ without any changes at the intermediate dose $0.05mg.kg^{-1}$. CCK-8s at $0.0005mg.kg^{-1}$ and $0.002mg.kg^{-1}$ decreased the numbers of total, open and closed arm entries, with the intermediate dose of 0.001 not showing a significant decrease in numbers. This dose response profile however is not observed in CCK-8us treated groups. A hypothesis describing this phenomenon is largely conjecture, given the novel finding. It might be viable, however that an increase in anxiety level is induced at a lower dose, while at the higher dose an inhibition of locomotor activity might be evident. At the intermediate dose however, anxiety level may diminish while inhibition of locomotor activity may not yet be manifest, thus a loss

of any significant decrease in total arm entries or other measures. This however is not supported directly by percentage time spent measures, which indicate that a purported anxious state is observed at the intermediate dose for CCK-4 but is not observed at either higher or lower doses. Regarding CCK-8s, the supposed anxious state occurs at the intermediate and higher dose but not at the lower dose. These observations infer that CCK-8s and CCK-4 possibly act via different mechanisms with regard to these effects. The aforementioned hypothesis may be apparent for CCK-4 given a purported anxiety state induced at the lower dose and no effects upon time spent at the higher dose. With regard to CCK-8s, this relationship may be in reverse whereby at the lower dose locomotor activity is inhibited via an anxiety independent factor and at the higher dose, anxiety dependent reduction in activity are observed. The intermediate dose of CCK-8s would thus be at a post-independent and pre-dependent stage. The CCK_2 specific antagonist, CI-988 also reverses the effects of chlordiazepoxide on the total number of entries, interestingly also adhering to a profile of two separate dose response troughs, identical to that of CCK-4 and CCK-8s. Therefore the active elements underlying these phenomena probably are restricted to CCK_2 receptors. The observed profile for CI-988 is comparable with that of CCK-8s, given that the higher dose is not active with regard to open arm time. The underlying physiological mechanism surrounding these phenomena is mostly open solely to conjecture. One possible mechanism is that of a four way model of receptor activation. Firstly where a cholecystokinin peptide is at the maximal dose (altering open arm time), activation of one receptor subsite occurs, assigned here as the CCK_{2A} site. At the specific dose altering entry number, this is negated by activation of both CCK_{2A} and CCK_{2B} sites. At the higher entry number specific dose then CCK_{2B} sites are activated to a greater degree than CCK_{2A} sites, possibly via activation at the non-peptide site on the CCK_{2A} receptor (thus deactivating the CCK_{2A} receptor), hence secondary arm entry effects. At an even higher dose, inactive in all measures, the non-peptide site on CCK_{2B} receptors is also activated, thus deactivating both sites. A CCK_2 antagonist such as CI-988, therefore, may deactivate both subtypes selectively. For example CCK_{2A} deactivation occurring at the lower dose and at the

higher dose CCK_{2B} is deactivated. This deactivation of both receptor subtypes inhibits locomotor activity within the plus-maze model. At the intermediate dose, however, deactivation of both subtypes would not be enough to produce locomotor inhibition, which would only occur at higher levels. Given hindsight, this hypothesis should have been tested within the open field locomotor activity paradigm. Although tests were performed using doses active upon time spent in the open arms, and the preceding dose; higher doses at which total entries are inhibited were not tested.

Dose response studies of CI-988 with DMSO (vehicle) in the absence of chlordiazepoxide showed no significant effects on any indices, probably due to floor effects, i.e.: inability to force the effect downwards any further from an already low level.

Nevertheless, no significant differences were recorded during locomotor activity testing. Certain trends were observed. However, standard deviation within drug groups was unusually high, thus negating possibility of significant analysis. An explanation for this variation cannot be easily proposed apart from perhaps differing sensitivities of individual animals to cholecystokinin doses determined by their distinct endogenous anxiety levels.

A combination of cholecystokinin forms at both half and maximally effective doses, with regard to attenuation of chlordiazepoxide effects upon time spent in the open arms (figure 3.6.1.), yielded quite exceptional results. The higher dose inhibited the activity of chlordiazepoxide (by decreasing open arm time), whereas the lower dose was either inactive or at least only partial in effect. Despite the maximal dose of CCK-8us ($0.01 mg.kg^{-1}$) increasing time spent on the central platform in this study, this was not observed in the previous dose response study, and thus perhaps can be disregarded. Another interesting phenomena was observed here whereby, a combination of CCK-4 and CCK-8 (either form) at either half or at their maximally effective doses failed to elicit inhibition of chlordiazepoxide activity. It might be expected that combining two different forms of cholecystokinin peptide at half their

maximally active doses would also produce a maximal effect, if they were efficacious via the same receptor site. However data to the contrary was observed. Furthermore, a combination of CCK-8us and CCK-8s at these doses did maintain the expected inhibition of chlordiazepoxide activity. These phenomena may be explained by either of the following hypotheses:

1. Binding site/ receptor interactions are specific for CCK-4 and CCK-8, at two distinct binding sites on either separate or on the same receptor that counteract the action of one another. These receptors would exist either centrally or peripherally.

2. Distinct populations of receptors present at different regions of the central or peripheral nervous system (where cholecystokinin forms differ in their bioavailability) due to either differential blood brain barrier permeability or other peripheral bioavailability factors.

It may be proposed that the CCK-4 induced anxiety-like activity is gated at the CCK_2 receptor and is counteracted by CCK-8s at the CCK_1 receptor. However, subsequent studies showed that, specific CCK_2 antagonists, CI-988 and LY-288513, attenuated a reversal of chlordiazepoxide activity, by each cholecystokinin form. This would indicate that solely CCK_2 receptor activation underlies these effects in all cholecystokinin forms tested. This hypothesis of CCK_2 receptor subpopulations is further supported by evidence from a study by Derrien et al., (1994) that a systemically administered, selective CCK_2 agonist BC-197 induced anxiogenic-like effects in the plus-maze, whereas the CCK_2 agonist BC-264 had no observed activity. BC-197 induced effects were also blocked by CI-988 but BC-264 produced anxiogenic-like dose dependent effects when administered with L-365260. Binding competition with [^{3}H]pBC-264 revealed that BC-197 exhibits differing affinities for two distinct receptor sites and BC-264 exhibits a very similar affinity for these sites. This implies that BC-264 binds to both peptide and non-peptide receptor sites with equal affinity, therefore, exhibiting activity via the former and negating this activity via the latter. Addition of L-365260, binding solely to the non-peptide site will exert no efficacy, and thus confers activity

to BC-264 at the peptide site, hence anxiogenesis. BC-197 is probably efficacious only at the peptide site and will only be antagonised in effect by CI-988 at the non-peptide site, thus negating any activity.

Numbers of arm entries were modified only in the CCK-8s group at the maximally responding dose and in the CCK-8us (0.01) / CCK-8s (0.002) combined group. This differed from profiles in the preceding dose response curves, where CCK-4 and CCK-8us both achieved significant differences. This loss of significance may be explained by a relatively low number of entries by the chlordiazepoxide group in this study (a mean of 9.375 for open entries) compared to that within previous CCK-4 and CCK-8us (a mean of 12.69) dose response studies. Experimental and housing conditions for both batches were identical. Differences between numbers of open arm entries between batches missed significance with a p-value of 0.98. Closed arm entries showed no observed trends whereas numbers of total arm entries between batches were significantly different ($F_{1,21}$=4.80, $p<0.05$). A cause of this variation cannot be easily proposed apart from differences between batches of animals and their specific locomotor activity.

Dose response studies of the CCK_2 antagonist, LY-288513, in DMSO (vehicle) demonstrated a broadly dose dependent increase in time spent and in number of entries onto the open arms without the previously dominant inverse bell shaped dose response profile. This might occur where LY-288513 not exhibiting selectivity for either subtype, and thus at a suitable dose rebalancing any endogenous imbalance between receptor subtypes. This would thus exert an anxiolytic like response. Despite total and closed arm entries remaining unaffected across the dose range, doses, $0.1mg.kg^{-1}$ and $10mg.kg^{-1}$, open arm entries were significantly increased. Yet again, however, an intermediate, $1mg.kg^{-1}$ dose was without effect. This phenomenon of dual peak or trough profiles across a dose response was observed for measures of open or total entries in other tested agonists and antagonists. A possible hypothesis elucidating this

phenomenon was proposed earlier.

Studies of cholecystokinin peptide antagonism by CI-988 and LY-288513 showed a general attenuation of effects, whereby co-administration of CI-988 with both the CCK-4/chlordiazepoxide and CCK-8s/chlordiazepoxide combinations, restores chlordiazepoxide activity. Perhaps a relatively extraneous observation is that CI-988 co-administered with CCK-8us/chlordiazepoxide only partially attenuating CCK-8us decreased open arm time, compared to chlordiazepoxide alone. With this comparison, CI-988 with CCK-8us/chlordiazepoxide maintains a significant difference compared to an unusually high open arm time measure in the chlordiazepoxide group (mean 61.37). However, at least partial attenuation of the CCK-8us effect by CI-988 is observed where, compared to the CCK-8us/chlordiazepoxide group, showing a significantly increased open arm time ($F_{8,83}$=23.36, $p<0.05$). Measures for number of entries follows a similar pattern to that of time spent within each sector thus can be assumed to adhere to the same mechanism.

The effects of cholecystokinin forms on chlordiazepoxide increased open arm time were attenuated by LY-288513 (1.0mg.kg^{-1}), with the exception of the CCK-8s combination narrowly missing significance. This partial inhibition of CCK-8s effect is interesting given that the other CCK_2 antagonist tested, CI-988, only partially inhibited CCK-8us. An explanation for this effect remains enigmatic. Measures for number of arm entries did not show such a clear distribution.

Interestingly, a chance prior observation was made that a dose of LY-288513 at 10mg.kg^{-1} in combination with chlordiazepoxide, resulted in marked inhibition in number of entries on the plus-maze. Furthermore, locomotor activity was visibly observed to be impaired within the apparatus, in addition to a clear lack of muscle tone in LY-288513/chlordiazepoxide treated rats. A dose of 10mg.kg^{-1} of LY-288513 with chlordiazepoxide was initially selected for antagonism but was shelved after these preliminary findings. These observations strongly

imply that chlordiazepoxide and LY-288513 at this higher dose produce sedation in combination, much the same as a high dose of chlordiazepoxide alone. Whether, the sedative activity of chlordiazepoxide is augmented by LY-288513 or whether LY-288513 produces discrete sedative properties alone requires further investigation. Doses of $20mg.kg^{-1}$ LY-288513 suffered from some solubility difficulties, thus preventing study.

An experiment to determine whether CI-988 was able to inhibit activity of FG-7142 produced data which was indistinguishable between groups. The aim of this study was to determine whether the anxiogenic activity of FG-7142 was upstream of the cholecystokinin anxiogenic system. This was therefore observed to not be the case. This study indicated that FG-7142 induced anxiogenesis is either independent or downstream from cholecystokinin-induced anxiogenesis. It is more likely that the benzodiazepine component is downstream of the cholecystokinin component. This would account for attenuation of cholecystokinin anxiogenic activity by chlordiazepoxide (in that order) rather than cholecystokinin attenuating chlordiazepoxide activity. If cholecystokinin antagonists were to attenuate activity of FG-7142 this would strongly imply that cholecystokinin components lie upstream of benzodiazepine components, which appears to not be the case.

Further study of locomotor activity within the open field for these rats, subsequent to plus-maze testing, showed that in both groups, activity is significantly inhibited. There appears to be a trend however that the CI-988/FG7142 inhibits locomotor activity to a greater extent than FG-7142 alone. CI-988 alone at this dose is observed to significantly inhibit total entry number in the plus-maze.

Social Isolation

While Vasar *et al.*, (1993a) and Janowska *et al.*, (1991) observed that social isolation of male rats significantly reduced their exploratory activity in the elevated plus-maze, compared with those group housed, this was not observed in this study. Chlordiazepoxide however was

observed to exhibit activity in group-housed but not in socially isolated animals. This implies that floor effects may have been observed for nondrugged animals, where the level of anxiety cannot be measured at any greater degree within the model. Floor effects can be considered whereby a model provides no extension for a possibility of further measurement, that the measurement within the model has reached its' maximal or minimal point, despite not reaching a figure close to zero. In this model at a maximal level of anxiety, it is not assumed that this is equivalent to an approximation of zero in measurement. LY-288513 was also observed to achieve this effect, whereby, in group-housed animals, increasing open arm time, and lacking activity in the isolated group. This would indicate that social isolation can affect cholecystokinin induced anxiety. The succeeding series of experiments showed that floor effects within the model appear to explain previous findings such as in the abovementioned study by Vasar *et al*., (1993a), where caerulein at a high dose significantly decreased the exploratory behaviour of rats housed in groups, but not in the isolated rats. A lack of effect was determined through analysis with regard to significant differences between caerulein in isolated rats and vehicle in isolated rats. This study was undertaken in order to test a hypothesis that floor effects, produced by isolation-induced decreases in indices within the vehicle group, were responsible for an observed lack of activity. Interestingly, where social isolation of the rats increased the number of [^{3}H]pCCK-8 binding sites in the frontal cortex (Vasar *et al*., 1993a) it may merely be attributed to a simple increase in anxiety level and not to some more fundamental change in efficacy of cholecystokinin with regard to behavioural models. It also cannot be assumed that an increase in receptor binding will prevent any further activation, apart from merely shifting the starting point upwards. No changes were observed in the density of benzodiazepine receptors. This implies that an increase in anxiety-like behaviour might be independent of the benzodiazepine component. This is not given credence however by findings in this study where, in the vehicle group, there are no observable differences between group-housed and socially isolated animals. In agreement with this finding is another by Frussa-Filho *et al*., (1991) which shows that housing isolation produces no effect.

Significant changes between group housed and isolated animals treated with chlordiazepoxide and the cholecystokinin antagonist are observed however, which implies that both benzodiazepine and cholecystokinin systems provide active components in this behavioural observation.

It may be quite possible that a differing endogenous level of anxiety underlies differences in findings within a vehicle group between studies. Where animals exhibit a higher level of endogenous anxiety then floor effects may be observed, whereas lowered endogenous anxiety levels and those pharmacologically manipulated (by benzodiazepines, for example) are able to demonstrate significant differences.

Housing effects upon cholecystokinin activity were examined using maximally effective doses of CCK-4, CCK-8us and CCK-8s, as described in earlier studies (see figures 3.2 to 3.4). All remaining treatment groups showed significant decreases in time spent in the open arms compared with chlordiazepoxide and specific housing regimes. This finding provides further evidence that floor effects were responsible for lack of effects observed by caerulein in the aforementioned study. Despite not testing these cholecystokinin forms in the absence of chlordiazepoxide, which would provide almost unfailing evidence, were there to be no effects observed in isolated animals, this hypothesis of floor effects, would be strongly supported by the available data.

Thus an explanation of the range of differing results using cholecystokinin specific ligands, may either be explained by hypotheses described within, or by experimental, strain, or housing differences. Given that these hypotheses highlight a far greater subtlety regarding the underlying mechanisms, it is also likely that subtle differences in the aforementioned factors are also able to alter this cholecystokinin receptor balance. Therefore, a range of studies with often quite contradictory findings regarding the same ligand doses and route of administration, but differing in their experimental procedures are apparent.

CHAPTER FOUR - ANIMAL MODELS OF CONDITIONED FEAR

Introduction

Clinically defined phobias are thought to correspond to animal models of the conditioned fear paradigm. Classically pairing an electric shock with a light cue and eliciting some form of anxious response is a commonly utilised model of such. Relating Pavlovian conditioning to the underlying nature of human phobic conditions however may seem rather contrived. Commonly reported clinically defined phobias are concentrated within a restricted number of fear-inducing stimuli (Davey 1995). These stimuli, would ordinarily represent a minor threat, yet in phobic conditions produce disproportionately high fear associations. Expectancy bias is an estimate of the degree of perceived threat that any stimulus might represent. The level at which expectancy bias is influenced by either ontogenetic experiences (conditioned fear) or innate alarm signals (unconditioned fear) underlies two major groups of animal model. The shock-light classical Pavlovian conditioning paradigm, under these criteria may seem quite artificial in that an animal will not display an innate fear of electric shock. It may be therefore proposed that as Pavlovian fear conditioning is solely characterised by ontogenetic experience, it is not representative of a majority of characterised and clinically defined phobia. In the previous chapter, the pharmacological nature of the elevated plus-maze was examined. Anxiety-like behaviour exhibited upon the maze is believed to be generated by an innate fear response elicited by one or more variables within the apparatus itself. However, on subsequent exposures to the plus-maze, the pharmacological profile of benzodiazepine anxiolytics, which exhibit well defined efficacy on an initial exposure, is inactivated. It may be proposed that while the first exposure elicits an innate neophobic-like response, subsequent exposure evokes a conditioned fear-like response. This might be confirmed in face validity arguments as having consistency with that of human phobic development. Within the process of this conditioning procedure, there is an exposure phase leading to an active avoidance phase. Development of phobia in humans additionally is believed to exhibit these two phases. This chapter focuses on

characterisation and validation of the two trial elevated plus-maze as a putative model of phobia and interactions with the cholecystokinin system. In addition, the question of whether cholecystokinin possesses activity in a classical fear potentiated startle paradigm is examined.

The Two Trial Elevated Plus-maze: A Putative Model of Phobia

A discovery by Lister (1987) that a single prior exposure to the plus-maze renders a mouse relatively insensitive to the anxiolytic effects of chlordiazepoxide on further trials, has provided scope for a great wealth of research into the area. File (1990) confirmed this phenomenon in rats, claiming a similar insensitivity to chlordiazepoxide on trial two. File (1990) noted that this phenomenon of "one-trial tolerance" persisted even when the two trials were separated by as much as fourteen days, and according to File *et al*., although disputed by other authors, the scores of control animals remain stable over repeated tests. In addition, they found that chlordiazepoxide was able to block the development of this phenomenon with a very high dose given prior to the initial trial. This however, is almost certainly due to either amnestic activity or state induced attentional deficits. This study also showed that interestingly, the conditioning phase on trial one was not specific to any individual plus-maze apparatus. This phenomenon was observed even when the mazes utilised on both trial one and two, were of different materials.

File (1993) suggested that the phenomenon of chlordiazepoxide inactivity on trial two is due to a mechanism by which during the first 5 minutes in the elevated plus-maze, rats are conditioned to fear of heights, associated with the plus-maze aversive stimulus, and it is, a phobic anxiety state that is measured during the second 5-minute trial. It would thus follow that chlordiazepoxide, which like other benzodiazepines is clinically inactive in treatment of phobic conditions, will be inactive in this animal model of phobia.

Broadly in confirmation of this observation, Treit *et al*., (1993a) demonstrated that although diazepam-treated rats avoided the open arms less than vehicle-controls on the first test, this

difference faded away across subsequent trials.

File *et al*., (1993) observed that attenuation of diazepam activity on trial two occurs only when the trials are short (5 minutes); with longer exposure (10 minutes) diazepam retains anxiolytic efficacy. File *et al*., concluded that during a brief (5 minute) trial in the plus-maze, rats acquire a specific phobic anxiety (conditioned fear), which is relatively resistant to benzodiazepines. With a longer exposure to the plus-maze this form of fear extinguishes. Interestingly though, the phenomenon is not seen in unhandled rats, in rats given longer trials in the plus-maze, or with those administered an amnestic treatment on trial one (Andrews and File 1993).

Holmes and Rodgers (1999) demonstrated that, while extending the duration of initial exposure to 10 minutes did not prevent the loss of chlordiazepoxide ($10mg.kg^{-1}$) efficacy in a standard-duration second trial, increasing the duration of both trials to ten minutes reinstated an anxiolytic profile for the compound.

File and Zangrossi (1993) examined this two trial phenomenon in other aversive models of anxiety, observing that diazepam ($5mg.kg^{-1}$) increased the number of shocks accepted by rats on two successive trials in the punished drinking test. Hence, this phenomenon of tolerance to benzodiazepines within the second trial is possibly restricted to the plus-maze and like protocols. Furthermore, File and Zangrossi (1993) observed that chlordiazepoxide also retained its ability to counteract anxiogenic effects in the plus-maze induced by prior exposure to cat odour, over successive trials. This implies that the specific anxiety component to which benzodiazepine tolerance is developed within a two trial paradigm, is exclusively due to fear conditioning factors within the plus-maze itself. Furthermore, the anxious response observed within the plus-maze with exposure to cat odour is multifaceted, whereby two or more components underlie the total anxious response on the successive trials. File and Zangrossi, in addition, observed that chlordiazepoxide (5 and 10 $mg.kg^{-1}$) was also ineffective against the behavioural responses of rats during exposure to cat odour, another possible animal test of

phobia.

These observations again imply that several factors are underlying the observed anxiety measures within this paradigm. Hence, the anxiety component, which underlies development of chlordiazepoxide tolerance, is fundamental to behaviour during exposure to cat odour, and during trial two of the plus-maze. These may be described as an innate phobic and a conditioned phobic response respectively. Another type of anxiety-like behaviour is that observed in the plus-maze subsequent to cat odour exposure, which is resistant to chlordiazepoxide tolerance, and possibly the same as that observed in the initial plus-maze exposure of naïve rats. This might be described as a generalised neophobic anxiety. However, it was noted by Hogg and File (1994), that laboratory-bred rats can be divided into those showing clear innate behavioural responses to the odour of a predator (a cat) and those showing no response, these two groups differed neither in their responses to a neutral odour, nor in the elevated plus-maze tests of anxiety. Furthermore, within trial two in the plus-maze, two clear groups of responders and non-responders could be identified (but not on trial one or in the social interaction test). Interestingly however, the distribution of cat odour responders did not correspond to the distribution within plus-maze trial two. This suggests that it is possible to identify bimodal populations of rats in tests of both innate and acquired simple phobias, and that these populations are quite distinct.

Dawson *et al*., (1994) re-examined the phenomenon of "one-trial tolerance" in the elevated plus-maze and observed that unlike previous experiments, nondrugged pre-exposure to the maze resulted in habituation with a consequential reduction in time spent on the open arms. Habituation was measured by recording the actual distance travelled by the rats in the maze, which was found to be significantly reduced by pre-exposure. Dawson *et al*., observed that chlordiazepoxide administration prior to trial one resulted in an inhibition of "anxiolytic-like" effects of chlordiazepoxide, on trial two. Dawson *et al*., found contrary to previous findings that, although the time spent on the open arms was reduced by pre-exposure, chlordiazepoxide

significantly increased the time spent on the open arms by rats pre-exposed under a non-drugged state. The authors inferred that rats do not become tolerant to the effects of chlordiazepoxide, but rather the reduced response to chlordiazepoxide after pre-exposure is due to habituation of exploratory behaviour. This is perhaps a valid point, given that chlordiazepoxide is plainly ineffective in promoting motivation for exploration in addition to its inactivity in phobia. This question forms part of the following study and is elaborated further here.

Rodgers *et al*., (1996) confirmed in mice, some of the findings of Dawson *et al*., (1994) where re-exposure to the maze, produces an increased avoidance of the open arms and a corresponding preference for the enclosed sections of the apparatus. Rodgers *et al*., (1996) conducted further ethological analyses of behaviour during trial two and confirmed marked differences to that seen on initial exposure. These changes furthermore are independent of the duration of trial one (2 vs. 5 minutes). Trial two specific behavioural changes included a decrease in entry latencies, open arm entries, time on the open arms and central platform, lower levels of exploratory head dipping, and increased entries into and time spent in the closed arms. Subsequent time lapse analyses of the behaviour of naïve animals within the initial trial showed that behaviour during the first minute is characterized by high levels of risk assessment based at the central platform and relatively low, but equal, levels of open- and closed-arm exploration. Subsequent exploration showed increasing open arm/central platform avoidance, increasing closed-arm preference, and decreasing levels of central platform risk assessment and exploratory head-dipping. These analyses appear to reveal a fear conditioning process within the initial exposure.

Within my own studies I can confirm some of these observations (data not shown) whereby most open arm exploration occurs within the first period of the trial, and animals spend more time in the closed arm in the later period. In addition, Rodgers *et al*., (1996) demonstrated that post-trial administration of the muscarinic antagonist and putative amnestic agent, scopolamine

(0.1-1.0 $mg.kg^{-1}$), failed to significantly alter the behavioural changes seen between trials.

Holmes and Rodgers (1998) observed upon exposure of mice to the plus-maze for 5 minutes for three consecutive days, that a single prior nondrugged exposure to the maze increases behavioural indices of anxiety and that these changes are either maintained or further enhanced on subsequent trials. Espejo (1997) has confirmed this observation. Furthermore, the behavioural profile evident by trial three was largely unchanged when animals were re-exposed to the maze 10 days later. Detailed time based analyses of behavioural patterns within trials demonstrated that open arm avoidance is acquired by the third minute of trial one, and that the behavioural profile evident by the end of trial one is markedly different to that seen at the beginning of that trial. This late trial behaviour is generally maintained or even accentuated on trials 2 and 3 (Holmes and Rodgers 1998).

The Aversive Nature of the Plus-maze

File *et al*., (1992) observed that handling of rats produced insensitivity to chlordiazepoxide in the plus-maze. This phenomenon was not seen in unhandled rats. Chlordiazepoxide-induced decreases in cortical GABA release were abolished by prior plus-maze experience. Hippocampal GABA release in response to chlordiazepoxide was decreased in unhandled and increased in handled rats. This would thus imply that the phobic conditioning of the first plus-maze trial is driven by both animal handling and plus-maze exposure.

Treit *et al*., (1993a) attempted to examine the precise nature of the anxiogenic stimuli associated with phobic conditioning within the plus-maze. Although it has often been assumed that rats avoid the open arms because of their novelty, height, or open field, the anxiogenic role of these stimuli in the plus-maze had not previously been methodically examined. The experimenters observed that firstly, with rats repeatedly exposed to the elevated plus-maze, open-arm avoidance increased on the second trial and showed no evidence of habituation after eighteen trials. Furthermore, three 30-minute sessions of confinement to the open arms

("flooding") failed to decrease rats' open-arm avoidance. Instead, rats that had received flooding avoided the open arms significantly more than control rats during the first test. However, in contrast to this observation, Bertoglio and Carobrez (2000) noted that rats confined to either open or enclosed arms failed to show an increased avoidance of open arms in a subsequent trial. Holmes and Rodgers (1999) observed that trial one confinement to an open arm did not compromise chlordiazepoxide efficacy when mice were subsequently allowed to freely explore the maze, whereas closed arm confinement during initial exposure abolished the drug's anxiolytic action upon retest. The authors suggest that the experientially induced loss of benzodiazepine efficacy in the mouse plus-maze depends rather critically upon prior discovery and exploration of relatively safe areas of the maze (i.e. closed arms).

Treit *et al*., (1993a) further demonstrated that variation of plus-maze height from 50 to 6 cm, did not change open-arm activity. Height cues were manipulated further by placing a "floor" 8 cm beneath one open arm while leaving the floor of the other open arm at 50 cm. Rats did not avoid the "low" open arm less than the "high" open arm. It was thus concluded that height of the plus-maze was not the anxiogenic factor in the efficacy of this model.

In a further attempt to elucidate the aversive nature of the plus-maze, Fernandes and File (1996) tested rats in a plus-maze, with or without small ledges on the open arms. Chlordiazepoxide had significant anxiolytic-like effects on trial 1 only in the maze without ledges, and on trial 2 only in the maze with ledges; thus, the presence of ledges differentially affected anxiolytic sensitivity on trials 1 and 2. The authors suggest "both types of plus-maze may be measuring the same type of anxiety with different sensitivities on trial one (e.g., generalised anxiety or fear of open spaces), different types of anxiety on trial 2 (without ledges--phobia/fear of heights; with ledges--not known)".

Neuropharmacology of the Two Trial Phenomena

In an attempt to validate this model further, File *et al*., (1994) demonstrated that rats given one or two 5-minute trials in the elevated plus-maze had plasma corticosterone concentrations significantly higher than the home cage control group. Furthermore they observed that in rats given two plus-maze trials, corticosterone responses were significantly higher in the group given 10-minute rather than 5-minute trials, and that chlordiazepoxide habituation was not observed in the ten minute trial group. Moreover, previous experience of cat odour (1 week earlier) had no effect on the plasma corticosterone level, but did have an anxiogenic-like effect that could be detected by a decrease in the percentage of time spent on the open arms of the plus-maze.

Chacur *et al*., (1999) observed that a receptor autoradiography of rat brains after a single drug-free experience in the elevated plus-maze (with sacrifice post exposure) revealed that radiolabelled flunitrazepam binding was significantly elevated in several amygdaloid and hippocampal nuclei. Tritiated muscimol binding in adjacent sections was not significantly altered. The authors suggest that benzodiazepine receptors are able to alter binding affinity / receptor density very rapidly in response to anxiogenic conditions. Moreover, the findings that flunitrazepam binding is increased by maze exposure, is ostensibly the opposite to that which might be expected, and additionally, the rapidity with which a change in binding is detected is curious. It may be possible that affinity of individual benzodiazepine receptors, and not down-regulation of receptors, is altered in response to the aversive stimulus. This would both increase the efficacy of the endogenous benzodiazepine species and increase observable binding affinity. An example of where this might also occur is with the various affinity states of the cholecystokinin$_2$ receptor subtype (Huang *et al*., 1994)

File and Gonzalez (1996) observed that direct administration of the 5-HT$_{1A}$ receptor agonist 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) into the Medial Raphe Nuclei had significant

anxiolytic effects in all three test situations examined (social interaction, plus-maze trials 1 and 2). These anxiolytic effects were antagonised by the 5-HT_{1A} receptor antagonist WAY-100635.

Functional Neuroanatomy of the Two Trial Phenomena

File *et al*., (1998) observed that rats that had received reversible bilateral lesions of the basolateral amygdala (by lidocaine injection) immediately after trial one responded with an anxiolytic response to chlordiazepoxide when tested forty-eight hours later on trial two. Those that received vehicle injections after trial 1, showed the usual lack of response to chlordiazepoxide on trial 2. The authors consequently propose that "the basolateral amygdala plays a crucial role in the consolidation of information that leads to the formation of a proposed phobia and subsequent insensitivity to benzodiazepines".

In addition, rats receiving lidocaine infusions, into the dorsomedial hypothalamus immediately before trial 2, responded with an anxiolytic response to chlordiazepoxide. Those injected with control infusions into the dorsomedial hypothalamus showed the usual lack of response to chlordiazepoxide ($5mg.kg^{-1}$, intraperitoneal). Since the lidocaine injections were without anxiolytic effects, the authors suggest that this region of the hypothalamus regulates the functional state of benzodiazepine receptors in other brain regions (File *et al*., 1999).

Ouagazzal *et al*., (1999), examined the effects of a wide dose range of systemically administered (-)-nicotine on trials 1 and 2 in the plus-maze. Higher doses of nicotine had anxiogenic-like effects on both trials. Infusion of nicotine bilaterally into the dorsal hippocampus was without effect on trial one, but a dose of 1 microgram had an anxiolytic effect on trial two. In addition, in trial 1, lateral septal administration of nicotine has anxiogenic-like effects, while in trial two it has no effect (File *et al*., 2000).

A Motivational Deficit Underlying Two Trial Tolerance

Pereira *et al*., (1999) examined whether this phenomenon of "one-trial tolerance" resulted from a motivational deficit on trial two. A hypothesis arises that whereas there is a motivational conflict on trial one in relation to the open arms (exploration drive versus natural fear of open spaces), accordingly, there is no "reason" for an animal to explore on trial two. A motivational conflict was introduced in this study on trial two by rendering the enclosed arms of the apparatus aversive on trial one. Upon entry into the closed arms, an aversive stimulus (hot air puff) was produced until the animal left the arm. On trial two, rats did not receive this aversive stimulation (extinction procedure). Chlordiazepoxide significantly increased open arm exploration indices on trial two in rats that had been submitted to aversive conditioning, but was ineffective in rats which had been exposed to a standard maze conditions on trial one. In addition, there was no difference in the open exploration on trial two between saline-treated rat groups. Furthermore, the aversive conditioning in trial one did not modify the number of total arm entries on trial two. The authors suggest that the anxiolytic effect of chlordiazepoxide in the elevated plus-maze depends on the presence of a motivational conflict situation. An attempt to elucidate this as an underlying mechanism was made in a study within this chapter.

Shock Potentiated acoustic Startle

The fear potentiated startle paradigm was first described by Brown *et al*., (1951). This groundbreaking study demonstrated that the magnitude of the startle reflex in response to an acoustic stimulus was potentiated when presented with a shock-paired stimulus. The fear conditioning trials provide a light cue serving as a conditioned stimulus thus signalling an incoming presentation of a footshock (the unconditioned stimulus). This model is a classic example of Pavlovian fear conditioning.

Face Validity

From this and other studies, it is clear that the startle reflex is potentiated during experimentally induced anxiety (i.e.: fear-potentiated startle). In satisfying the face validity criteria for this model, it is also increased in various phobic anxiety disorders. Clinical studies have observed that stimuli previously associated with phobia are able to potentiate startle responses. In a study by Grillon *et al*., (1993) the eye-blink component of the acoustic startle reflex was measured in a paradigm involving the anticipation of electric shocks in normal human subjects. The individual anxiety level related to anticipation of the shock was assessed using the State-Trait Anxiety Inventory. The magnitude of fear-potentiated startle was increased in the high-anxiety group when compared to the low-anxiety group. The time-course of startle modulation suggested a longer duration of anticipatory anxiety in the high-fear group. Trait anxiety, which was assessed with the trait portion of the STAI, did not relate to individual differences in either baseline or fear-potentiated startle (Grillon *et al*., 1993). Difficulties may arise in comparison of this model with phobia in the clinical condition specifically with regard to the nature of the aversive stimulus.

Predictive Validity

The history of study of the pharmacological nature of fear-potentiated startle has been fraught with questionable methodology and validity. In one of the earlier studies of the pharmacology of fear potentiated acoustic startle, Davis (1979) found that diazepam produced a dose-dependent decrease of the potentiated startle effect (with a shock paired light stimulus). The effect was thought to be selective since the same doses did not depress baseline startle amplitude measured in non-conditioned apparatus naïve animals. In order to rule out whether diazepam exhibited state-dependent effects of learning during the acquisition trials, rats were trained under diazepam and were found to exhibit the same dose-dependent decrease in startle amplitude in the retention trial as those trained with saline. Therefore, Davis proposed that "the

primary effect of diazepam was to block expression of, rather than acquisition of fear, as measured by potentiated startle". State dependent effects on learning may arise from possible attentional deficits produced by any pharmacological intervention. These data however, fail to show whether or not diazepam is acting upon amnestic mechanisms and thus causing inhibition of memory eduction or whether exhibiting an anti-anxiety effect. The control study of state dependence failed to rule this out, whereby any alteration of the acquisition phase of the conditioning trials would be determined but possible effects on memory recall would be masked. Hence, studies utilising pharmacological manipulation of this model at the retention trial will undoubtedly remain somewhat equivocal in their conclusions.

Using this perhaps flawed methodology it has been shown that a range of anxiolytic agents such as diazepam, midazolam, and flurazepam are able to produce dose dependant decreases in the level of fear potentiated startle (Davis, 1979; Hijzen and Slangen, 1989). Benzodiazepines have also been shown to exhibit an "anxiolytic" effect in inhibition of the potentiated startle amplitude without affecting the basal startle level of the acquisition session (Davis, 1993). In a study by Berg and Davis (1984) it was demonstrated that this inhibitory effect of diazepam on potentiated startle was attenuated by flumazenil, a benzodiazepine receptor antagonist, when startle was electrically initiated at the brainstem. Another study by Hijzen and Slangen (1989) using a benzodiazepine receptor inverse agonist showed an enhancement of the potentiated startle response.

Following cocaine withdrawal, humans commonly experience an abstinence syndrome accompanied with high levels of anxiety. Chronic cocaine administration in rats produces lasting increases in conditioned fear, measured by fear-potentiated startle. Repeated cocaine pre-exposure can effect acoustic startle differently depending on whether fear conditioning occurred and whether cocaine was administered in the testing chamber (Gordon and Rosen 1999).

Construct Validity

A series of studies have indicated that the amygdaloid complex is necessary for acquisition and expression of conditioned fear and that an efferent amygdalofugal pathway to the primary startle circuit, at the level of the caudal pontine reticular formation, mediates the expression of conditioned fear (Fendt *et al*., 1996a). Pre- and post-training lesions of the midbrain central grey (CG) totally blocked the potentiation of the acoustic startle amplitude. This was not observed in sham-lesioned rats, indicating that CG lesions affect the expression of conditioned fear. Baseline startle amplitude was not influenced by CG lesions (Fendt *et al*., 1996b).

Plus-Maze Exposure and Potentiation of Acoustic Startle

It has been observed that significant negative correlations were observed among inbred mouse strains between the percent time spent in the open arms of the elevated maze and amplitude of an acoustic startle response (Trullas and Skolnick 1993). Adamec *et al*., (1999a & 1999b) have shown that increases in anxiety-like behaviour in rats on the elevated plus-maze and in the acoustic startle paradigm, are produced by a single 5-min exposure to a cat and last for 3 weeks afterwards. However, despite the aversive environment of the elevated plus-maze producing fear and apprehensive behaviour in rodents, the effects of plus-maze exposure on anxiety levels measured in other models of anxiety has been seldom, if at all, studied. My own study has shown that plus-maze exposure has indeed an effect on acoustic startle.

Cholecystokinin and Fear Potentiated Startle

To date only one study has explored the effects of cholecystokinin ligands upon fear potentiated startle. Josselyn *et al*., (1995) observed that pre-treatment with the CCK_2 antagonist L-365260 (0.1 -10.0 $mg.kg^{-1}$, I.P.) did not affect baseline acoustic startle amplitudes, but dose-dependently decreased fear-potentiated startle. The present findings are consistent with the effects of CCK_2 antagonists in other tests measuring anxiety in animals.

Materials and Methods

Animals

Male Sprague Dawley rats (Laboratory Animal Centre, National University of Singapore, Singapore), weighing 250-320g were housed in groups of five to seven and maintained with free access to food and water *ad libitum*, in an animal room for one week prior to experimentation. Room lights were on from 07:00 to 19.00 hours. The test room and housing block were maintained at a standard temperature throughout the experiments. Animals were randomly assigned treatment groups using a simple computer program random number sequence generator (Random Number Generator V.1.0 by Colin Greengrass, 1998). Usage of this number listing is described in chapter two methodology.

Test Room Conditions

Animals were tested in each apparatus within a particular test room with constants of temperature at 25^{O}C, lighting intensity and background white noise generation (at 70dB).

Drugs

A Cholecystokinin peptide antagonist, LY-288513 ([trans-N-(4-bromophenyl)-3-oxo-4, 5-diphenyl-1-pyrazolidinecarboxamide]) at 10.0 mg/ml (kindly donated by Eli Lilly and Company, USA), chlordiazepoxide hydrochloride at 3.0mg/ml (A generous gift from Roche) and/or scopolamine hydrobromide at 0.15 mg/ml (Sigma, USA) were dissolved in dimethylsulphoxide (DMSO; Merck). Cholecystokinin peptide agonists, CCK-4 (Trp-Met-Asp-Phe-NH_2); CCK-8us (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2; Biotechnology Centre, National University of Singapore) CCK-8s (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; American Peptide Company and Princeton Biomolecules) were dissolved in DMSO. The drugs or vehicle were administered by intraperitoneal injection in a volume of 1ml per kg body weight. Drugs were administered 30 minutes prior to testing. Cholecystokinin peptide was

stored under desiccation at -70^{o}C. Cholecystokinin peptide solutions were prepared freshly each day approximately two hours prior to testing. Powdered peptide was dissolved in DMSO and specific concentrations prepared through serial dilution with thorough vortex mixing at each stage.

Apparatus

Elevated Plus-maze:

With the exception of the plus-maze multiple exposure experiments, the protocol is identical to that described in chapter three, for one trial testing.

Acoustic Startle Apparatus:

A computer controlled auditory startle chamber was used (SR-LAB, San Diego Instruments Inc.). Further details of this apparatus are described in chapter two; methods and materials. Motion data was recorded via an interface assembly to an IBM computer with relevant software (San Diego Instruments, PSR2 Software program). Light levels within the chamber remained constant throughout the acquisition and retention procedures. The chamber was thoroughly cleaned with 70% ethanol between sessions and allowed to dry. The interval between the acoustic stimulus and peak amplitude of each startle reflex movement, and the peak amplitude itself were recorded. Behavioural experiments were performed between 14:00-18:00hours.

Shuttle Box Shock Chamber:

A single compartment of a computer-controlled two-compartment shuttlebox (San Diego Instruments, Inc.) was used as a shock chamber. The Gemini II shuttle box is a two compartment acrylic chamber of dimensions: 53cm (width) x 53cm (height) x 32cm (depth) with a steel grid floor and a steel, computer controlled, sliding door between compartments.

Animals were placed in a lighted compartment (40-W bulb) with the main door closed. The scrambled sine-wave electric shock was delivered through the steel grid floor. The shock intensity is adjusted manually and calculated by the following formula: $y = mx + b$; where y = shock intensity (mV); m = slope (0.004); x = animal weight (g); and b = y-axis intercept (- 0.4)

Experimental Procedure

Experiment 4.1: Two Trial Elevated Plus-maze

Animals were administered, 30 minutes prior to maze exposure, a single intraperitoneal injection either of; DMSO, chlordiazepoxide (3.0mg.kg^{-1}) or LY-288513 (10.0mg.kg^{-1}) and underwent near identical handling procedures. Animals were then exposed to the elevated plus-maze for either a five-minute or a ten-minute session. A subgroup of rats administered with DMSO were then injected with 0.3mg.kg^{-1} Scopolamine Hydrochloride intraperitoneally immediately after a five minute exposure to the plus-maze. Treatment groups defined are represented in table 4.1

Table 4.1 Protocol Group Classification Two Trial Elevated Plus-maze (Experiment 1)

Group Number	Drug Administration (dose in mg.kg^{-1})			
	Pre-Trial 1 (5 minute)	Pre-Trial 1 (10 minute)	Post-Trial 1 (5 minute)	Pre-Trial 2 (5 minute)
1	DMSO			
2	chlordiazepoxide (3.0)			
3	LY-288513 (3.0)			
4	DMSO			DMSO
5	DMSO			chlordiazepoxide (3.0)
6	DMSO			LY-288513 (10.0)
7		DMSO		DMSO
8		DMSO		chlordiazepoxide (3.0)
9		DMSO		LY-288513 (10.0)
10	DMSO		Scopolamine (0.3)	DMSO
11	DMSO		Scopolamine (0.3)	chlordiazepoxide (3.0)
12	DMSO		Scopolamine (0.3)	LY-288513 (10.0)

Twenty-four hours later the animals were again exposed to the plus-maze. 30 minutes prior to this session animals were administered either DMSO, chlordiazepoxide at 3.0mg.kg^{-1} or LY-288513 at 10.0mg.kg^{-1}. This further group division is represented in table 4.1

Experiment 4.2: Prevention of Development of Chlordiazepoxide Insensitivity within the Two Trial Elevated Plus-Maze by LY-288513

Animals were exposed to the elevated plus-maze for a five-minute test session. 30 minutes prior to this session the animals were administered a single intraperitoneal injection of DMSO and underwent near identical handling procedures. The rats were subdivided and randomly assigned into two groups, one administered DMSO post-exposure and the other administered 10.0mg.kg^{-1} LY-288513 immediately post- exposure. Twenty-four hours later the animals were again exposed to the plus-maze after injection of chlordiazepoxide at 3.0mg.kg^{-1} (30 minutes before the session).

Experiment 4.3: Elevated Plus-maze Reward Manipulation

Experimental manipulations of the elevated plus-maze were carried out in hope of introducing a sweetened foodstuff as an incentive onto the open arms.

4.3.1: Rats after one week fed *ad libitum* with standard maize pellets, were supplied either a fixed number of sugar lumps or a sweetened maize cereal coated in a honey, sugar and cinnamon paste. The time of consumption was measured in order to determine both preference for each type and ease of consumption (given the relatively short time of the plus-maze session). The maize cereal was consumed within a home cage of five rats more rapidly than sugar lumps. Therefore, the cereal was chosen as a food incentive for use upon the elevated plus-maze. Both food types were consumed eagerly within home cages.

4.3.2: Rats after one week plus feeding *ad libitum* standard maize pellets, were tested on the elevated plus-maze with six cereal pellets placed on each open arm. The placement of these pellets was such that one was placed at a fastening screw position extending about 15cm from the central platform, and the remaining five were placed in a floret pattern surrounding another fastening screw 15cm from the end. It was hoped that the first pellet, within distance of any stretch posture from the central platform would provide an initial incentive, whilst the outlying pellets would bring the animal onto the open arm entirely. The animals failed to consume any pellets, despite entry onto the open arm. The rats would sniff and then ignore any pellets.

4.3.3: Rats, after one-week food deprivation, were tested on the elevated plus-maze with six cereal pellets placed on each open arm. Food deprivation encompassed a schedule of 80% normal chow consumption, calculated by measuring weight of food consumed per each cage housing five rats in 24 hours. These cages were then provided with 80% of this weight in chow each day for one week. Water was provided *ad libitum*. Sixteen rats were tested on the plus-maze yet only three pellets were consumed by three individual rats (one pellet each). Moreover, these were the pellets lying proximal to the central platform, and were carried by each rat to the central platform before consumption. These animals were tested again, 24 hours later on the elevated plus-maze, as described in experiment one, with either DMSO or chlordiazepoxide ($3.0mg.kg^{-1}$) administered intraperitoneally 30 minutes pre-trial.

4.3.4: Rats underwent the same protocol as in experiment 4.3.3 (above) except that the experimenter handled them for one minute immediately prior to feeding time for one week before the plus-maze exposure. These animals failed to consume any pellets within the plus-maze.

4.3.5: Rats underwent the same protocol as in experimental 4.3.3 (above) except that they were allowed a 30-minute exposure to the plus-maze. A similar pellet consumption was observed to that of a five-minute session.

Experiment 4.4: Elevated Plus-maze Induced Fear Potentiated Startle

Several different protocols were carried out in order to ascertain whether elevated plus-maze exposure exerts a "fear potentiated startle" like activity. Animals naïve to the plus-maze or those immediately following either a first or second exposure, were placed in the acoustic startle chamber. Animals underwent acclimatisation for three minutes in the presence of constant white noise (70dB). Following this period each animal was immediately exposed to twenty-five 100-millisecond bursts of white noise at 120dB punctuated with an eight second period of 70dB white noise between each burst. The peak amplitude of the startle response measure was taken. This procedure measures auditory startle habituation over the twenty-five noise burst stimuli that are plotted on a chart versus the sequential 120dB noise burst. Program listings are recorded in appendix C-2.

Table 4.2 Protocol Group Classification Two Trial Elevated Plus-maze exposure effects on acoustic startle (Experiment 4)

	Acoustic Startle Trial 35 minutes After Drug Administration (dose in $mg.kg^{-1}$)			
Group Number	Plus-maze Naive	Post-Trial 1 (5 minute)	Pre-Trial 2	Post-Trial 2 (5 minute)
1	Saline			
2		Saline		
3	DMSO			
4		DMSO		
5			DMSO	
6				DMSO
7	chlordiazepoxide (3.0)			
8		chlordiazepoxide (3.0		
9			chlordiazepoxide (3.0)	
10				chlordiazepoxide (3.0)
11		LY-288513 (10.0)		
12			LY-288513 (10.0)	
13				LY-288513 (10.0)

Rats were randomly assigned each drug treatment group from within each group housed cage. Number randomisation was achieved via use of the aforementioned computer program. All animals were administered drugs or vehicle 35 minutes prior to acoustic startle sessions. The

thirty-five minute period was chosen in order to emulate a profile whereby animals are tested post plus-maze. Thirteen different protocols were examined concurrently and are summarised in table 4.2.

Experiment 4.5: Shock induced Fear Potentiated Startle

4.5.1: Apparatus naïve animals were placed in the acoustic startle chamber and underwent identical procedures to that described for experiment 4.4. Immediately after the acoustic startle trial the animal was placed in a single shuttle box chamber (as described in chapter 5). The animal was placed in a lit compartment and left for a ten second adaptation period. Subsequently a scrambled sine wave shock was delivered to the cage floor continuously for a period of thirty seconds. The shock intensity to be delivered is calculated by the following formula; $y = mx + b$; where y = shock intensity (milliamps); m = slope (0.004); x = animal weight (g); and b = y-axis intercept (- 0.4). This calculation allows a pseudo dosing effect of shock intensity with regard to animal weight. Immediately after this shock period the animal was removed and injected intraperitoneally under a series of protocols detailed below. In order to maintain experimental controls, the animals post-shock were placed in a holding cage in order to isolate them from the other animals prior to experimentation. It is observed that shocked animals exhibit high levels of aggression immediately afterwards. Only when all the animals within a home cage had been tested were they placed back into their home cages and immediately returned to the animal housing block. Seventy-two hours later, animals underwent an identical acoustic startle procedure. This test attempted to measure a conditioning response whereby the startle apparatus is associated with an impending shock. An index of retention startle amplitude divided by acquisition startle amplitude was calculated. A series of experiments carried out are detailed below.

4.5.2: An experiment was performed whereby an animal post acoustic startle testing on trial one was either submitted to electric shock as per the aforementioned protocol, or was placed in

the shuttle box chamber for the 40-second period without administration of electric shock. Subsequently, immediately following shuttle box exposure the animals were administered DMSO intraperitoneally and returned to holding cages. Seventy-two hours later the two groups were retested for acoustic startle. The acoustic startle index was calculated.

4.5.3: A repeat of experiment 4.5.1 was performed with the introduction of scopolamine hydrochloride (0.3mg.kg^{-1}) administration. Four groups were tested: DMSO administered post exposure without shock; DMSO administered post shock; Scopolamine administered post exposure without shock; Scopolamine administered post shock.

4.5.4: Effects of a dose range of CCK-4, 0.0125 to 0.1mg.kg^{-1}, on shock potentiated acoustic startle. Animals post acoustic startle testing on trial one were submitted to electric shock as per the aforementioned protocol. Immediately following shock exposure, the animals were administered either DMSO or CCK-4 (specified dose range) intraperitoneally and returned to holding cages under the aforementioned schedule. Seventy-two hours later the two groups were retested for acoustic startle. An acoustic startle index was calculated.

4.5.5: Effects of a dose range of CCK-8us, 0.001 to 0.1mg.kg^{-1}, on shock potentiated acoustic startle. An identical procedure to that in experiment 4.5.4 was performed.

4.5.6: Effects of a dose range of CCK-8s, 0.0005 to 0.01mg.kg^{-1}, on shock potentiated acoustic startle. An identical procedure to that in experiment 4.5.4 was performed.

Statistical Analysis

Statistical analysis was performed using statistical software (SPSS V9.0, SPSS inc. USA). The data were subjected to a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc analysis of group differences. Probability ($p < 0.05$) was considered statistically significant.

Results and Data

Experiment 4.1

Figure 4.1.1 – Percentage Time Spent

Time Spent on Open Arm:

Administration of Chlordiazepoxide, at 3.0 $mg.kg^{-1}$ and LY-288513, at 10.0 $mg.kg^{-1}$, produced a statistically significant increase in time spent on open arms of naïve rats compared to the DMSO vehicle (group 1); chlordiazepoxide (group 2); 3.0$mg.kg^{-1}$, ($F_{11,90}$=13.66, $p<0.01$); LY-288513 (group 3); 10.0$mg.kg^{-1}$, ($F_{11,90}$=13.66, $p<0.05$). However, both drugs when administered to rats with prior maze experience were unable to exact these effects in a subsequent plus-maze trial; groups 5 and 6 respectively. Administration of scopolamine, at 0.3$mg.kg^{-1}$, immediately following first plus-maze exposure, was able to restore the significant increases in percentage time spent in the open arm by chlordiazepoxide (group 11) in trial two, 3.0 $mg.kg^{-1}$ ($F_{11,90}$=13.66, $p<0.01$) but not for LY-288513 groups (group 12).

Group comparison between each drug treatment versus plus-maze exposure protocol, yielded the following differences with regard to percentage time spent on the open arms. With DMSO administered rats it was observed that prior plus-maze exposure exerted inhibition of open arm exploration, although failing significance. Significant differences within the chlordiazepoxide-administered protocols were observed. Chlordiazepoxide in trial one exerted a significant increase in open arm exploratory behaviour but failed to produce any changes in plus-maze trial two; group two versus groups five and eight ($F_{11,90}$=13.66, $p<0.001$). Administration of scopolamine immediately post-trial one, reinstated chlordiazepoxide sensitivity within trial two, (increased exploratory activity in the open arm).

An increase in open arm exploratory behaviour observed for LY-288513 in trial one was not

observed in trial two; significant difference between group three and groups six and nine ($F_{11,90}$=13.66, p<0.001). Administration of scopolamine immediately post trial one failed to reinstate an increased exploratory activity in the open arms produced by LY-288513 upon maze naïve rats; group three versus group twelve ($F_{11,90}$=13.66, p<0.001).

Time Spent on the Central Platform:

No significant differences were observed between either drug groups or between protocols.

Time Spent on the Closed Arms:

Significant increases in closed arm exploratory behaviour (percentage time spent) were observed between the groups (5 & 8) administered chlordiazepoxide (figure 4.1.1) on trial two compared to the group administered chlordiazepoxide on trial one (maze naïve rats; $F_{11,90}$=4.26, p<0.05). Closed arm exploratory behaviour was significantly increased in group 12 (administered LY-288513 on trial two with scopolamine administration) compared to group 3 (LY-288513 in maze naïve rats; $F_{11,90}$=4.26, p<0.05).

Scopolamine administration was able to reinstate chlordiazepoxide activity at trial two (group five versus eleven; $F_{11,90}$=13.66, p<0.05).

Figure 4.1.2 - Number of Arm Entries

Open Arm Entries:

Administration of Chlordiazepoxide, at 3.0 $mg.kg^{-1}$ and LY-288513, at 10.0 $mg.kg^{-1}$, produced a statistically significant increase in number of entries into the open arms compared to the DMSO control, with an initial exposure of naïve rats to the elevated plus-maze (Figure 4.1.2); chlordiazepoxide; 3.0$mg.kg^{-1}$, ($F_{11,90}$=9.34, p<0.01); LY-288513; 10.0$mg.kg^{-1}$, ($F_{11,90}$=9.34, p<0.01). However, both drugs when administered in a subsequent plus-maze trial after prior

maze exposure, (either five or ten minutes), were unable to exact these effects. Administration of scopolamine, at 0.3mg.kg^{-1}, immediately following first plus-maze exposure (5 minute trial), was not able to restore the significant increases in number of entries in the open arm by chlordiazepoxide, 3.0 mg.kg^{-1} ($F_{11,90}$=9.34, p<0.001) that was observed for percentage time spent. DMSO vehicle showed no differences between protocols except in a trend observed, whereby plus-maze exposure exerted inhibition of number of open arm entries. Significant differences were observed between chlordiazepoxide control (group 2), versus chlordiazepoxide on trial two (Groups 5 & 8; $F_{11,90}$=9.34, p<0.001). Administration of scopolamine immediately post trial one (group 11) failed to reinstate the increased open arm entries produced by chlordiazepoxide within trial one ($F_{11,90}$=9.34, p<0.001).

An increase in open arm exploratory behaviour produced by LY-288513 was not observed in trial two (group 3 versus group 6; $F_{11,90}$=9.34, p<0.001) and (group 3 versus group 9; $F_{11,90}$=9.34, p<0.01). Administration of scopolamine failed to reinstate, increased exploratory activity in the open arms (group 3 versus group 12; $F_{11,90}$=9.34, p<0.001).

Closed Arm Entries:

Plus-maze exposure exerted inhibition of open arm exploration (number of open arm entries) in DMSO groups; group 1 versus group 4 ($F_{11,90}$=7.67, p<0.001), but not with group 7. Scopolamine administration exerted no effect on closed arm entries (group 1 versus group 10; $F_{11,90}$=7.67, p<0.001). Number of closed arm entries were significantly decreased upon exposure of chlordiazepoxide administered groups; (group 1 versus group 5 and versus group 8; $F_{11,90}$=7.67, p<0.001). Scopolamine failed to reinstate increased closed arm entries produced by chlordiazepoxide within trial one (group 1 versus group 11; $F_{11,90}$=7.67, p<0.001). Number of closed arm entries were significantly decreased upon exposure of LY-288513 administered groups; (group 1 versus group 6; $F_{11,90}$=7.67, p<0.001). Scopolamine failed to reinstate increased closed arm entries produced by LY-288513 within trial one (group 1 versus group

12; $F_{11,90}$=7.67, p<0.001). Chlordiazepoxide showed no significant differences between exposure protocols, except in a trend observed (p=0.054), comparing group 2 with group 8. LY-288513 showed no differences between protocols, in number of closed arm entries.

No significant differences were observed for number of closed arm entries with scopolamine administration .

Total Arm Entries:

No differences between drug groups were observed with regard to total number of arm entries. Rats pre-exposed to a five minute plus-maze session showed a significant decrease in total arm entries on trial two versus total entries observed in maze naïve rats under the same drug protocol; DMSO ($F_{11,90}$=9.09, p<0.001), chlordiazepoxide ($F_{11,90}$=9.09, p<0.001), LY-288513 ($F_{11,90}$=9.09, p<0.01). Administration of scopolamine produced a significant decrease in total arm entries on trial two compared to total entries observed in maze naïve rats under the same drug protocol; DMSO ($F_{11,90}$=9.09, p<0.001), chlordiazepoxide ($F_{11,90}$=9.09, p<0.001), LY-288513 ($F_{11,90}$=9.09, p<0.01). Significant differences within drug groups between rats pre-exposed to a five minute plus-maze session with administration of scopolamine following immediately, versus those pre-exposed to a five minute plus-maze session without scopolamine were not observed.

Experiment 4.2:

No statistically significant differences were observed (figures 4.2.1 and 4.2.2), except for a trend, narrowly missing statistical significance. Percentage open arm time in trial two for the LY-288513 post-exposure administered group was increased with chlordiazepoxide administration when compared to a group undergoing the same protocol less LY-288513 post-exposure administration ($F_{1,14}$=4.43, p<0.054).

Experiment 4.3:

No statistically significant differences were observed in any of the sampled variables.

Experiment 4.4:

No significant differences or any trends were observed for saline versus DMSO in either maze naïve or in the single maze exposure group (figure 4.4.1). Chlordiazepoxide (3.0mg.kg^{-1}), in maze naïve rats, (figure 4.4.1 and 4.4.6) produces a significant decrease in startle amplitudes compared to DMSO in maze naïve rats; $F_{12,106}$=7.82, $p<0.05$ (Trial block 1). This is no longer observed with maze exposure. LY-288513 does not exhibit any significant effects in maze naïve rats upon startle amplitudes.

No significant differences are observed between saline administered groups naïve to the plus-maze and post five-minute exposure (figure 4.4.2).

A second exposure to the elevated plus-maze, DMSO administered, produced a statistically significant increase in startle amplitudes compared to the plus-maze naïve DMSO control (Figure 4.4.3); $F_{12,106}$=7.82, $p<0.05$ (Trial block 1).

Analysis of differences between chlordiazepoxide administered protocols (figure 4.4.4), demonstrates a significant increase in startle amplitudes after the second plus-maze exposure when compared to maze naïve chlordiazepoxide administered rats; $F_{12,106}$=7.82, $p<0.001$ (Trial block 1). This is also observed when compared to amplitudes post first exposure; $F_{12,106}$=7.82, $p<0.05$ (Trial block 1) although this is of questionable value given the latter measures drugged exposure.

Analysis of differences between LY-288513 administered protocols (figure 4.4.5), also demonstrates a significant increase in startle amplitudes after the second plus-maze exposure when compared to amplitudes 24 hours post first exposure in LY-288513 administered rats;

$F_{12,106}$=7.82, p<0.05 (Trial block 1).

No significant differences are observed between drug treatments for animals post-initial exposure to the plus-maze. Chlordiazepoxide exhibits a trend towards a decrease in startle amplitudes when compared to DMSO but this misses a significant analysis.

Experiment 4.5

A series of preliminary validation experiments were conducted. Effects in rats of electric shock induction (post conditioning trial) on a retention index of; acoustic startle peak amplitude in the retention session divided by that in the conditioning (acquisition) session, for individual rats, over twenty-five trials. Dimethylsulphoxide (DMSO) was administered post acquisition session as control. Five consecutive trial blocks are shown for each protocol. These each represent the means of five individual retention index values for response to acoustic stimuli. Induction of shock produced a statistically significant increase in retention index compared to the nonshocked control (Figure 4.5.1); Shocked group; $F_{1,13}$=49.03, p<0.001 (Trial block 1); $F_{1,13}$=7.36, p<0.05 (Trial block 2), $F_{1,13}$=10.18; p<0.01 (Trial block 4).

Effects in rats of scopolamine (0.3mg.kg^{-1}) on the aforementioned retention index with both shocked and nonshocked rats showed significant differences (figure 4.5.2). Scopolamine, administered post-shock was shown to significantly decrease the index values compared to DMSO administered post-shock, $F_{3,30}$=14:00, p<0.001 (Trial block 1), $F_{3,30}$=4.05, p<0.05 (Trial block 2). The nonshocked DMSO control group showed a significant decrease in index value from the shocked DMSO group, $F_{3,30}$=14:00, p<0.001 (Trial block 1), $F_{3,30}$=4.05, p<0.05 (Trial block 2), $F_{3,30}$=4.06, p<0.05 (Trial block 4). The nonshocked scopolamine group also showed significant decreases in index value from the DMSO shocked group, $F_{3,30}$=14:00, p<0.001 (Trial block 1 only), but did not when compared to the scopolamine shocked group nor the DMSO nonshocked group. Perhaps significantly, index values for the scopolamine-unshocked

group approached a mean of 1 (with <9.6% standard error).

Administration of the cholecystokinin form; CCK-4 (figure 4.5.3), at doses, 0.0125, 0.025, 0.05 and 0.1 $mg.kg^{-1}$) failed to produced any significant changes in index values when compared to DMSO (control) although a decrease in index value at 0.0125 $mg.kg^{-1}$ narrowly missed significance in trial block 1, $F_{4,34}=2.51$, $p=0.052$.

CCK-8us at doses, 0.001, 0.005, 0.01, 0.05 and 0.1 $mg.kg^{-1}$ (figure 4.5.4) failed to produce any significant changes in index value or any trends.

CCK-8s at doses, 0.0005, 0.001, 0.005 and 0.01 $mg.kg^{-1}$, (figure 4.5.5) also failed to produce any significant changes in index value but showed a trend towards an increase in index value at 0.005 $mg.kg^{-1}$, $F_{4,32}=2.33$, $p=0.074$.

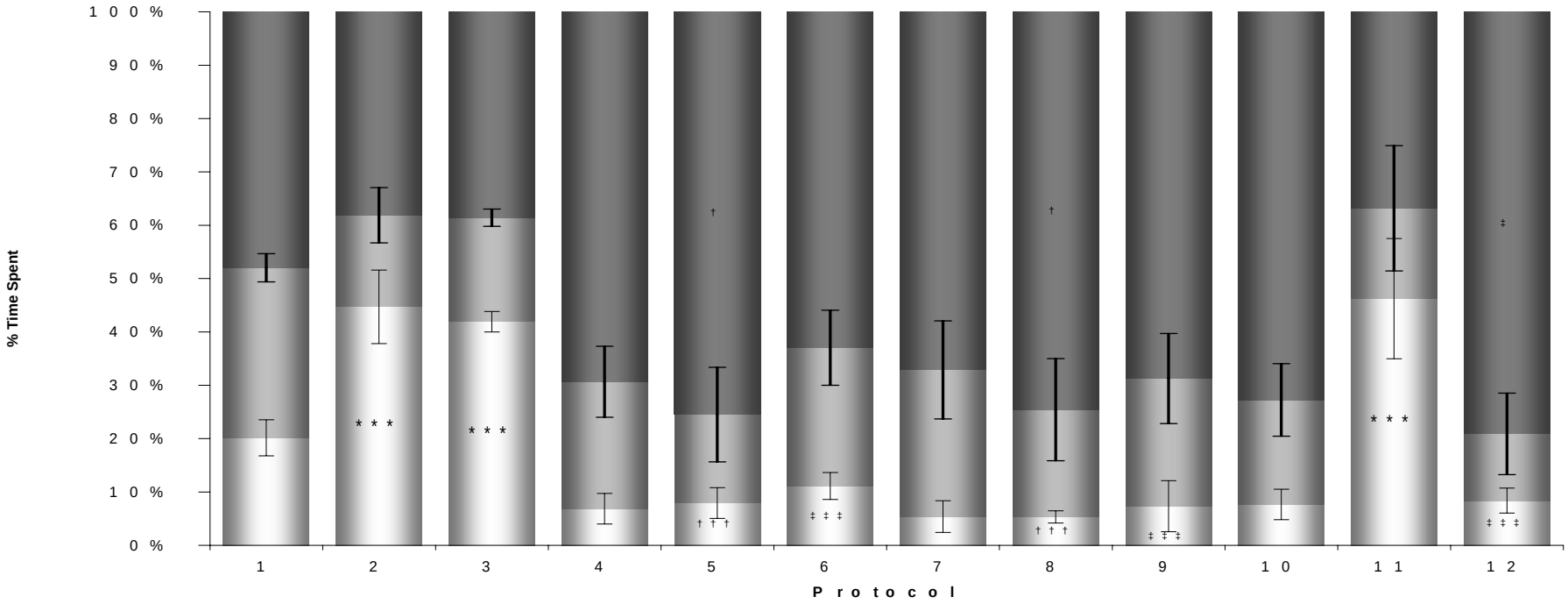


FIG 4.1.1.: Effects on behaviour within the elevated plus-maze of a prior pre-exposure to the maze. Group numbers correspond with protocols detailed in table 4.1. Measures of time spent in specific areas of the maze as a percentage of total trial duration are shown. Lightly shaded portions, bottommost section, correspond to percentage time spent in open arms; Grey shaded portions, middle section, correspond to percentage time spent in the central platform area; Dark grey shaded portions, uppermost section, correspond to percentage time spent in the closed arms. Results are presented as mean with light bars representing standard error of the mean (SEM). SEM bars atop the open arm time section represent SEM for percentage time spent in the open arm. SEM bars beneath the closed arm time section represent SEM for percentage time spent in the closed arm. SEM bars for percentage time spent on the central platform not shown. Significant differences from the each specific drug administered to naïve rats are compared with the same drugs administered post-trial; ***$p<0.001$ for DMSO, $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$ for chlordiazepoxide; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$, $^{\ddagger\ddagger\ddagger}p<0.001$ for (Bonferroni post hoc analysis after significant one-way ANOVA).

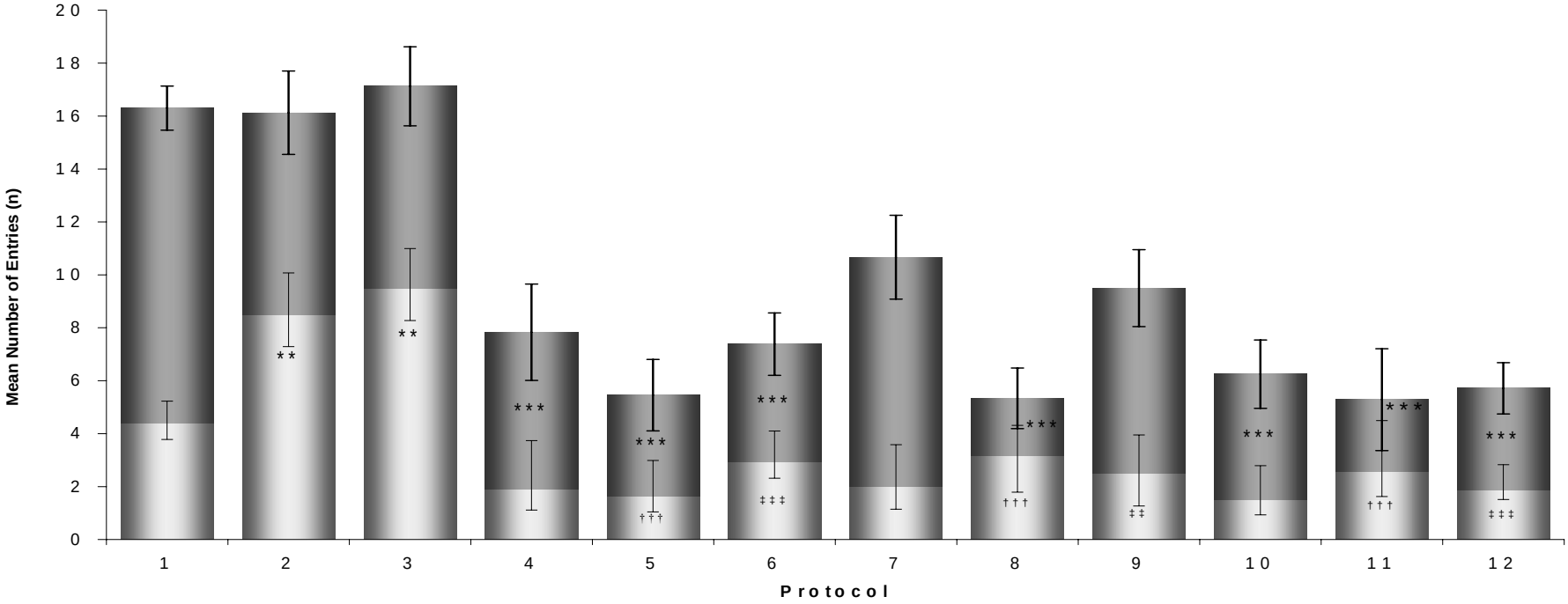


FIG 4.1.2: Effects on behaviour within the elevated plus-maze of a prior pre-exposure to the maze. Group numbers correspond with protocols detailed in table 4.1. Measures of numbers of entries into specific areas of the maze are shown. Lightly shaded portions, lower section, correspond to numbers of entries into open arms; dark grey shaded portions, upper section, correspond to numbers of entries into the closed arms. Results are presented as mean with light bars representing standard error of the mean (SEM). SEM bars atop the open arm entries section projecting downward represent SEM for number of open arm entries. SEM bars atop the open arm entries section projecting upwards represent SEM for number of entries into the closed arm. SEM bars atop the closed arm entries section projecting both upward and downward for total number of entries into both arms. Significant differences from the each specific drug administered to naïve rats are compared with the same drugs administered post-trial; ***$p<0.001$ for DMSO, $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$ for chlordiazepoxide; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$, $^{\ddagger\ddagger\ddagger}p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

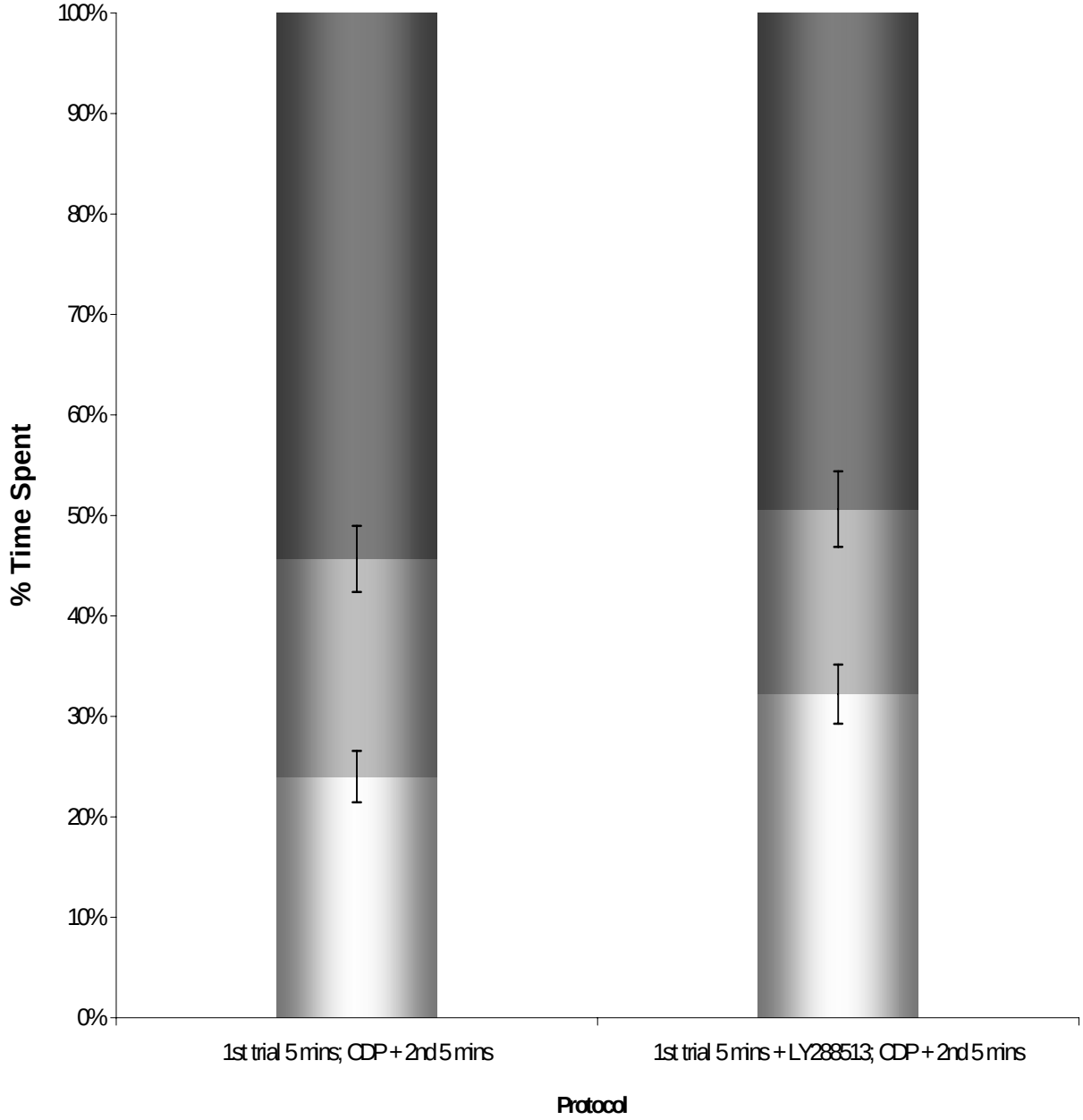


FIG 4.2.1: Effects on behaviour within the elevated plus-maze of a prior pre-exposure to the maze. Protocol groups represented are as follows: chlordiazepoxide [CDP], , ($3.0mg.kg^{-1}$) pre-trial at second exposure after a five minute pre-exposure; chlordiazepoxide pre-trial at second exposure after a five minute pre-exposure followed immediately with administration of LY-288513 ($10.0mg.kg^{-1}$). Measures of time spent in specific areas of the maze as a percentage of total trial duration are shown. Lightly shaded portions, bottommost section, correspond to percentage time spent in open arms; Lighter grey shaded portions, middle section, correspond to percentage time spent in the central platform area; Dark grey shaded portions, uppermost section, corresponds to percentage time spent in the closed arms. Results are presented as mean with light bars representing standard error of the mean (SEM). SEM bars atop the open arm time section represent SEM for percentage time spent in the open arm. SEM bars beneath the closed arm time section represent SEM for percentage time spent in the closed arm. Significant differences from the chlordiazepoxide pre-trial two group were not found.

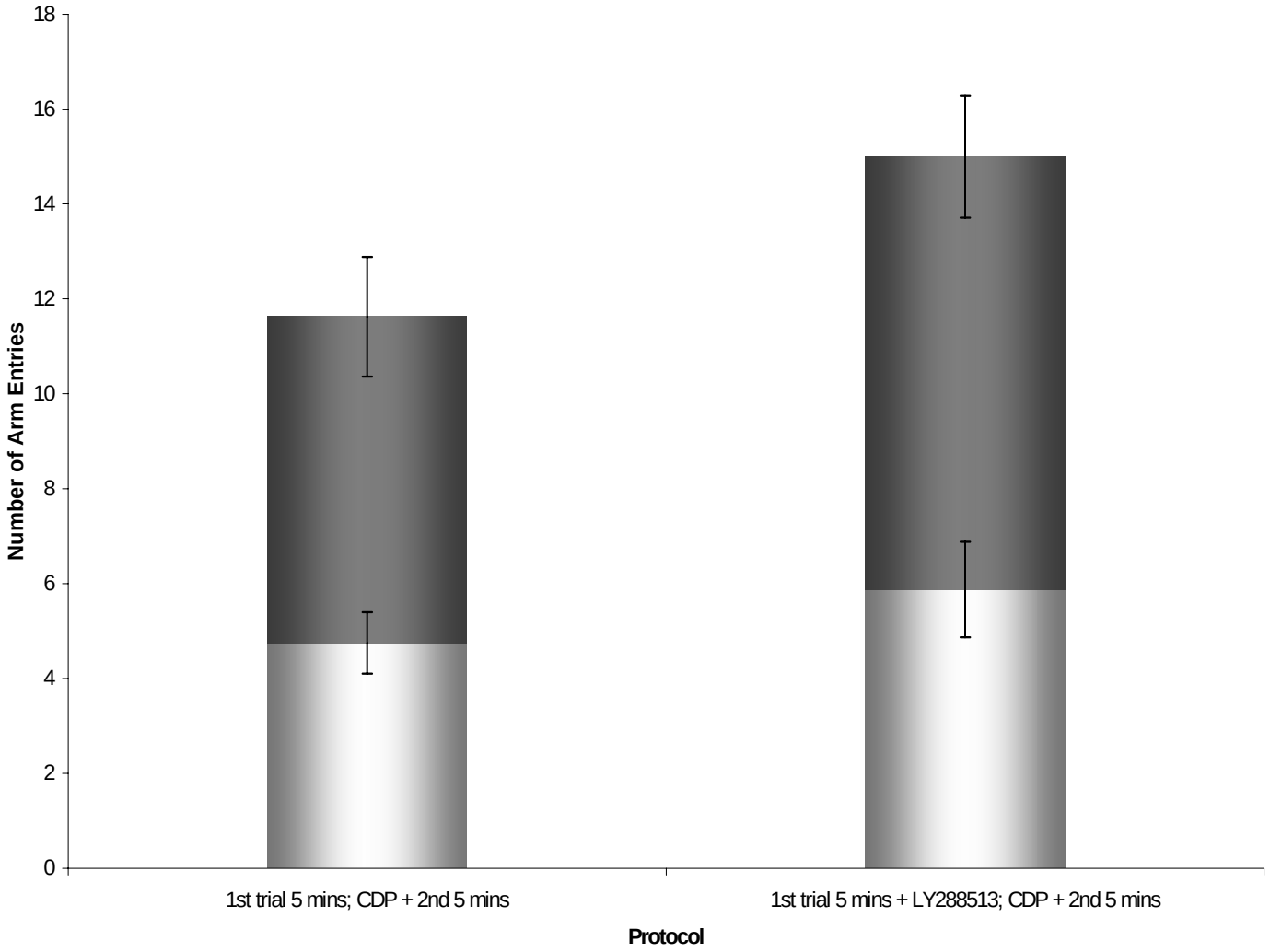


FIG 4.2.2: Effects on behaviour within the elevated plus-maze of a prior pre-exposure to the maze. Protocol groups represented are as follows: chlordiazepoxide [CDP], ($3.0mg.kg^{-1}$) pre-trial at second exposure after a five minute pre-exposure; chlordiazepoxide pre-trial at second exposure after a five minute pre-exposure followed immediately with administration of LY-288513 ($10.0mg.kg^{-1}$). Measures of arm entries within the maze are shown. Lightly shaded portions (bottommost section) correspond to number of entries into the open arms; dark grey shaded portions (uppermost section) correspond to number of entries into the closed arms. The results are presented as mean with light bars representing standard error of the mean (SEM). SEM bars atop the open arm time section represent SEM for percentage time spent in the open arm. SEM bars beneath the closed arm time section represent SEM for percentage time spent in the closed arm. Significant differences from the chlordiazepoxide pre-trial two group were not found.

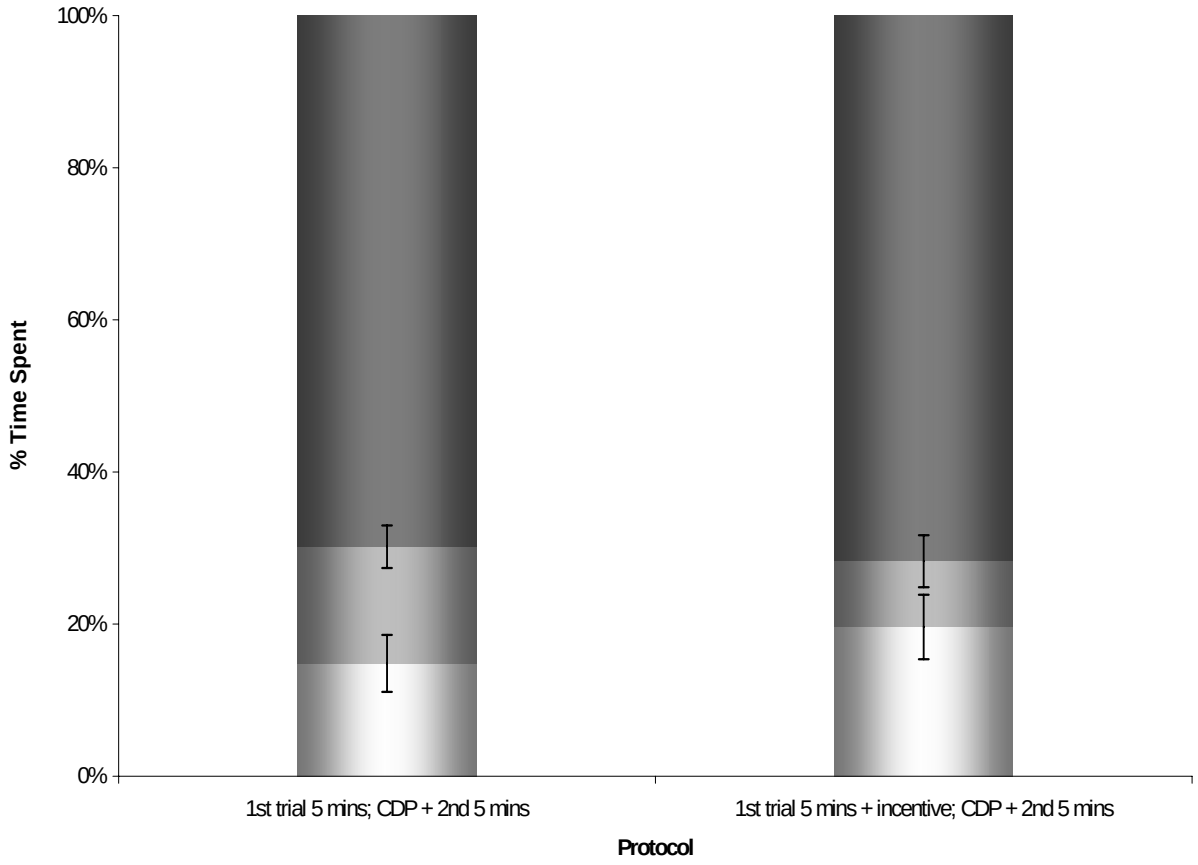


FIG 4.3.1: Effects on behaviour within the elevated plus-maze of a prior pre-exposure to the maze. Two distinct groups are represented are as follows: chlordiazepoxide, [CDP], ($3.0mg.kg^{-1}$) pre-trial at second exposure after a five minute pre-exposure to a clear plus-maze; chlordiazepoxide, ($3.0mg.kg^{-1}$) pre-trial at second exposure after a five minute pre-exposure to a plus-maze containing a food incentive. Measures of time spent in specific areas of the maze as a percentage of total trial duration are shown. Lightly shaded portions, bottommost section, correspond to percentage time spent in open arms; Lighter grey shaded portions, middle section, correspond to percentage time spent in the central platform area; Dark grey shaded portions, uppermost section, corresponds to percentage time spent in the closed arms. Results are presented as mean with light bars representing standard error of the mean (SEM). SEM bars atop the open arm time section represent SEM for percentage time spent in the open arm. SEM bars beneath the closed arm time section represent SEM for percentage time spent in the closed arm. No significant differences from the chlordiazepoxide pre-trial two group after exposure to a clear plus-maze were found.

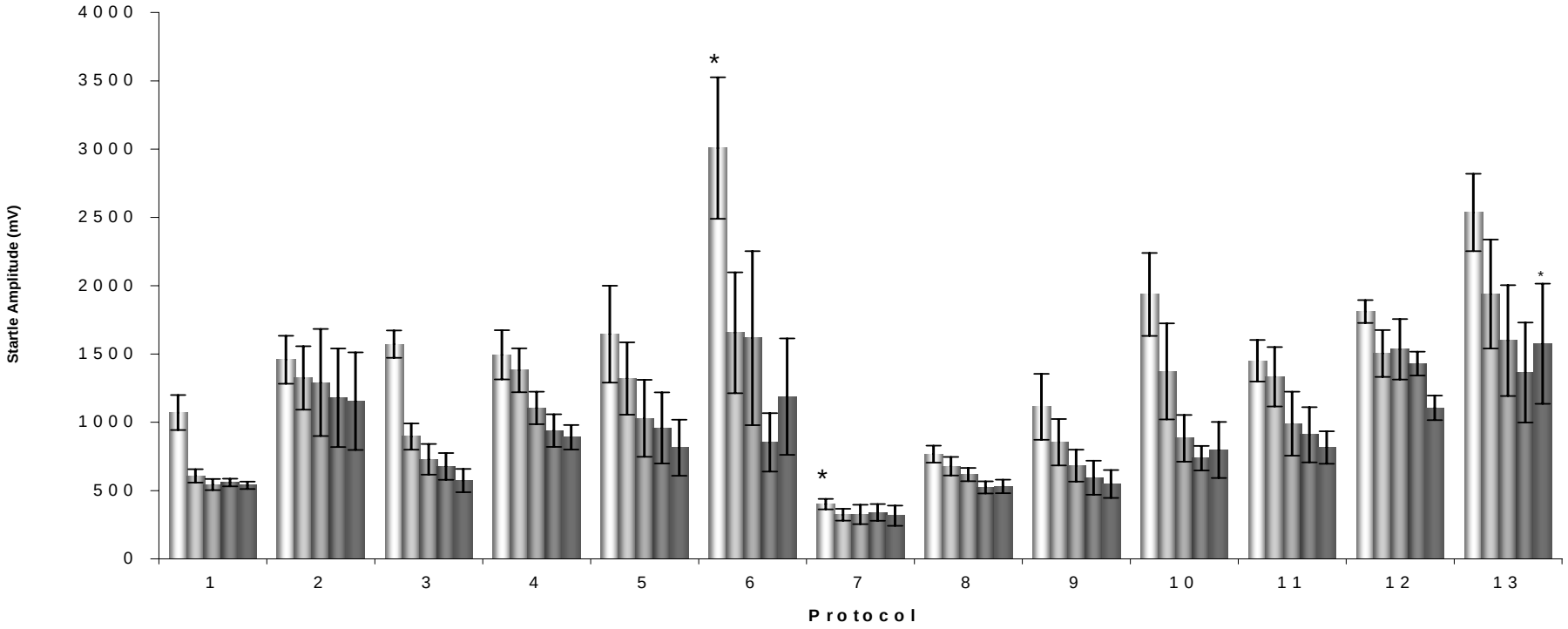


FIG 4.4.1: Startle amplitudes are shown for animals either naïve to the plus-maze (groups 1, 3 and 7), following an initial exposure to the plus-maze (groups 2, 4, 8, 11), twenty four hours after initial plus-maze exposure (groups 5, 9, and 12), and following a second plus exposure; twenty-four hours after the first (groups 6, 10, 13). Animals were administered either; saline (groups 1 and 2), DMSO (groups 3 to 6), chlordiazepoxide [CDP] at 3.0mg.kg^{-1} (groups 7 to 10) or LY-288513 at 10.0mg.kg^{-1} (11 to 13) prior to acoustic startle sessions. The effects of these group protocols (summarised in table 4.2) on acoustic startle peak amplitude over twenty-five trials are shown. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the plus-maze naïve DMSO group (3) are shown; *$p<0.05$; (Bonferroni post hoc analysis after significant one-way ANOVA).

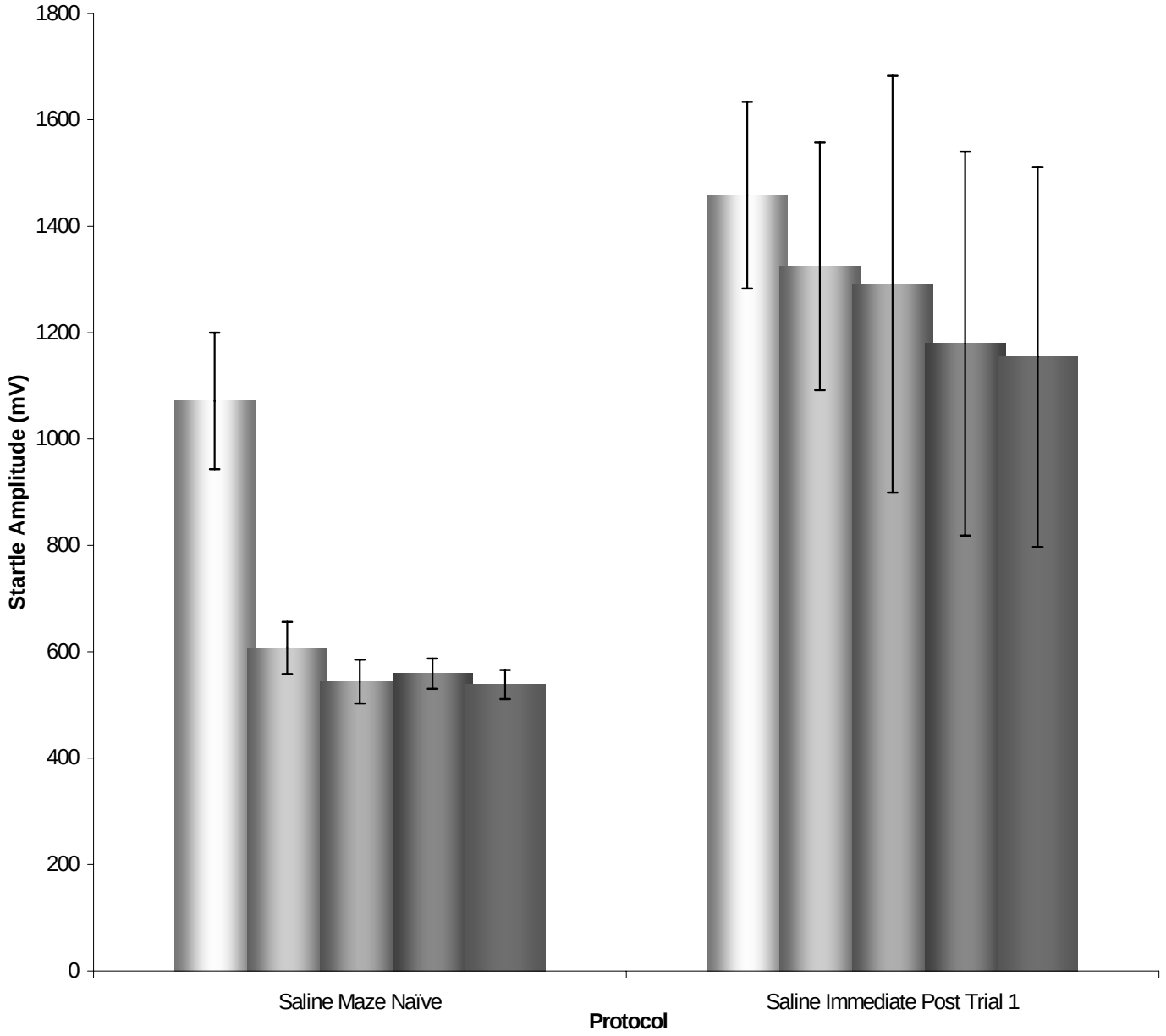


FIG 4.4.2: Startle amplitudes are shown for vehicle - administered animals either naïve to the plus-maze or following an initial exposure to the plus-maze. The effects of plus-maze exposure on acoustic startle peak amplitude over twenty-five trials are shown. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increased shading. No significant differences from the plus-maze naïve saline group (3) are observed (Bonferroni post hoc analysis after significant one-way ANOVA).

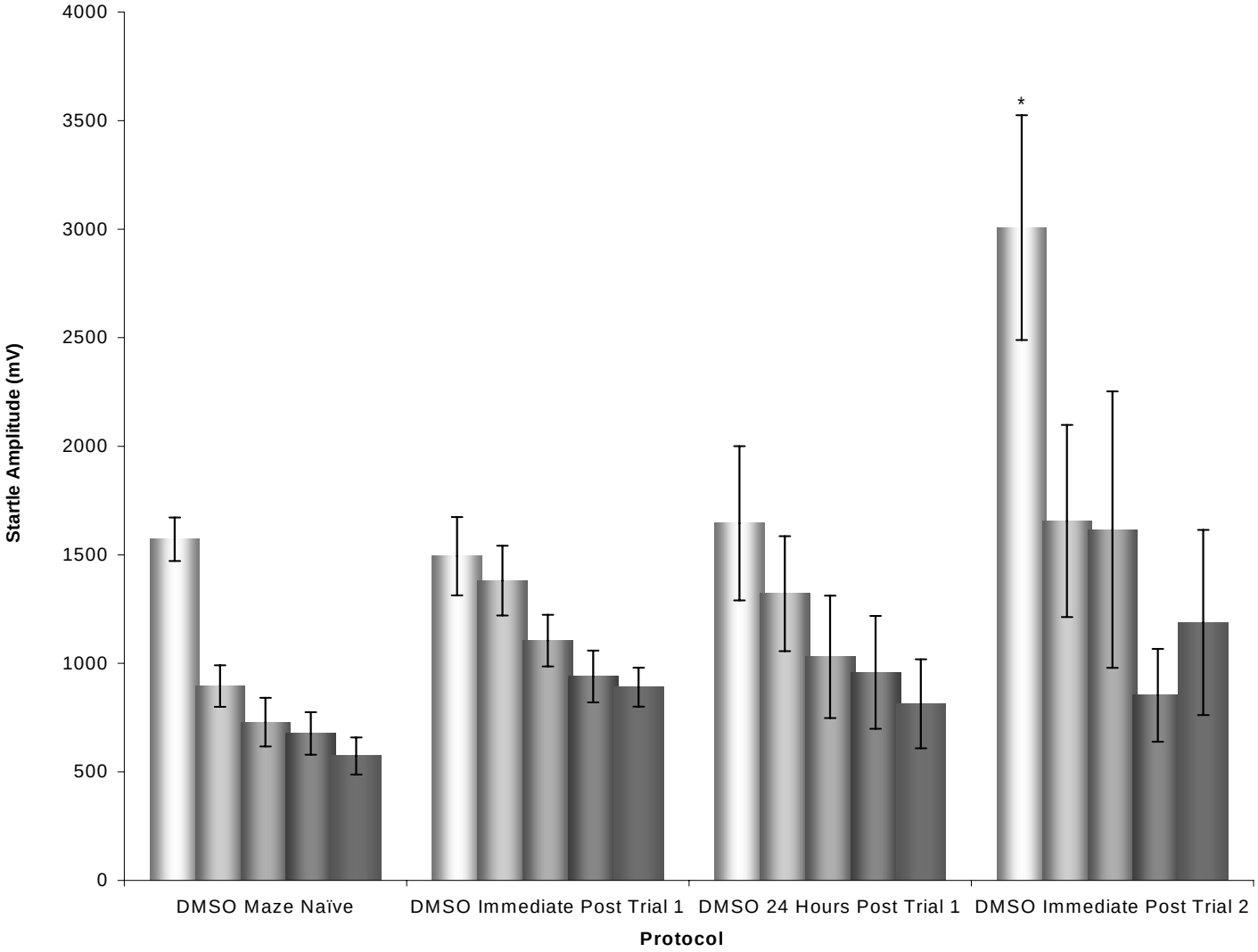


FIG 4.4.3: Startle amplitudes are shown for animals either naïve to the plus-maze, following an initial exposure to the plus-maze, twenty four hours after initial plus-maze exposure and following a second plus-maze exposure; twenty-four hours after the first. Animals were administered DMSO prior to acoustic startle sessions. The effects of these group protocols on acoustic startle peak amplitude over twenty-five trials are shown. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the plus-maze naïve DMSO group (3) are shown; *$p<0.05$, (Bonferroni post hoc analysis after significant one-way ANOVA).

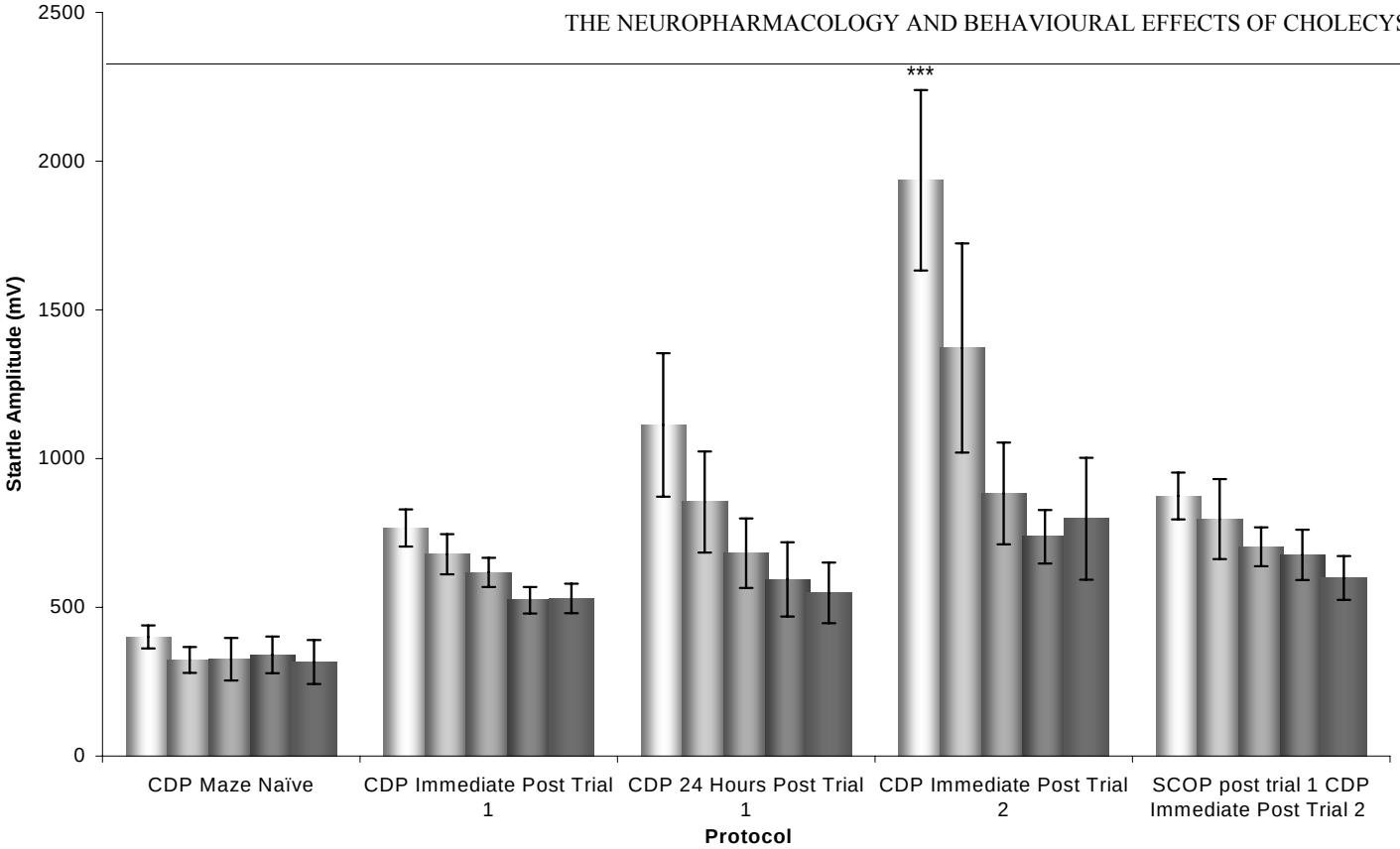


FIG 4.4.4: Startle amplitudes are shown for animals either naïve to the plus-maze, following an initial exposure to the plus-maze, twenty four hours after initial plus-maze exposure and following a second plus-maze exposure; twenty-four hours after the first. Animals were administered chlordiazepoxide [CDP] ($3.0mg.kg^{-1}$) prior to acoustic startle sessions. The effects of these group protocols on acoustic startle peak amplitude over twenty-five trials are shown. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increased shading. Significant differences from the plus-maze naïve chlordiazepoxide group (3) are shown, ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

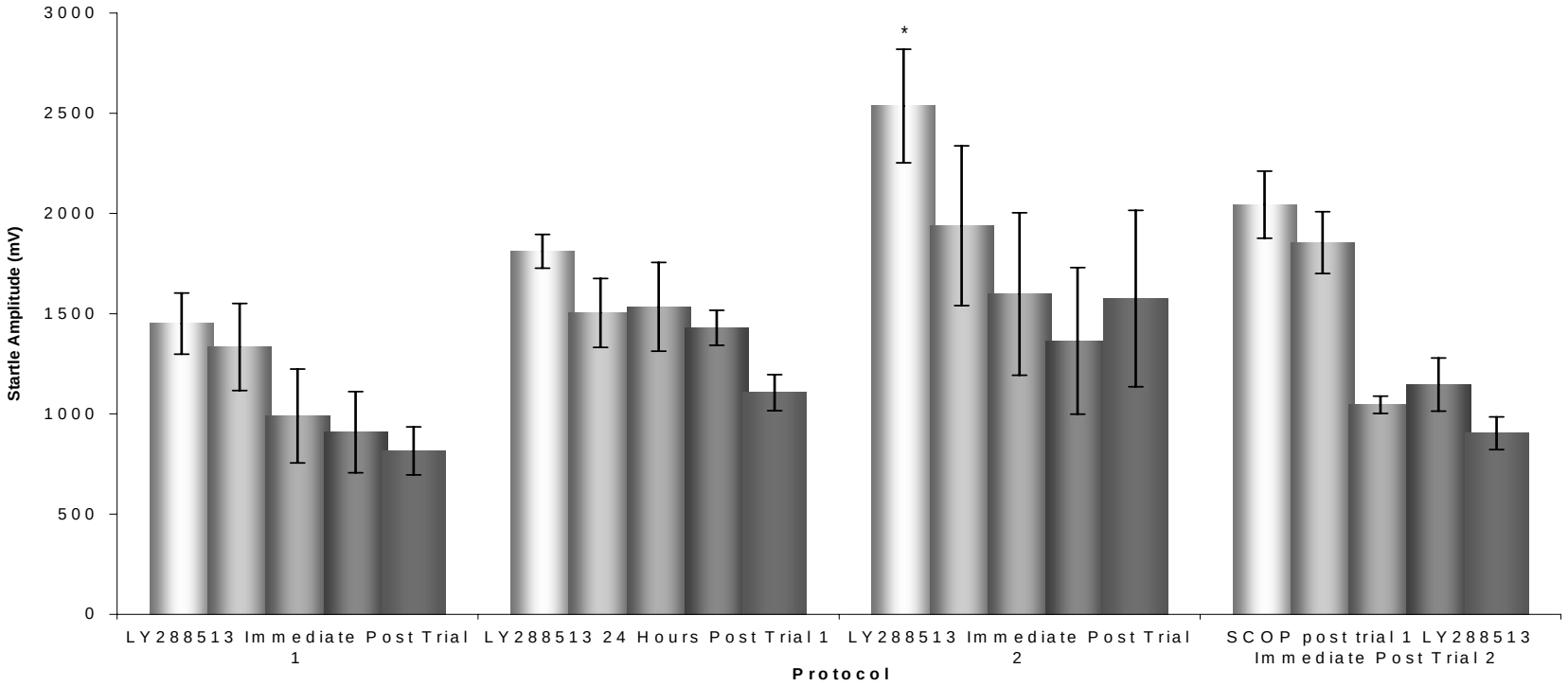


FIG 4.4.5: Startle amplit-udes are shown for animals either following an initial exposure to the plus-maze, twenty four hours after initial plus-maze exposure and following a second plus-maze expos-ure; twenty-four hours after the first. Animals were admin-istered LY-288513 (10.0mg.kg^{-1}) prior to acoustic startle sessions. The effects of these group protocols on acoustic startle peak amplitude over twenty-five trials are shown. Five consecutive trial blocks are shown for each treatment. These each represent the means of five individual responses to acoustic stim-uli (results presented as mean with SEM). Consec-utive trial blocks are represented from left to right and with increased shading. Signif-icant differences from the post first exposure LY-288513 group (3) are shown; *$p<0.05$, (Bonferr-oni post hoc analysis after significant one-way ANOVA).

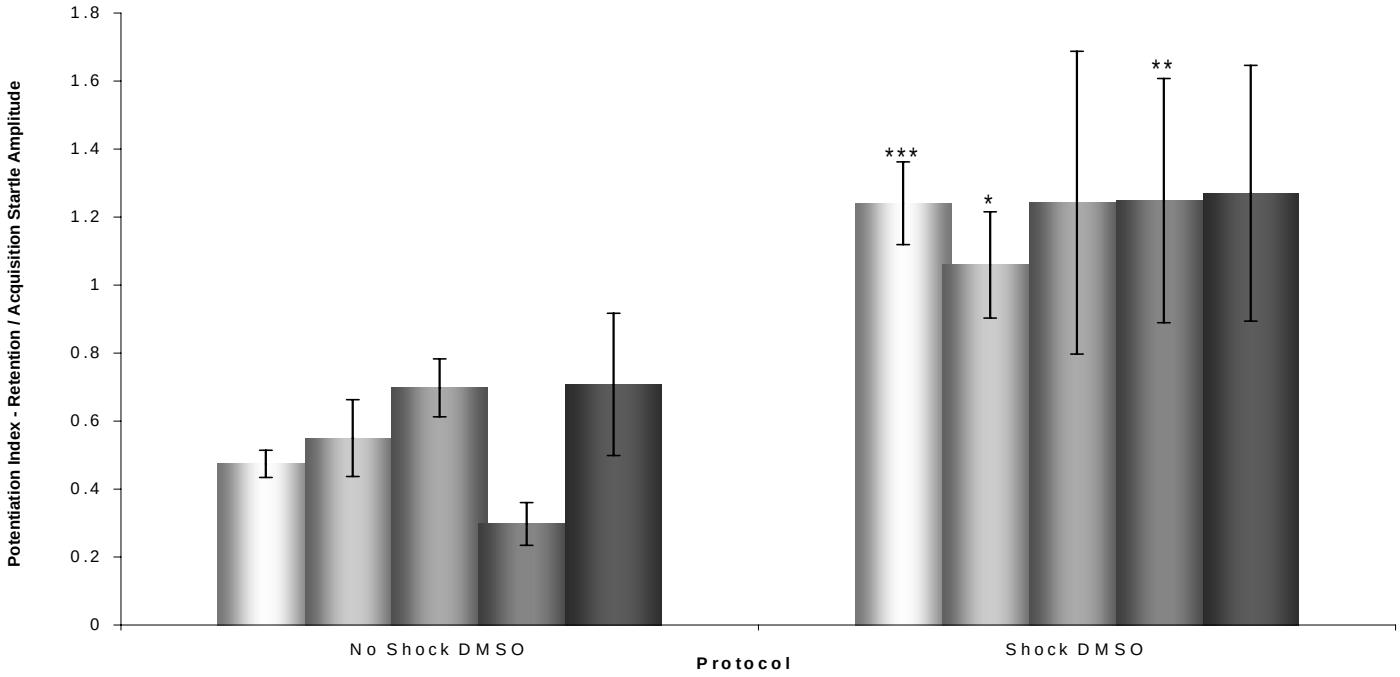


FIG 4.5.1: Effects in rats of electric shock induction (post conditioning trial) versus non-shocked exposure to the shock chamber on a retention index of; acoustic startle peak amplitude in retention session divided by that in conditioning (acquisition) session, for individual rats, over twenty-five trials versus DMSO administered post exposure. Five consecutive trial blocks are shown for each protocol. These each represent the means of five individual retention index values for response to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increasingly darkened shading. Significant differences from the non-shocked group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

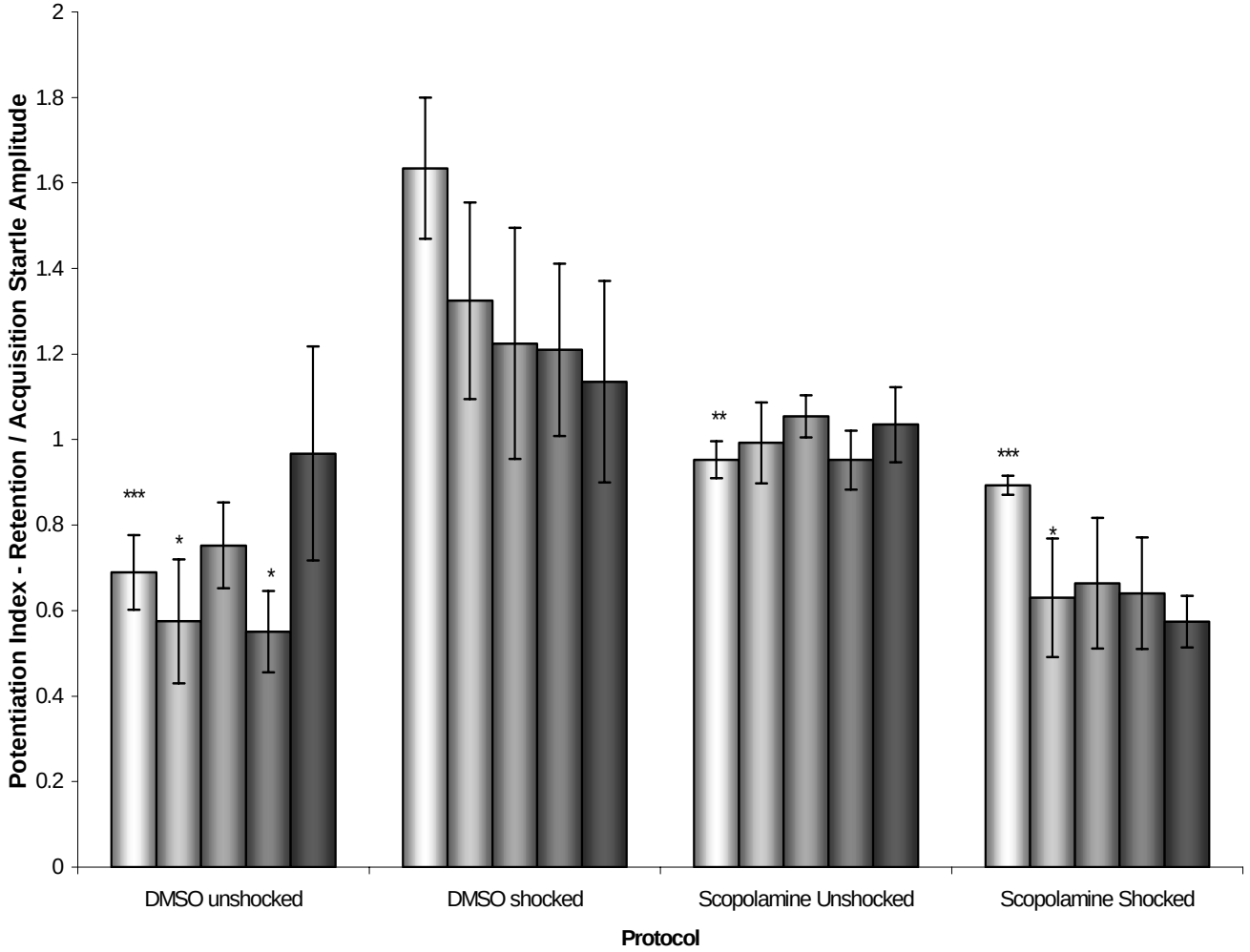


FIG 4.5.2: Effects in rats of electric shock induction (post conditioning trial) on a retention index of; acoustic startle peak amplitude in retention session divided by that in conditioning (acquisition) session, for individual rats, over twenty-five trials versus scopolamine ($0.3mg.kg^{-1}$) administered post shock. Five consecutive trial blocks are shown for each protocol. These each represent the means of five individual retention index values for response to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increasingly darkened shading. Significant differences from the DMSO shocked group are shown; *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

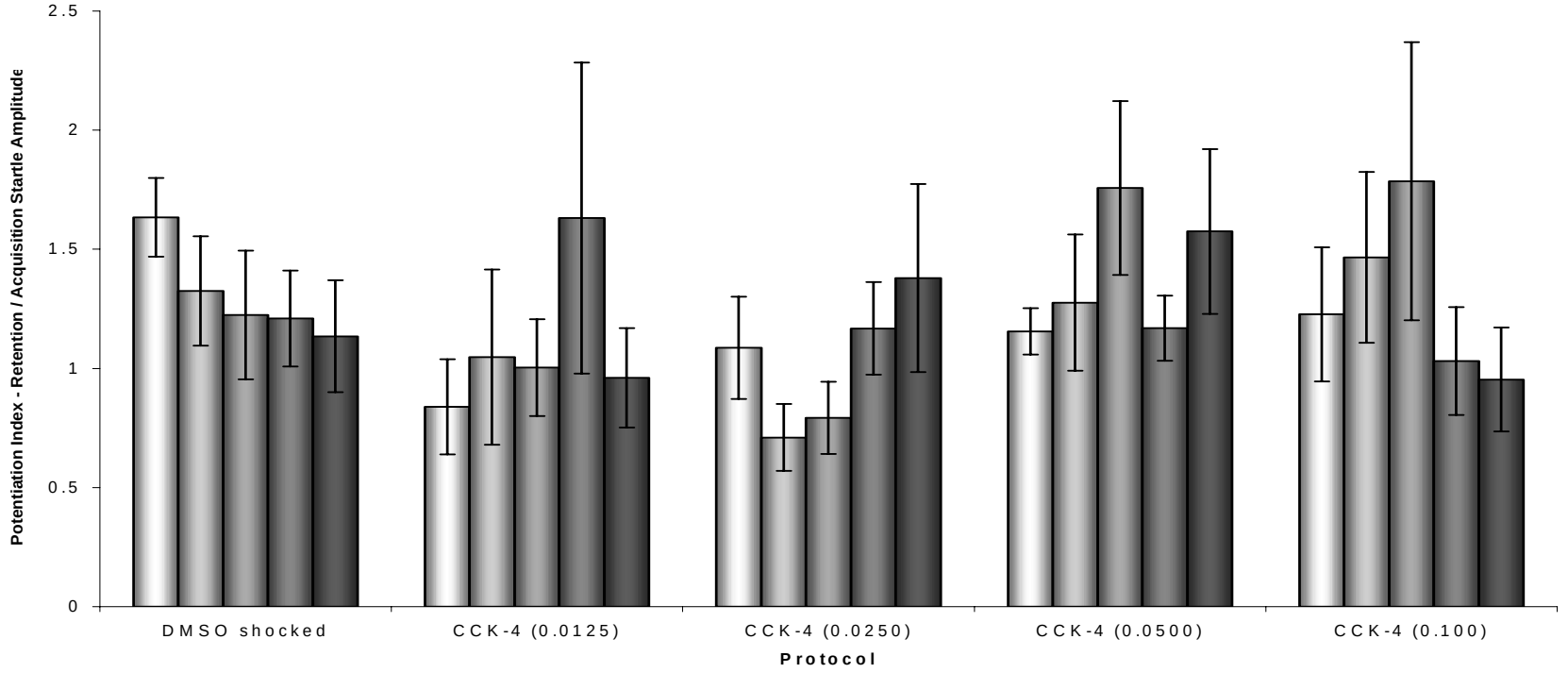


FIG. 4.5.3: Effects in rats of a range of doses (in mg kg^{-1}) of CCK-4 with DMSO vehicle, on a retention index of; acoustic startle peak amplitude in retention session divided by that in conditioning (acquisition) session, for individual rats, over twenty-five trials, versus the effect of DMSO alone; drugs administered post shock. Five consecutive trial blocks are shown for each protocol. These each represent the means of five individual retention index values for response to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increasingly darkened shading. Significant differences from the DMSO group were not observed. (Bonferroni post hoc analysis after one-way ANOVA).

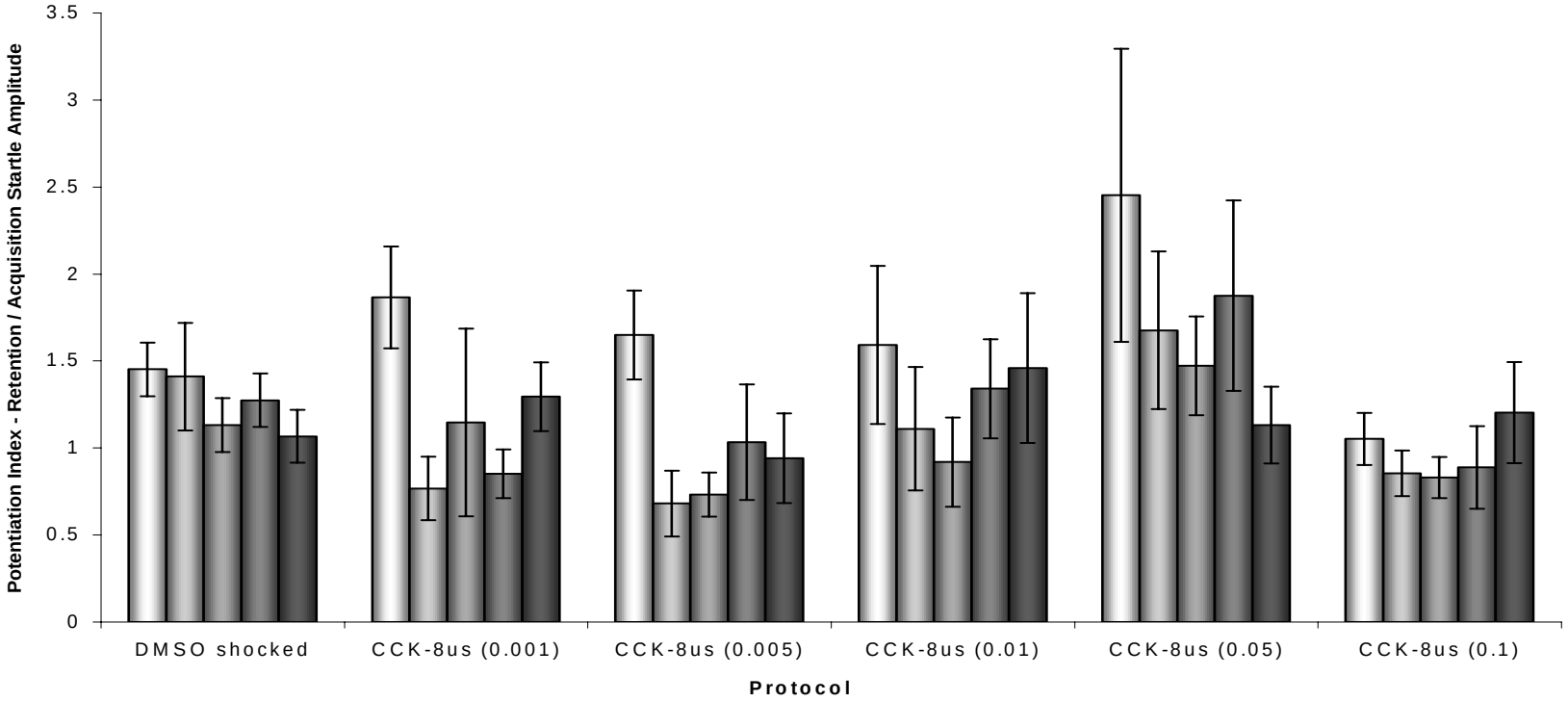


FIG. 4.5.4: Effects in rats of a range of doses (in mg kg^{-1}) of CCK-8us with DMSO vehicle, on a retention index of; acoustic startle peak amplitude in retention session divided by that in conditioning (acquisition) session, for individual rats, over twenty-five trials, versus the effect of DMSO alone; drugs administered post shock. Five consecutive trial blocks are shown for each protocol. These each represent the means of five individual retention index values for response to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increasingly darkened shading. Significant differences from the DMSO group were not observed. (Bonferroni post hoc analysis after one-way ANOVA).

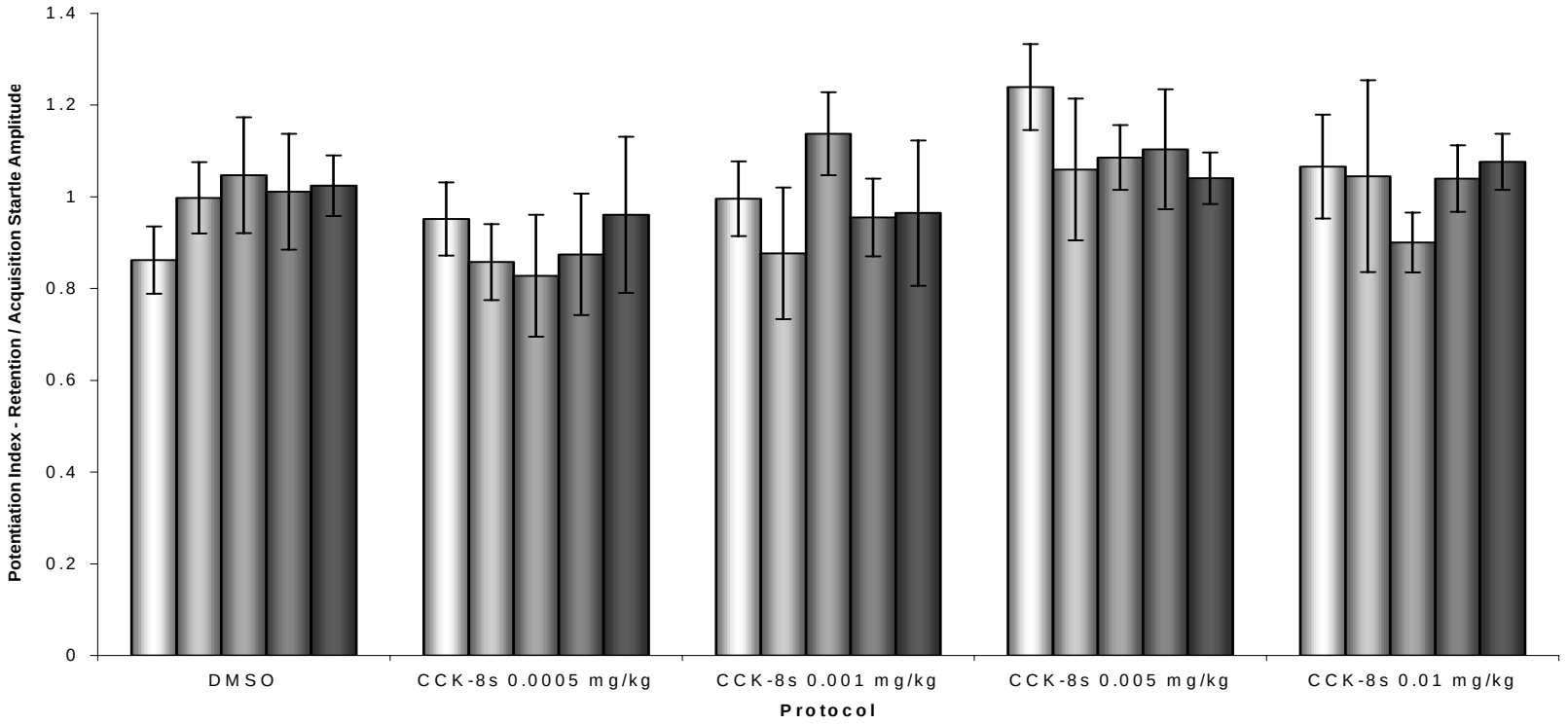


FIG. 4.5.5: Effects in rats of a range of doses (in mg kg^{-1}) of CCK-8s with DMSO vehicle, on a retention index of; acoustic startle peak amplitude in retention session divided by that in conditioning (acquisition) session, for individual rats, over twenty-five trials, versus the effect of DMSO alone; drugs administered post shock. Five consecutive trial blocks are shown for each protocol. These each represent the means of five individual retention index values for response to acoustic stimuli (results presented as mean with SEM). Consecutive trial blocks are represented from left to right and with increasingly darkened shading. Significant differences from the DMSO group were not observed. (Bonferroni post hoc analysis after one-way ANOVA).

Discussion

Chlordiazepoxide, at 3.0mg.kg^{-1} was able to produce a statistically significant increase in time spent on open arms compared to the DMSO control. This is a commonly reported activity for major benzodiazepines.

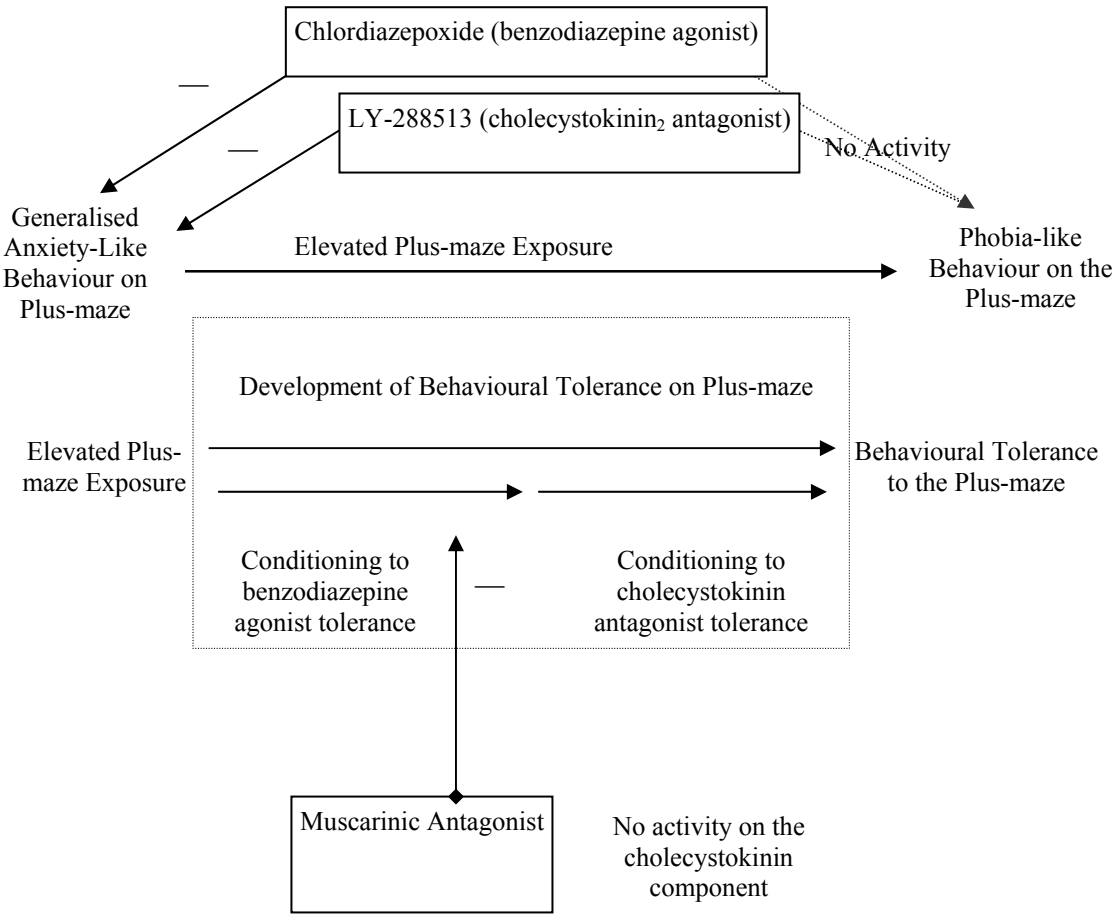
Previously I have shown that LY-288513 has some anxiolytic activity at high doses within the plus-maze (see chapter 3 - unconditioned fear). The dose of 10mg.kg^{-1} was chosen in the present study because of the stability of its' anxiolytic property. In confirmation of the previous study, LY-288513 at the prescribed dose produced a significant increase in both percentage time spent within and entries into the open arms. These are proposed to be measures of anxiolytic-like behaviour within the maze.

Both drugs when administered in a subsequent plus-maze trial after the initial maze exposure, with the initial exposure either five or ten minutes in duration, were unable to produce these "anxiolytic" effects. It has been observed in previous studies that chlordiazepoxide is no longer active in subsequent trials after an initial exposure to the plus-maze of two or more minutes (Lister, 1987; File, 1990; File *et al*., 1990). This is however a first report of LY-288513, a cholecystokinin$_2$ receptor specific antagonist, having similar effects.

Administration of scopolamine following the initial plus-maze exposure was able to restore the significant increases in percentage time spent in the open arm by chlordiazepoxide but not LY-288513 or control groups. Scopolamine appears therefore to restore sensitivity to chlordiazepoxide but not to LY-288513. This leads to an inference that the site at which benzodiazepine driven anxiolysis is gated, lies upstream of a site gating cholecystokinin gated anxiolysis within the neuronal organisation. According to this hypothesis, this upstream site would be in connection to neuronal organisations influenced by the cholinergic system, particularly muscarinic antagonists, whereas the downstream site (cholecystokinin organisation) would not be susceptible to muscarinic influences (i.e.: not connected to

cholinergic organisations). Furthermore, it may be proposed that chlordiazepoxide and possibly other benzodiazepine agonists achieve their anxiolytic profile through activity upon the cholecystokinin system.

Figure 4.6 A proposed mechanism underlying development of One trial tolerance to the anxiolytic activity of benzodiazepine agonists and cholecystokinin-2 antagonists upon the elevated plus-maze. (–) = inhibition



An experiment in which a blockade of the cholecystokinin organisation by an antagonist, LY-288513 was performed. This protocol attempted to prevent development of desensitisation to chlordiazepoxide following the aforementioned hypothesis. However, this was deemed a flawed protocol. Given the anxiolytic activity of specific CCK_2 antagonists, administration of such a ligand subsequent to the first plus-maze exposure, as was scopolamine, would produce effects that would interfere with activity upon the aforementioned neuronal organisation. For example, LY-288513 might produce an anxiolytic state wherein the anxiety inducing experience produced within the plus-maze, would be repressed. It is very likely that this anxious state allows development of chlordiazepoxide insensitivity, and without such a state, this phenomenon would not occur. Logically development of a phobia would not occur if the anxiety-producing stimulus was unable to elicit an anxious state. Therefore, the findings of the second experiment were deemed pre-experimentally as of questionable validity. The fact that LY-288513 administration post exposure was able to partially prevent (trend observed, just missing significance) development of tolerance to chlordiazepoxide is analysed and remains inconclusive. The blockade of the cholecystokinin component of tolerance development is alas perhaps unachievable.

In confirmation of studies by File *et al*., (1993), the scores of control animals remain stable over repeated tests. The control group in this study (DMSO) showed no differences between protocols. However, some credence must also be given to the conflicting reports regarding either presence or absence of differences, given that a non-significant trend was observed whereby prior plus-maze exposure exerted some inhibition of open arm exploration (time spent). With measurement of variables pertaining to ostensibly the same behaviour, yet differing in approach, for example recording percentage time spent on the open arm (as by File *et al*., 1993) and quantification of the actual distance travelled by the rats in the maze (as by Dawson *et al*., 1994), this trend observed with time spent, within my own study, might reach statistical significance with determination of the distance travelled variable. Therefore, these

seemingly incompatible observations may not be contradictory per se. However, the persistence of chlordiazepoxide sensitivity observed within trial two by Dawson *et al*., (1994) is not confirmed by nor is implied by this study or by a majority of data.

In contrast to that previously reported by File *et al*., (1993), chlordiazepoxide increased open arm exploratory behaviour was not observed in trial two post ten-minute initial exposure. This is however, in accordance with the study by Holmes and Rodgers (1999) where trial two chlordiazepoxide insensitivity was maintained following the aforementioned protocol. Only when the second trial was increased to ten minutes in duration was the anxiolytic profile of chlordiazepoxide restored to a statistically significant degree in the Holmes and Rodgers study. This restoration of sensitivity was also not observed in the LY-288513 group.

Fernandes and File (1996) observed that chlordiazepoxide had significant anxiolytic effects on trial one only in a maze without ledges, and on trial two only in the maze with ledges. A behavioural mechanism underlying the aversion elicited by an absence of ledges, compared to a proposed lack of phobic conditioning in the presence of ledges may be offered. Whereby absence of ledge elicits a phobic conditioning experience, analogous to walking the plank, offering a real fear of danger from falling, the motivation for investigation, inhibited by fear, within this arm will be repressed in late trial one and within whole of trial two. The presence of ledges offers an environment of relative safety analogous to "walking upon a veranda". Consequently, a plus-maze with ledges provides an environment with fundamentally different aversive cues. The multidimensional aspects of the fear experience present upon the un-walled plus-maze may be in part, a combination of a fear of "open spaces" and a fear of falling. A suitable hypothesis for elucidation of mechanisms underlying the sensitivity to chlordiazepoxide tolerance within the enwalled maze, might be whereby the generation of a proposed phobic state is initiated by fear of falling and not by fear of open spaces. Furthermore it may be noted that the implications of each fear are somewhat different. Cognitive processes will dictate that falling will deliver a possible injury, and is thus a realistic

fear. Fear of open spaces, is perhaps, a fallaciously perceived threat, driven by an innate fear of predation within the open field. The likelihood from each fear of a catastrophic outcome may therefore differ in intensity.

Interestingly, a disparity with the Fernandes and File (1996) study and my own is noted. Their study claimed that rats pre-exposed to a maze, with 0.5cm ledge surrounding the open arm, did not develop chlordiazepoxide insensitivity on a subsequent trial, whereas a ledge free maze induced such an occurrence. My own studies have constantly used a maze with open arms surrounded by a 1cm clear Perspex™ ledge. This was used in order to minimise falls from the open arm, which were at a surprisingly high level during preliminary trials. However, my own studies have shown that chlordiazepoxide insensitivity on trial two is produced using a maze with ledges, and that chlordiazepoxide sensitivity is indeed exhibited during trial one. This might perhaps be seen as an irreconcilable difference, however, I believe it to be significant that the Fernandes and File maze platforms are constructed with wood, whilst my maze platforms were constructed from Perspex™, as adapted from Lister (1987). This difference may explain both the initial high level of falling from the maze and the development of chlordiazepoxide insensitivity. Speculation of reasoning processes underlying behaviour of an individual rat may lie in a perceived probability of falling. The Perspex™ platform undoubtedly presents less friction than a wooden platform. Thus the perceived probability of slipping and falling would be deemed greater in the Perspex™ constructed platform. In addition, it may also be of significance that the ledges constructed on my maze were composed of transparent Perspex™, whereas within the Fernandes and File study it is not noted. A transparent construction may indeed confer distinct cues with regard to the nature of the open space, whereas an opaque material may impart a perception of a more enclosed area. This, in turn, might alter any perceived threat of falling.

No significant differences were observed between either drug groups or between exposure protocols with regard to central platform time. Time spent on the central platform may be

indicative of risk assessment behaviour. This probing pre-empts entry onto the arms of the maze, particularly onto the open arms.

Measures of arm entries showed broad similarity regarding group distribution to that of time spent, with some notable exceptions. As with time measures, maze naïve rats, administered chlordiazepoxide or LY-288513 produced statistically significant increases in the number of entries into the open arms compared to the control. This is thought to confirm the anxiolytic properties of both drugs.

Once again, both drugs when administered in a subsequent plus-maze trial after prior maze exposure, were unable to exact these effects, hence supporting the hypothesis of drug insensitivity. However, unlike with time measures, administration of scopolamine, immediately following initial exposure failed to restore the increases in open arm entries by chlordiazepoxide with maze naïve rats. Furthermore, the total number of entries for the scopolamine-chlordiazepoxide group remains significantly inhibited. These measures reveal the relative lack of exploration between arms whilst spending time within each arm. This difference appears to highlight a peculiarity, where these two measures are generally assumed to represent the same or very similar aspects of the anxious response within the maze. Moreover, the total number of entries of all the pre-exposed groups is significantly inhibited. This indicates therefore, either an inhibition of locomotor activity or an increase in anxiety directed towards the maze in its entirety. An underlying cause of this phenomenon however, might lie in motivational changes with regard to exploration. Furthermore, motivational factors underlying an open arm entry would be a combination of incentive driven motivation and fear aversion. Factors underlying total entry numbers would be to a lesser degree fear aversion, and with a far greater component of incentive driven motivation. Scopolamine activity upon conditioning processes involved in plus-maze exposure, may prevent development of phobia (conditioned fear) but is unable to preclude reward circuit conditioning. It might be proposed that decreased exploration (arm entries) apparent in trial two, and late trial one as observed by

Rodgers *et al*., (1996) may be as a result of a lack of incentive value tied to the maze. The animal will undoubtedly learn that the plus-maze contains no incentive value and is partly aversive in nature (with particular reference to the open arm), thus exploration within subsequent trials will be inhibited. This argument is given some credence whereby the number of total entries is significantly decreased in maze pre-exposed animals, and accordingly a decrease in total maze exploration. Furthermore, although chlordiazepoxide has been shown clinically to lack efficacy in phobic disorders, it is also plainly not efficacious in restoring motivation. This matter was hoped to be elucidated upon with a protocol in which open arms of the maze on the first exposure, contained a sweetened cereal pellet, as an incentive. It was anticipated that, alike the food incentive driven radial arm maze, the plus-maze open arm would be awarded incentive value with regard to motivation surrounding exploration in subsequent trials. Several revisions of this protocol were performed, without a successful training trial. Rats were averse to consuming sweetened food incentives within the plus-maze in any sector, even when food deprived. Of a fairly large sample group (n = 32 rats) not more than a single pellet was consumed upon the plus-maze, and even then was carried from the open arm to the central platform. Upon retesting these animals, unsurprisingly, no differences in exploration were observed from the other pre-exposed groups. This protocol, in hindsight, was condemned to failure given previous findings by Holson (1986) who observed a feeding neophobia of rats in maze conditions, particularly pertinent to unhandled animals. However, despite extensive animal handling, rats were still averse to foodstuff consumption within the maze. It may appear logical that an animal will be reluctant to feed within a perceptually "perilous" environment with other factors underlying their motivation, and this I believe is the underlying cause. In a recent study, by Pereira *et al*., (1999), pairing an aversive stimulus to presence within the closed arm retained chlordiazepoxide sensitivity on the subsequent trial. The saline administered groups were not affected on the second trial. This study provides fairly strong evidence as to the nature of the chlordiazepoxide tolerance phenomenon. However, it does not take account of the possible amnestic efficacy or ability to produce

attentional deficits of chlordiazepoxide, as does my own protocol. In addition, their study did not produce avoidance of the closed arm in any group which is unexpected, given that conferring an aversive stimulus to the closed arm, might be predicted to balance the aversive properties of both the open and closed arms. This suggests that the aversive experience of the open arm is at a far greater intensity than that of the closed arm plus air puff. Therefore, I tried to achieve the same feat using an opposite methodology. Introducing an incentive value, I had hoped to partially withdraw the aversive component from the open arm, rather than introduce one to the closed arm. In addition, this was hoped to increase exploratory motivation within the maze rather than decreasing this component as in the Pereira study. With an increase in motivation to explore, the inhibition of total arm entries observed previously was hoped to be reversed, and the question of, "does a decreased motivation to explore underlie the one trial tolerance to chlordiazepoxide?" would be addressed. Nevertheless, this study by Pereira *et al*., (1999) whose inspiration lay contemporaneous and independent from myself, has provided some interesting scope for further investigation.

The absence of neophobic feeding, with regard to inducing incentive value within the maze, is possibly resolved by using female rat odour within the open arms of the maze. This procedure however was not achievable at the time of the experiment with regard to logistical concerns.

Effects of Elevated Plus-Maze Exposure on Acoustic Startle

The absence of any significant differences observed for saline versus DMSO in either maze naïve or in the single exposure group demonstrates firstly that use of DMSO, as a vehicle is acceptable. In line with plus-maze studies, chlordiazepoxide produces an anxiolytic-like response in maze naïve rats. However, quite unlike its profile within the plus-maze, LY-288513 does not exhibit any significant effects compared to DMSO, upon startle amplitudes.

Interestingly, for each drug group, neither an initial exposure to the plus-maze nor testing twenty-four hours later produced any significant increases in startle amplitudes. However, with

startle sessions conducted after a second exposure to the plus-maze, a statistically significant increase in startle amplitudes was observed in all three drug administered groups. This is noteworthy in that, firstly an anxiety-like state observed is conferred by only a second exposure to the plus-maze, and secondly, neither chlordiazepoxide nor LY-288513 are unable to counteract this effect. This reproduces findings within the plus-maze with regard to anxiolytic drug effects, and also demonstrates that a considerable increase in anxiety-like behaviour can be induced by a second exposure to the plus-maze. This increase in anxiety-like behaviour, indicative of a phobic state, is thought to underlie the nature of chlordiazepoxide and related benzodiazepine insensitivity on trial two of the plus-maze. This is supported with these findings. Furthermore, given that chlordiazepoxide fails to exhibit an anxiolytic profile after trial one onwards, it may be proposed that development of chlordiazepoxide insensitivity occurs within trial one, as is proposed by Rodgers *et al*., (1996). It may also be notable that a trend toward lowering of startle amplitudes by chlordiazepoxide can be observed for all exposure groups. This might imply that some anxiolytic activity is observed for chlordiazepoxide in post trial two startle amplitudes, although this missing statistical significance by quite a degree. Perhaps we can ignore the fact that LY-288513 fails to produce any anxiolytic like effects within the startle paradigm after exposure to the plus-maze. In experiments detailed within chapter two, it was demonstrated that LY-288513, despite producing an anxiolytic-like activity within the plus-maze at specific doses, was unable to produce a like effect within the acoustic startle paradigm at these doses.

It is perhaps also of note that significant increases were not observed in startle responding twenty four hours post trial one. The animals underwent precisely identical procedures as those tested after trial two, with the exception of a second plus-maze exposure of course. This shows that it is not the experience of handling, test room exposure and injection that gates the increase in startle amplitudes observed post trial two. It can be inferred that the anxiety-state observed is provoked to a very large degree by a second plus-maze exposure in itself.

Data observed for animals post trial one for chlordiazepoxide and LY-288513 administered groups, is provided through flawed methodology, in that, the animals, by nature of the protocol itself, did not undergo an nondrugged exposure to the plus-maze. This data was merely collected as an indicator of possible anomalies. Statistical analyses must therefore examine comparisons between either maze naïve or post maze (after twenty-four hours) with post trial two groups. However, although not reaching statistical significance chlordiazepoxide drugged exposure to the plus-maze produced a slight increase in startle responding which may indicate that an anxious state may not be required in order to produce development of phobia. This would have to be investigated further with drugged exposure to the plus-maze. In summary this experiment was able to confirm an anxiety-like activity provoked by a second plus-maze exposure, which substantiates evidence for perhaps a more intense form of anxiety observed within trial two. This observation provides scope for further study regarding manipulation of maze exposure time as was performed by Rodgers *et al*., (1996). This evidence does seem to support that the animal perceives the plus-maze as a highly aversive environment. This might indicate that indeed there is some phobic development upon multiple exposures. However this also does not discount motivational deficits producing subsequent plus-maze behaviour, but implies that multiple factors are underlying.

Shock Induced Fear Potentiated Startle

The series of validation experiments demonstrated that shock exposure within a separate chamber, immediately following the initial exposure to the acoustic startle apparatus and startle protocol, was able to produce a fear potentiated startle response when compared to rats undergoing exposure to the shock chamber in absence of the shock itself. In addition, this series also showed that scopolamine, a muscarinic acetylcholine antagonist, commonly used as an amnestic agent, was able to inhibit this fear potentiated startle response. Moreover, scopolamine may have extended its' amnestic properties to blocking habituation to the startle stimuli between trials one and two in the nonshocked rats, although significant differences

between the nonshocked groups are not observed.

Evidence against any activity upon anxiety by scopolamine has been provided in a range of studies. Scopolamine did not produce anti-conflict effects in the Vogel conflict test (Umezu 1999), nor any effects on anxiety-like behaviour in the elevated plus-maze (Thiel *et al*., 1999). Scopolamine also had no effects in the light-dark transition test of anxiety (Shimada *et al*., 1995), nor the shock probe/ burying test (Treit 1990). Although scopolamine does produce locomotor stimulation (Sansone 1980), this observation is probably irrelevant in indicating an alteration of anxious behaviour. Thus, it is probable that scopolamine acts via an amnestic mechanism rather than a mood alteration in blocking fear potentiated startle.

In a study by Tsutsumi *et al*., (1999) rats were individually subjected to 30 min of inescapable electric footshock. After a period of twenty-four hours the rats were again placed in the chamber and observed for a 5-minute period without shocks. It was observed that administration of the CCK_2 antagonist, PD-135158, 30-minutes prior to the conditioned fear retention session and 30-minutes prior to the conditioning (acquisition) session, significantly reduced freezing behaviour. However, administration of PD-135158 5-minutes after the acquisition did not significantly reduce freezing behaviour. Izumi (1998) observed that subcutaneous administration of the CCK_2 specific antagonist, LY-288513, 30-minutes before footshock (0.3 $mg.kg^{-1}$) and 30 min before conditioned fear stress (0.03-0.3 $mg.kg^{-1}$) reduced conditioned freezing in rats. A similar protocol to that used by Tsutsumi *et al*., (1999) was utilised here with the exception of a 5-minute shock exposure. The authors of these experiments (Izumi and Tsutsumi) stated that their studies "indicate that LY-288513 and PD-135158 blocked not only the acquisition but also the expression of conditioned fear". This conclusion however is highly questionable. Given that my own studies have shown that cholecystokinin agonists are unable to exert any effects on fear potentiated startle when administered after the shock session, as with PD-135158, administered post-conditioning session, the inference arises that cholecystokinin does not therefore exert an effect upon the

acquisition or expression of conditioned fear. It is also plausible that effects upon anxiety level thus, freezing behaviour itself, within the retention sessions, and that state effects on perhaps, either nociception or attention induced by PD-135158 / LY-288513 administered prior to the conditioning session, are responsible for the observations made by these authors. Freezing behaviour in response to environmental stress has been documented with cat exposure in rats (Farook *et al*., 2001) so thus would not be unexpected after shock exposure. Furthermore, it may also be possible that the first exposure initiates a coping mechanism that is activated within the retention session. This coping mechanism may involve habituation to anxiety effects, or nociceptive desensitisation, as a preparative measure for the ensuing shock or expectation of such. Particular drug treatments may as well manipulate these factors as they might alter mnestic properties. In order for a categorical statement, that an agent is able to effect processes involved in acquisition / retention of a conditioned fear, to be partially acceptable, the agent must be able to alter behaviour in the retention trial when administered after the acquisition trial only. With this method it would be hoped to ensure that effects of the drug on subjective experiences within the acquisition trial, and / or effects on activity within the retention trial are ruled out. This ideal had underlain my assessment of the paradigm upon beginning this series of experiments. However, recently (post validation) serious doubts had arisen as to the nature of observed differences within this protocol. It is possible that drugs administered after the acquisition session itself may also exhibit state effects on the paradigm. Rather than demonstrate a connection between the specific drug activity and neural pathways fundamental to the gating of a conditioned fear response, they may also add to the aversion experience proceeding the acquisition session. If for example, a drug, which produces an anxiogenic-like response, is administered subsequent to the acquisition, the anxious experience may further augment any anxiety-like behaviour within the retention session itself. This observation could then be incorrectly interpreted as a facilitation of memory rather than an intensification of the aversive experience. Likewise, an anxiolytic agent might lead to interpretation of an amnestic activity, where in effect a lessening of a deleterious experience by

reducing anxiety may be apparent. Unfortunately, the vast majority of studies using avoidance / conditioned fear paradigms have overlooked this seemingly fundamental issue of interpretation. All-encompassing statements that a particular putative agent has been characterised in animal studies, then to often fail at clinical examination. This issue may be the explanation behind the lack of success in this field.

The experiment carried out within this study, aimed to demonstrate whether, cholecystokinin forms, which have a well-characterised anxiogenic activity at specific doses, are able to augment the intensity of aversive experience subsequent to the acquisition session. The dose range itself was determined by my own studies of anxiogenic activity of cholecystokinin and those of proposed effects upon memory retention of cholecystokinin. A combination of the shock exposure and the anxious state might augment behaviour within the retention sessions when compared to shock exposure in isolation. This was found however, not to occur, at least significantly under statistical analysis, across the dose range tested. The trend observed for a decrease in index value by CCK-4 (0.0125 $mg.kg^{-1}$) which narrowly missed significance, may be of some importance given the proximity to the anxiety- provoking dose within the elevated plus-maze. In hindsight, it seems that study of pharmacological agents and their effects upon attenuation or augmentation of fear potentiated startle measures will be infeasible given the aforementioned argument. For any agent to have claim to activity upon developmental processes in conditioned fear, they must have been proven to not exhibit any activity on anxiety itself.

CHAPTER FIVE - CHOLECYSTOKININ EFFECTS UPON MEMORY ACQUISITION AND RETENTION

Introduction

Initial studies implicating a role for cholecystokinin in alteration of memory processes were conducted in the early nineteen-eighties. Fekete *et al*., (1981) observed that the sulphated cholecystokinin octapeptide, CCK-8s, and it's non-sulphated analogue CCK-8us, administered intracerebroventricularly, increased active avoidance retention latency, thus implying an amnestic activity. Further work by Fekete *et al*., (1982b) showed that the same effects are maintained with systemic administration, via a subcutaneous route. Cohen *et al*., (1982) observed these same effects with intraperitoneal administration of CCK-8s but only when administered within fifteen minutes prior to the retention trial. Intraventricular administration (Fekete *et al*., 1981) and subcutaneous administration (Fekete *et al*., 1982b), of CCK-8us and CCK-8s both impaired the acquisition of, and facilitated the extinction of active avoidance. CCK-8 antisera delayed the extinction of active avoidance and conditioned feeding response, while ineffective on acquisition and maintenance stages (Fekete *et al*., 1982a). Combined injection of CCK-8 (320 micrograms.kg^{-1} i.p.) and haloperidol (75 micrograms.kg^{-1} i.p.) reduced avoidance significantly more than either drug alone (Cohen *et al*., 1982). CCK-8 has been found to improve retention when administered intraperitoneally in normal but not in vagotomised mice whereas CCK-8 enhanced retention in mice with cortical lesions but not in mice with stria terminalis lesions (Flood *et al*., 1995).

Investigations of two-way step-through active avoidance have shown that CCK-8 depresses avoidance responding and that with chronic administration of CCK-8 within the intervening period, tolerance to CCK-8 is conferred (Cohen *et al*., 1983). Hadjiivanova *et al*., (1995) also observed that CCK-4, administered post-acquisition session, was not able to affect two-way active avoidance retention, twenty-four hours after a fifty trial acquisition session.

Concurrent studies however, generated some quite contrary observations. Kadar *et al*., (1981) demonstrated that CCK-8s and CCK-8us, administered intracerebro-ventricularly, at periods before or after acquisition and pre-retention trial, in contrast increased passive avoidance retention latency, indicative of improved retention. This was also confirmed with systemic intraperitoneal administration (Fekete *et al*., 1982). Subsequent, studies showed that a range of cholecystokinin analogues, from the tetrapeptide form (CCK-4), to CCK-5, -6, -7 and both forms of CCK-8, administered both intracerebroventricularly and intraperitoneally, also increased passive avoidance latency (Fekete *et al*., 1984). Hadjiivanova *et al*., (1995) also observed that CCK-4, administered post-acquisition session, was able to impair passive avoidance retention, three hours after a one trial acquisition session, but not when tested twenty-four hours or seven days afterward.

CCK-8 (30-500g) injected immediately following acquisition sessions produced an increase in the passive avoidance latency of rats. Proglumide, a non-selective cholecystokinin antagonist, ($5mg.kg^{-1}$) was able to block CCK-8 effects on rat passive avoidance conditioning. Proglumide by itself at a dose of 2 $mg.kg^{-1}$ decreased the latency to enter the darkened chamber. In addition, CCK-8 was able to prevent extinction within a passive avoidance paradigm (Deupree and Hsiao 1988). Passive avoidance behaviour was facilitated following subcutaneous administration of 10 micrograms of CCK-8s and CCK-8us administered up to 1 hour before a retention test. Administration into the nucleus accumbens (0.3 pg) however attenuated passive avoidance behaviour (Van Ree *et al*., 1983). An increase in passive avoidance latencies is indicative of enhanced memory retention, the antithesis of observations within the active avoidance paradigm.

These contrasting phenomena are complicated further by observations by Fekete *et al*., (1984) that while microinjection of CCK-8s or CCK-8us into the nucleus accumbens facilitated the extinction of active avoidance behaviour and attenuated the retention of passive avoidance behaviour, microinjection of these peptides into the central amygdaloid nucleus caused

opposite effects on these behavioural tests. The cholecystokinin tetrapeptide amide (CCK-4) injected into the lateral cerebral ventricle before the first retention test of a one-trial passive avoidance test caused a shortened latency to response. Chronic infusion into the lateral ventricle shortened the latency of the response to almost baseline level (Katsuura and Itoh 1986b). However, unilateral injection into the central nucleus of the amygdala (CeA) of CCK-8s (1 ng) or Boc-CCK-4 (20 ng), immediately post-trial, examined within a one-trial uphill avoidance task was found to improve the retention performance, whereas lower and higher doses had no effect, nor does injection performed 5 hours post-trial. This model is effectively a passive avoidance paradigm, whereby the animal placed facing downward, learns to avoid turning to face upwards for fear of shock. The intra-amygdala injections of Boc-CCK-4 and CCK-8s at these doses did not influence behaviour upon the elevated plus-maze model of anxiety nor upon the conditioned corral preference model of reinforcement (Huston *et al*., 1998). After bilateral 6-OHDA lesions to the central amygdala (Winnicka and Wisniewski 1999) and 6-OHDA lesions to the hippocampus (Winnicka and Wisniewski 2000) the facilitatory effect of CCK-8us and caerulein on retention of passive avoidance behaviour was abolished.

Interestingly while cholecystokinin octapeptides facilitated the extinction of active avoidance behaviour and retention of passive avoidance behaviour, both of which were reversed by anxiolytic chlordiazepoxide pre-treatment (Fekete *et al*., 1984).

Credence may however be added to the view that cholecystokinin exerts some effects upon memory retention for evidence from studies using an endogenous nonapeptide V-9-M (Val-Pro-Val-Glu-Ala-Val-Asp-Pro-Met). This peptide produced from procholecystokinin within the brain was observed to exert effects upon memory processes of rat performing a one-trial passive avoidance task and a platform jumping active avoidance task. The results indicate that injection of V-9-M into the lateral ventricle of the rat prevents experimental amnesia induced by electroconvulsive shock in passive avoidance testing and that it causes a long-lasting

enhancement of memory in the active avoidance task (Takashima and Itoh 1989). V-9-M may however be a distinct species from that of cholecystokinin given that their chemical structures are quite different, and thus receptors for these two peptides are probably not the same. This would certainly a wealth of differences between the two peptides both biochemically and functionally (Itoh and Takashima 1990). In addition, Itoh *et al*., (1989) observed that a single subcutaneous injection of caerulein (10-100ng.kg^{-1}) immediately after the learning trials of an active avoidance test, inhibited extinction of the acquired task by twice and at a dose of 1mg.kg^{-1} by thrice compared to the vehicle group. In the passive avoidance response, both electroconvulsive shock (ECS)-induced amnesia and scopolamine-induced amnesia was partially prevented by CCK-8 (0.1-1 μg.kg^{-1} s.c.) and attenuated in whole by caerulein (>0.1 μg.kg^{-1}).

Effects of cholecystokinin on experimentally induced Amnesia

Kadar *et al*., observed that both CCK-8s and CCK-8us appear to be able to partly attenuate amnesia when injected intracerebroventricularly into rats prior to electroconvulsive shock as measured in a one-trial step-through passive avoidance paradigm. Only the 0.8-picomole dose of CCK-8us, within a dose range, administered immediately after ECS, significantly prevent retrograde amnesia. CCK-8 administered prior to retention testing had no effects (Kadar *et al*., 1984).

Katsuura and Itoh (1986b), later observed in a passive avoidance paradigm that CCK-8 in doses from 1 ng to 1 microgram had no effect on the response when injected before the training trials, immediately after foot shock or before the first retention test. However, proglumide, a cholecystokinin receptor antagonist induced marked amnesia when injected prior to the training trials and before the first retention test, though not subsequent to foot shock. ECS given immediately after the foot shock caused amnesia in retention tests, which could be prevented by CCK-8 injected prior to the training trials, following ECS and before

the first retention test. Maurice *et al*., (1994) demonstrated caerulein (1-100 $\mu g.kg^{-1}$ s.c.) administered 30 min before carbon monoxide (CO) exposure, significantly prevented the CO-induced impairment of performance 7 days afterward using a step-down passive avoidance test. Caerulein exhibited the highest efficacy followed by CCK-8S, CCK-8us, and CCK-4 respectively. Caerulein was less effective when injected immediately after CO exposure.

Spatial Memory Paradigms

Ingestion of food in mice following training on T-maze footshock avoidance enhanced memory retention and is blocked by a specific cholecystokinin antagonist, L-364718, which in turn neither impaired or improved retention when given alone. This study provided evidence that activation of cholecystokinin receptors plays a physiological role in the mediation of meal-induced enhancement of memory retention, although perhaps only associated with spatial memory testing (Flood and Morley 1989).

Harro and Oreland (1993) examined cholecystokinin specific drugs given immediately after each training session in the radial arm maze with retention testing, drug-free, during a 2-week period. Proglumide (1-10 $mg.kg^{-1}$) and devazepide, (a selective CCK_1 receptor antagonist; 0.01-1.0 $mg.kg^{-1}$), caerulein (0.01-1 $\mu g.kg^{-1}$) and CCK-4 (a selective CCK_2 receptor agonist; 25 - 50 $\mu g.kg^{-1}$) had no reliable effects administered subcutaneously. Maurice *et al*., 1994 observed that caerulein (1-100 $\mu g.kg^{-1}$ s.c.) administered 30 min before carbon monoxide exposure, significantly prevented the CO-induced impairment of performance examined by using a Y-maze test 5 days afterward. Caerulein exhibited the highest efficacy followed by CCK-8S, CCK-8us, and then CCK-4. Caerulein was less effective when injected immediately after a single CO exposure.

Methods and Materials

Animals

Male Sprague Dawley rats (Laboratory Animal Centre, National University of Singapore, Singapore), weighing 280-350g were housed in groups of five to seven and maintained with free access to food and water *ad libitum*, in an animal room for one week prior to experimentation. Room lights were on from 07:00 to 19:00 hours. The test room and housing block were maintained at a standard temperature throughout the experiments. Animals were weighed daily at an appointed time for one week prior to experimentation and during the post acquisition period. This constituted animal handling whereby each animal was allocated the same handling style and weighing sequence. Animals were randomly assigned treatment groups using a simple computer program random number sequence generator (Random Number Generator for Q-Basic by Colin Greengrass, 1998). This simple program (see Appendix C.1 for listing) generated random whole numbers within a range corresponding to an assigned treatment group (see chapter 2 for further details).

Test Room Conditions

Animals were tested in each apparatus within a particular test room with constants of temperature at $25^{O}C$, lighting intensity, and background white noise generation (at 70dB). Animals were exposed within their home cages to the test room for two hours prior to each test session.

Drugs and Administration

Scopolamine hydrobromide (Sigma, USA) was dissolved in dimethylsulphoxide (DMSO; Merck). Cholecystokinin peptide agonists; CCK-4 (Trp-Met-Asp-Phe-NH_2); CCK-8us

(Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2; Biotechnology Centre, National University of Singapore); CCK-8s (Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; American Peptide Company and Princeton Biomolecules, USA) were dissolved in DMSO. The drugs or vehicle were administered by intraperitoneal injection in a volume of 1ml per kg body weight. Drugs were administered immediately following the avoidance acquisition sequence sessions. cholecystokinin peptide was stored under desiccation at -70^{o}C. cholecystokinin peptide solutions were prepared freshly each day approximately two hours prior to testing. Powdered peptide was dissolved in DMSO and specific concentrations prepared through serial dilution with thorough vortex mixing at each stage.

Apparatus

Shuttle Box Shock Chamber: A computer-controlled two-compartment step-through shuttlebox (San Diego Instruments, Inc.) was used. The Gemini II shuttle box is a two compartment acrylic chamber of dimensions: 53cm (width) x 53cm (height) x 32cm (depth) with a steel grid floor and a steel, computer controlled, sliding door between compartments. A scrambled sine wave electric shock is delivered through the steel cage floor. The shock intensity is adjusted manually and calculated by the following formula: y = mx + b; where y = shock intensity (mV); m = slope (0.004); x = animal weight (g); and b = y-axis intercept (- 0.4) therefore an animal weighing 350g, receives a shock intensity of (350 x 0.004) - 0.4 = 1.0 mV. This calculation allows a pseudo dosing effect of shock intensity with regard to animal weight. Gemini II operational program coding for these series of experiments is listed in appendix C.

Experimental Procedure

Experiment One: Passive Avoidance Paradigm

Acquisition Session: Tests were performed whereby an animal was placed in a lighted compartment (60 Watt bulb) for a 10- second adaptation period. After this period, a gate

separating the two compartments was opened thus allowing entry into the adjacent unlit compartment. Rats tend to exhibit an innate affinity for darkened areas over lit areas. Hence rats will have a propensity to enter the adjacent compartment. Upon entry the animal breaks an infrared light beam triggering the door to close automatically. After the gate closed a scrambled electric current was applied to the cage floor (see aforementioned formula). This constituted the end of a trial. Each individual trial session was held for a maximum duration of 60 seconds. Subsequent to each trial the animal was placed back into a holding cage for a period of 30 seconds. The animal was then retested in consecutive trials until they achieved the criterion necessary for completion of the trial, with a maximum of 15 trials. A definition of this criterion is that the animal remained in the lighted compartment for three consecutive 60-second trials. Once this criterion is satisfied the sequence of trials ends for that animal. Animals failing to satisfy this criterion were rejected. Animals were administered, either; DMSO, Scopolamine Hydrobromide ($0.3mg.kg^{-1}$), CCK-4 (0.0125 or $0.025mg.kg^{-1}$), CCK-8us (0.005 to $0.01mg.kg^{-1}$), or CCK-8s (0.0005 to $0.001mg.kg^{-1}$) either singularly or in combination, immediately subsequent to the last acquisition trial session.

Retention Trial: A retention test was performed 72 hours later where the animal was placed in the lit "safe" compartment. Immediately following an manual operation of an external button, the gate separating the compartments was opened. The door remained open for 300 seconds (5 minutes). The latency period, for which the animal to cross over to the shock compartment, was measured. The animals post session were placed in a holding cage in order to isolate them from the other animals prior to experimentation. Data is presented as number of trials fulfilling criterion (failure to traverse compartments within a 60 second period) in acquisition training and the crossing latency in retention testing (time taken to cross compartments).

Experiment Two: Active Avoidance Paradigm

Acquisition Session - Initiation Trial: with bright house lighting (60 watt bulb) on only in the

"safe" compartment and constant white noise within both chambers, the animal was placed in the unlit shock compartment (facing away from the door), and left for a ten second period. After this period, the gate separating the compartments was automatically opened. Simultaneously a scrambled sine wave shock was delivered to the steel cage floor for a period of 30 seconds (shock intensity in accordance with the aforementioned formula).

Foot-shock continued until the animal escaped through the open doorway. If the animal failed to escape during the shock period then it was gently actuated through. The animal subsequently remained in the lighted "safe" compartment for 30 seconds (defined as an inter-trial interval) before immediate removal and placement back into the unlit "shock" compartment.

The second and subsequent trials measure successful active avoidances (pre-shock period escape). The methodological differences between these and the initiation trial are necessary to prevent exploration driven crossing within the ten second pre-shock period, and consequently ensuring that shock is the primary motivation underlying crossing behaviour. The lighting arrangement is organised as such in order to induce an aversion to the safe compartment. Rodents exhibit aversive behaviour with regard to lit areas and prefer dimly lit or darkened chambers. In this regard the animal will be reluctant to explore the lit "safe" chamber but will be forced into entry by shock application within the unlit area. This innate aversion to lighted areas is thus utilised so that transfer to the safe compartment is driven by shock application alone and not through any other predilection.

Acquisition Session - Avoidance Trials: The animal was placed in the unlit shock compartment where immediately proceeding manual operation of an external push button, the door separating the compartments was opened. After a period of 10 seconds a scrambled sine wave shock was delivered to the cage floor for a period of 30 seconds. Shock intensity was determined as of the abovementioned formula. The footshock continued until the animal

escaped through the open doorway. If the animal failed to escape through the open doorway during the shock period then it was gently actuated through. If the animal was able to escape through the doorway within the 10-second period prior to the shock this fulfilled the criterion for avoidance. Immediately following entry into the "safe" compartment the inter-trial interval was initiated. The animal remained in the "safe" compartment for 30 seconds before placement back into the "shock" compartment. This active avoidance trial procedure was repeated fifteen times in succession.

After the final acquisition session, the animal was administered with a specified pharmacological agent and was placed in a holding cage with other animals that had been tested. The group housing of tested and naïve animals together is not permitted due to a heightened aggressive state of shocked animals. The percentage of acquisition avoidances for each animal must be above 33% (5 in 15 trials) in order for the animal to be retested. Several animals, around five percent, who failed to satisfy this criterion were not retested in retention tests and their data is not shown. Animals were administered, either DMSO, Scopolamine Hydrobromide ($0.3mg.kg^{-1}$), CCK-4 (0.0125 to $0.1mg.kg^{-1}$), CCK-8us (0.001 to $0.1mg.kg^{-1}$), or CCK-8s (0.0005 to $0.01mg.kg^{-1}$), immediately subsequent to the last acquisition trial session. Only when all the animals within a home cage had been tested were they placed back into their home cages and immediately returned to the animal-housing block.

Retention Test: A retention test was performed 72 hours later where the animal was placed in the unlit shock compartment where immediately following an external button operation the door separating the compartments was opened. The door remained open for 300 seconds (5 minutes) without application of the shock. The latency period, for which the animal to cross over to the safe compartment, was measured. Subsequent to crossing the "re-cross" latency, with a maximum 300 seconds, was measured (whereby an animal crosses back into the "shock" compartment). If the animal failed to traverse through the doorway during first the five-minute period then it was gently actuated through. The animals post session were placed

in a holding cage in order to isolate them from the other animals prior to experimentation.

Statistical Analysis

Statistical analysis was performed using statistical software (SPSS V9.0, SPSS inc. USA). The data were subjected to a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc analysis of group differences. Probability ($p < 0.05$) was considered statistically significant.

Results and Data

Passive Avoidance Paradigm

No differences were observed between means of trials to criterion in nondrugged rats within the one-way step through passive avoidance paradigm. The criteria are satisfied where within three sixty second sessions, held in succession, the animal fails to cross into the unlit shock compartment. The trial number at which the last of these consecutive sessions occurs is noted and this variable is analysed. No group differences were observed in nondrugged sessions between groups administered drugs post-trial. Administration of scopolamine hydrobromide (0.3 $mg.kg^{-1}$ i.p.) significantly shortened retention trial crossing-latency compared to the dimethylsulphoxide (DMSO) vehicle group (associated with amnestic activity; Figure 5.1); scopolamine hydrobromide; $0.3mg.kg^{-1}$, ($F_{13,98}=12.83$, $p<0.05$). Cholecystokinin tetrapeptide at $0.025mg.kg^{-1}$ (CCK-4) narrowly missed significance with an increase in crossing latency ($F_{13,98}=12.83$, $p<0.073$). Cholecystokinin sulphated octapeptide at $0.001mg.kg^{-1}$ (CCK-8s) alone and in combination with cholecystokinin unsulphated octapeptide (CCK-8us; $0.01mg.kg^{-1}$) produced a significant increase in crossing latency (Both $F_{13,98}=12.83$, $p<0.05$), associated with enhanced memory retention.

Active Avoidance Paradigm

Within studies of CCK-8us, CCK-8s or CCK-4 dose ranges, no significant differences were observed in acquisition sessions, between groupings pre-drug administration for mean number of avoidances This indicates an equivalent acquisition profile across all groups.

Figure 5.2.1: Significant inhibition of crossing latency, indicative of amnesia, at the active avoidance retention test was observed for scopolamine hydrobromide at $0.3mg.kg^{-1}$ ($F_{6,49}=8.84$, $p<0.001$), and for CCK-8us at doses $0.005mg.kg^{-1}$ ($F_{6,49}=8.84$, $p<0.001$); $0.01mg.kg^{-1}$ ($F_{6,49}=8.84$, $p<0.001$); $0.05mg.kg^{-1}$ ($F_{6,49}=8.84$, $p<0.01$); $0.1mg.kg^{-1}$ ($F_{6,49}=8.84$, $p<0.001$). The

lowest dose of 0.001mg.kg^{-1} failed to show any differences from the DMSO vehicle group.

Figure 5.2.2: Re-crossing latency was found to also be enhanced by scopolamine, indicative again of amnesia ($F_{6,49}$=2.75, $p<0.05$), while CCK-8us doses failed to alter re-crossing latency (implying lack of amnestic properties).

Figure 5.3.1: Significant inhibition of crossing latency, indicative of amnesia, at the active avoidance retention test was observed for CCK-8s at doses 0.001mg.kg^{-1} ($F_{4,35}$=31.57, $p<0.001$); 0.005mg.kg^{-1} ($F_{4,35}$=31.57, $p<0.001$); 0.01mg.kg^{-1} ($F_{4,35}$=31.57, $p<0.001$). The lowest dose of 0.0005mg.kg^{-1} failed to show any differences from the DMSO vehicle group.

Figure 5.3.2: CCK-8s doses failed to alter re-crossing latency (implying lack of amnestic properties).

Figure 5.4.1: Significant inhibition of crossing latency, indicative of amnesia, at the active avoidance retention test was observed for CCK-4 at doses 0.0125mg.kg^{-1} ($F_{4,35}$=6.74, $p<0.05$); 0.025mg.kg^{-1} ($F_{4,35}$=6.74, $p<0.01$); 0.05mg.kg^{-1} ($F_{4,35}$=6.74, $p<0.05$); 0.1mg.kg^{-1} ($F_{4,35}$=6.74, $p<0.05$) .

Figure 5.4.1: CCK-4 doses failed to alter re-crossing latency (implying lack of amnestic properties).

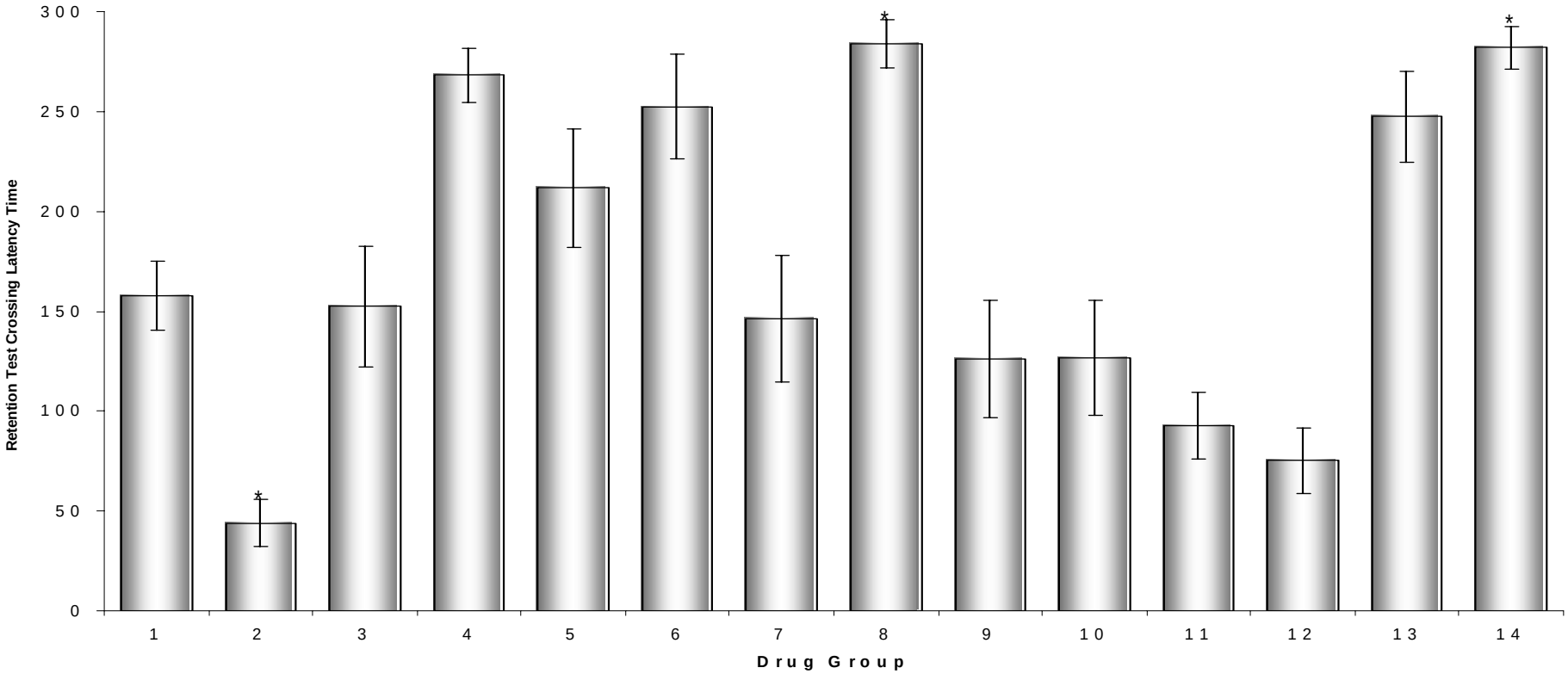


FIG 5.1: Means of crossing latency at passive avoidance retention test. Drug administration groups are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. Drugs tested are scopolamine hydrobromide at 0.3 $mg.kg^{-1}$ (2), cholecystokinin tetrapeptide (CCK-4) at 0.0125 (3) & 0.025$mg.kg^{-1}$ (4); cholecystokinin octapeptide (CCK-8us) at 0.005 (5) & 0.01$mg.kg^{-1}$ (6); and cholecystokinin sulphated octapeptide (CCK-8s) at 0.0005 (7) & 0.001$mg.kg^{-1}$ (8) are dissolved in dimethylsulphoxide (DMSO) vehicle (1). Drugs are administered both alone and in combinations; CCK-4 at 0.0125mg/g with CCK-8us at 0.005$mg.kg^{-1}$ (9); CCK-4 at 0.025mg/g with CCK-8us at 0.01$mg.kg^{-1}$ (10); CCK-4 at 0.0125mg/g with CCK-8s at 0.0005$mg.kg^{-1}$ (11); CCK-4 at 0.025mg/g with CCK-8us at 0.01$mg.kg^{-1}$ (12); CCK-8us at 0.005mg/g with CCK-8s at 0.0005$mg.kg^{-1}$ (13); CCK-8us at 0.01mg/g with CCK-8s at 0.001$mg.kg^{-1}$ (14). Results are presented as means with light bars representing standard error of the mean (SEM). Significant differences from the DMSO pre-trial one group (1) are shown; *$p<0.05$, (Bonferroni post hoc analysis after significant one-way ANOVA).

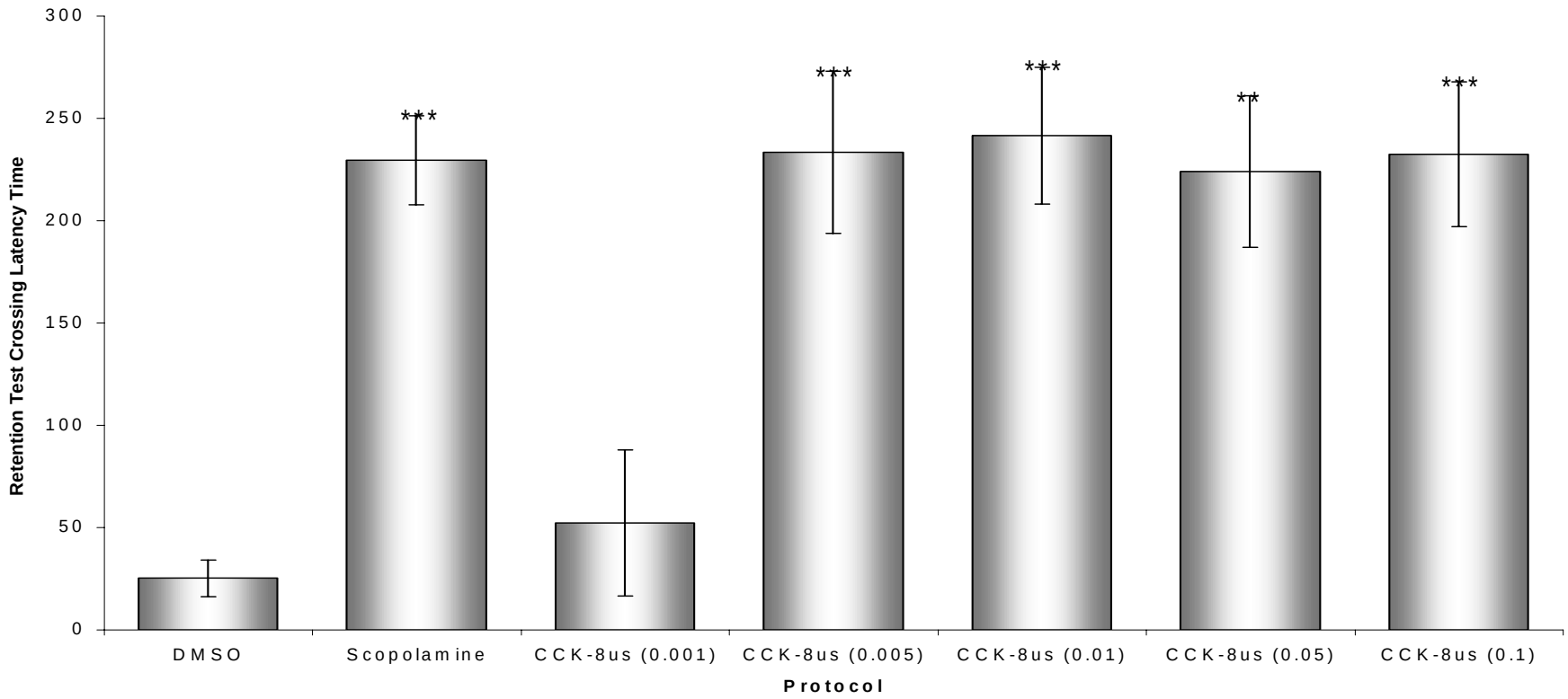


FIG 5.2.1: Means of crossing latency at active avoidance retention test. Drugs administrated are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. Drugs tested are Scopolamine Hydrobromide (0.3 $mg.kg^{-1}$), and cholecystokinin octapeptide at doses between 0.001 and 0.1$mg.kg^{-1}$ (CCK-8us) and are dissolved in dimethylsulphoxide (DMSO) vehicle. Results are presented as means with light bars representing standard error of the mean (SEM). Significant differences from the DMSO group are shown; **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

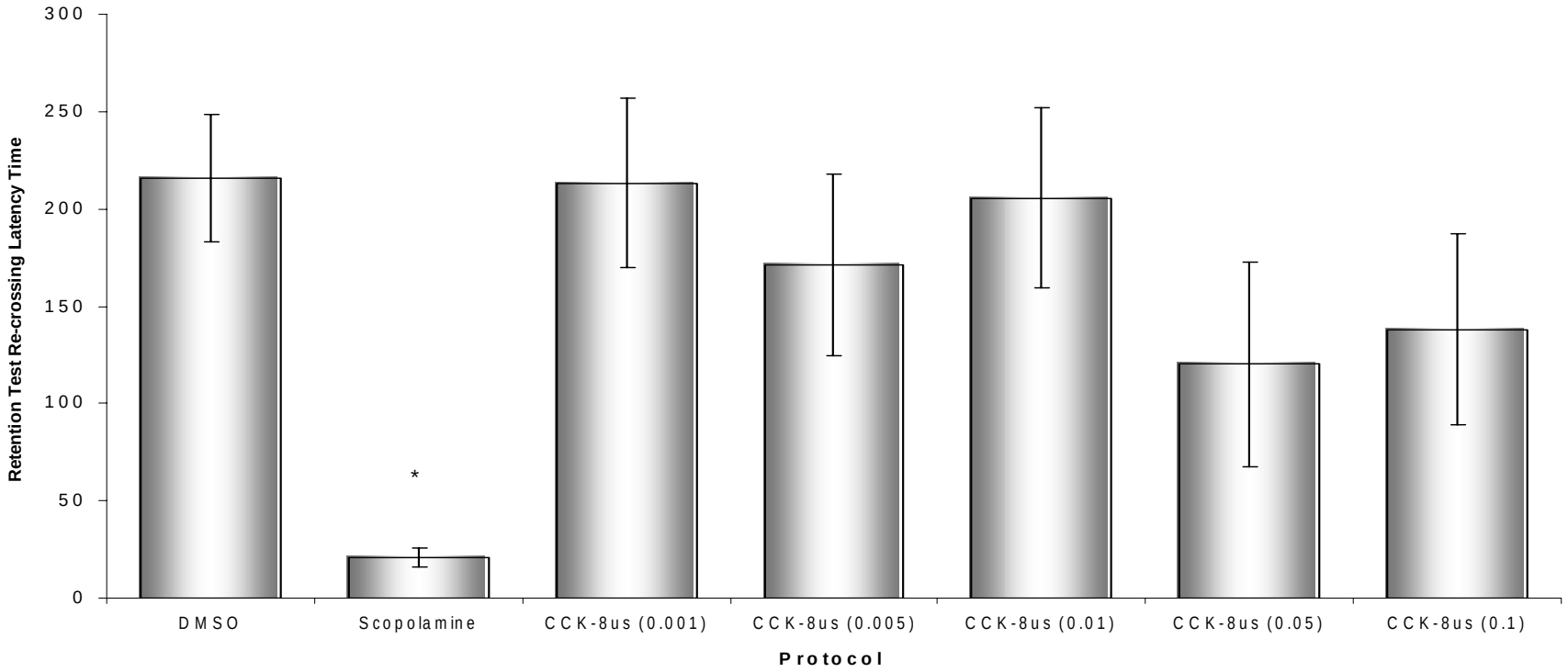


FIG 5.2.2: Means of re-crossing latency at active avoidance retention test whereby animals are initially placed in the non-shock compartment. Drugs administrated are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. Drugs tested are scopolamine hydrobromide (0.3 $mg.kg^{-1}$), and cholecystokinin octapeptide at doses between 0.001 and 0.1$mg.kg^{-1}$ (CCK-8us) and are dissolved in dimethylsulphoxide (DMSO) vehicle. Results are presented as means with light bars representing standard error of the mean (SEM). Significant differences from the DMSO group are shown; *$p<0.05$, (Bonferroni post hoc analysis after significant one-way ANOVA).

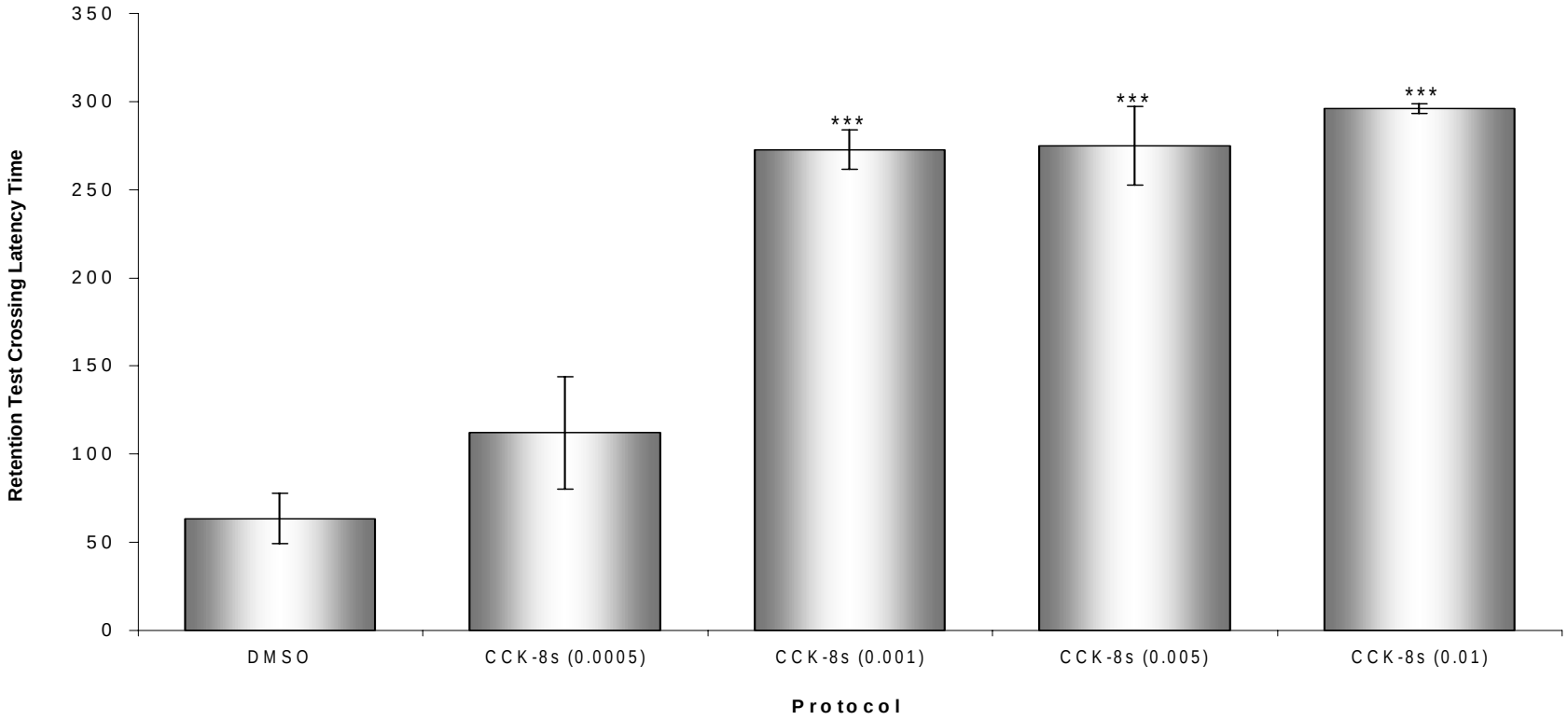


FIG 5.3.1: Means of crossing latency at active avoidance retention test. Drugs administrated are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. A range of doses of cholecystokinin sulphated octapeptide at doses between 0.0005 and 0.01$mg.kg^{-1}$ (CCK-8s) and are dissolved in dimethylsulphoxide (DMSO) vehicle. Results are presented as means with light bars representing standard error of the mean (SEM). Significant differences from the DMSO pre-trial one group (1) are shown; ***$p<0.001$ (Bonferroni post hoc analysis after significant one-way ANOVA).

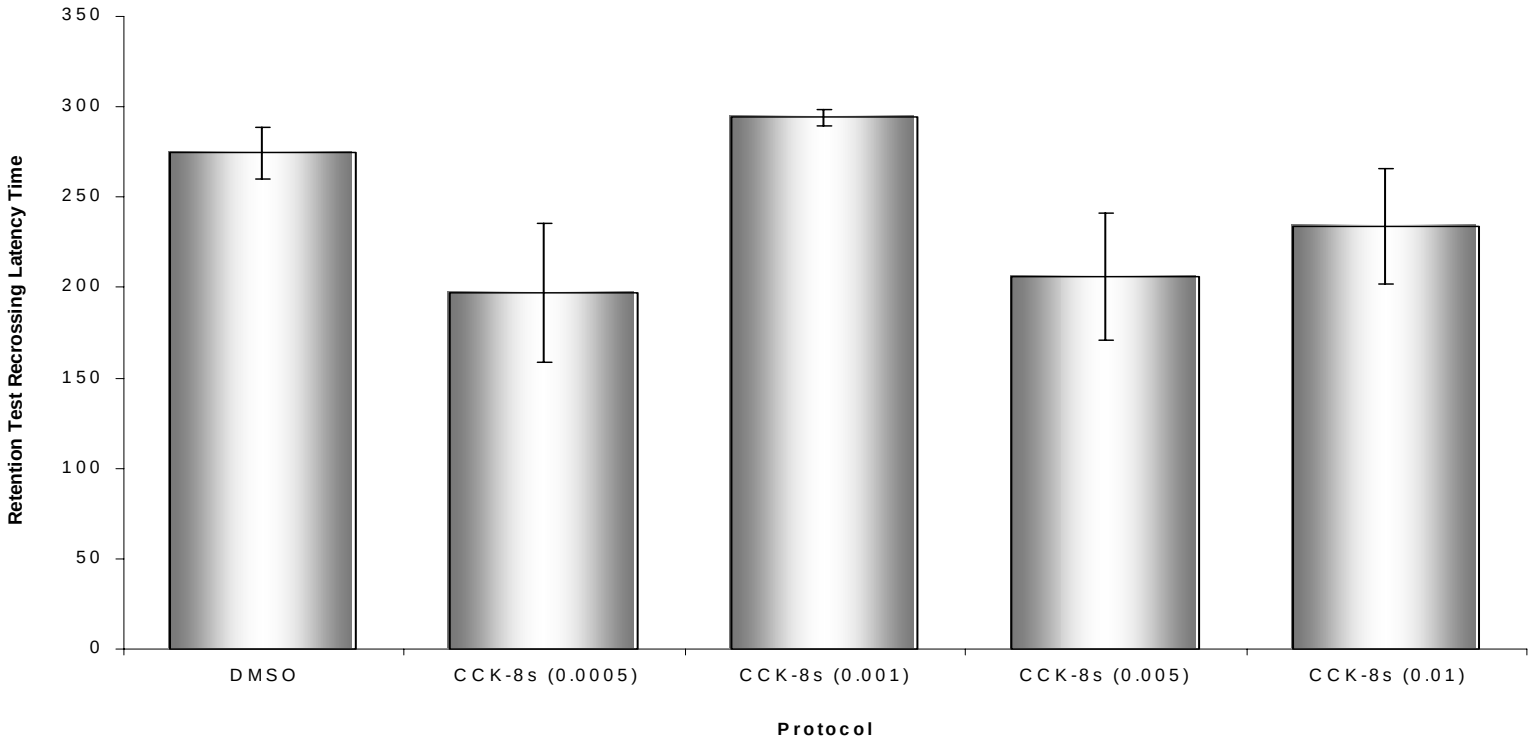


FIG 5.3.2: Means of re-crossing latency at active avoidance retention test whereby animals are initially placed in the non-shock compartment. Drugs administrated are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. A range of doses of cholecystokinin sulphated octapeptide at doses between 0.0005 and $0.01mg.kg^{-1}$ (CCK-8s) and are dissolved in dimethylsulphoxide (DMSO) vehicle. Results are presented as means with light bars representing standard error of the mean (SEM). No significant differences from the DMSO group are shown (Bonferroni post hoc analysis after significant one-way ANOVA).

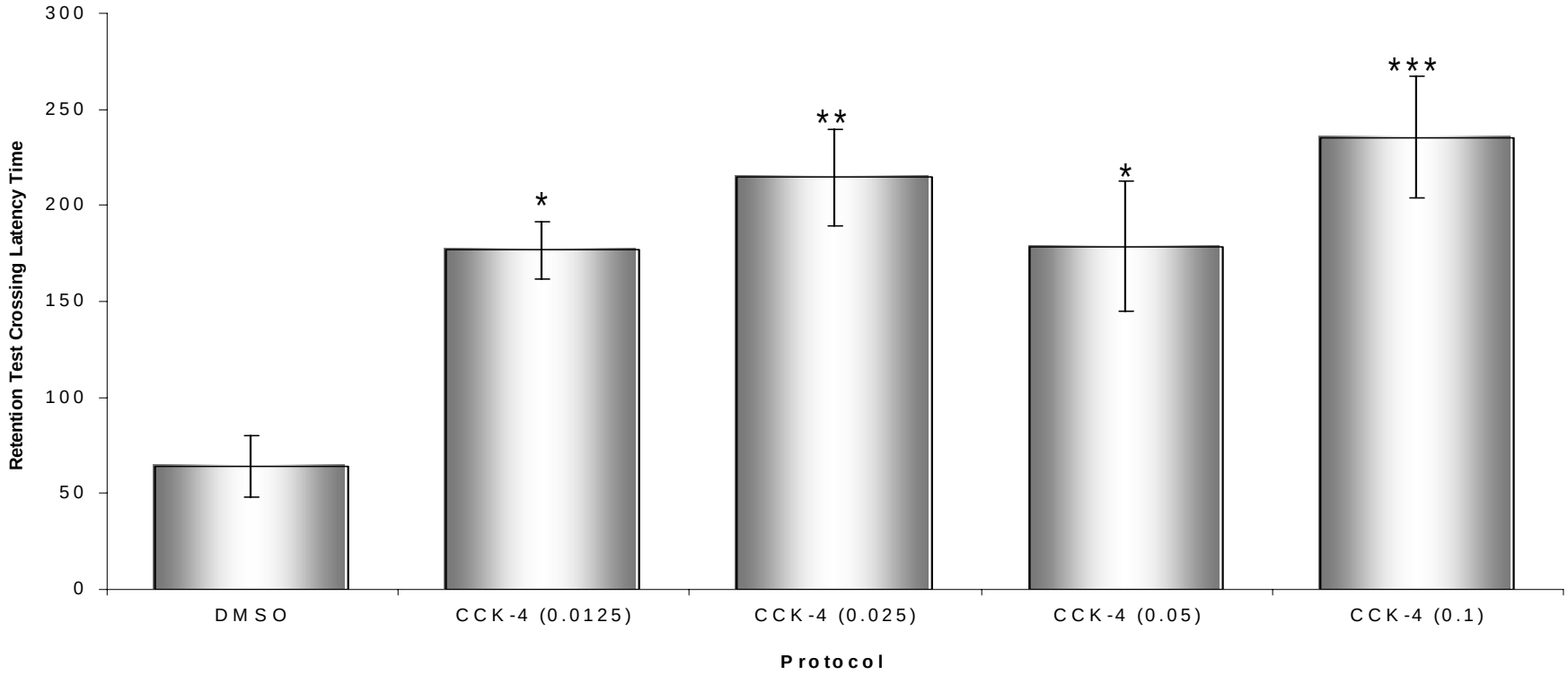


FIG 5.4.1: Means of crossing latency at active avoidance retention test. Drugs administrated are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. A range of doses of cholecystokinin tetrapeptide at doses between 0.0125 and $0.1mg.kg^{-1}$ (CCK-4) and are dissolved in dimethylsulphoxide (DMSO) vehicle. Results are presented as means with light bars representing standard error of the mean (SEM). Significant differences from the DMSO group are shown; *p<0.05, **p<0.01, ***p<0.001 (Bonferroni post hoc analysis after significant one-way ANOVA).

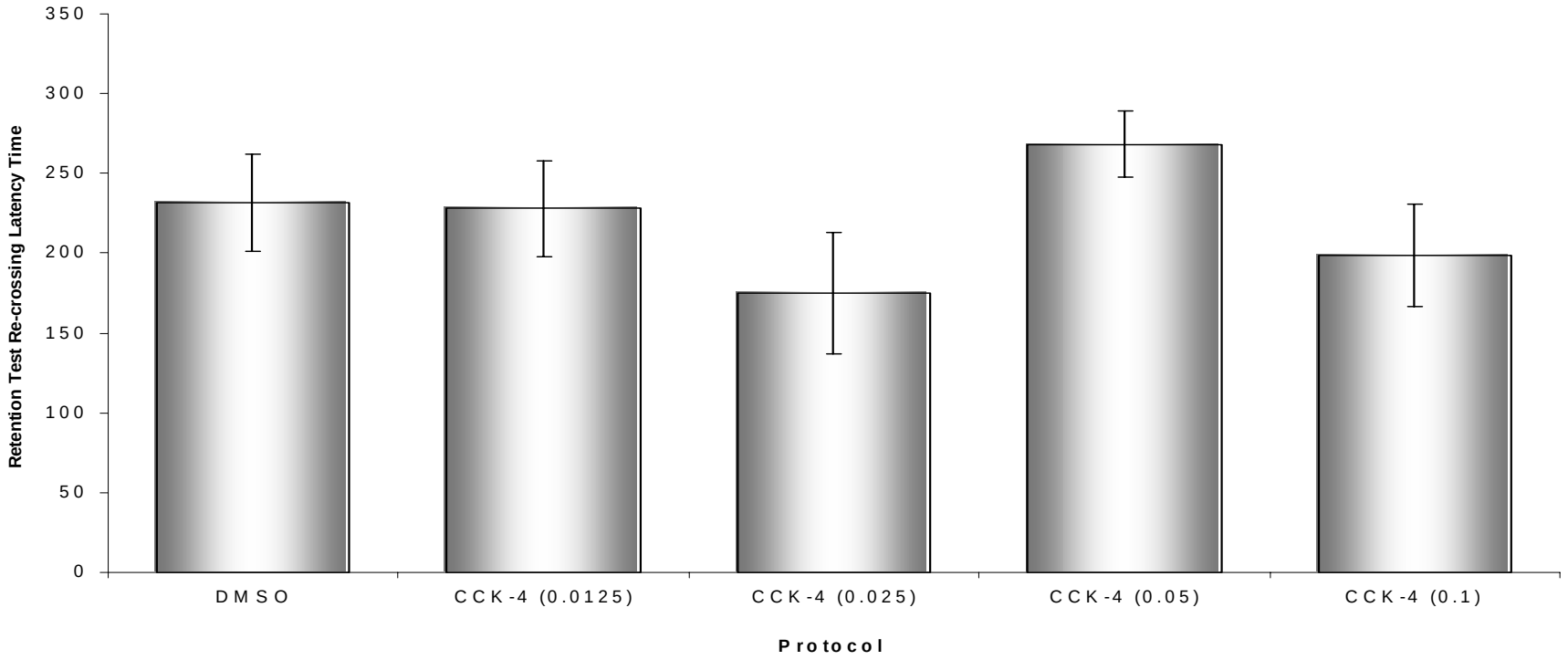


FIG 5.4.2: Means of re-crossing latency at active avoidance retention test. Drugs administrated are labelled on the x-axis; doses in $mg.kg^{-1}$ within parentheses. A range of doses of cholecystokinin tetrapeptide at doses between 0.0125 and $0.1mg.kg^{-1}$ (CCK-4) and are dissolved in dimethylsulphoxide (DMSO) vehicle. Results are presented as means with light bars representing standard error of the mean (SEM). No significant differences from the DMSO group are shown (Bonferroni post hoc analysis after significant one-way ANOVA).

Discussion

It was demonstrated in initial studies using a passive avoidance paradigm that doses corresponding to those exhibiting anxiogenic potency in the elevated plus-maze are able to induce an increased crossing latency. This increase might be interpreted as improved memory retention in isolation. Together with matching data for plus-maze anxiogenesis (chapter 3) at these doses, this supposition appears somewhat contradictory when tied in with improved memory retention. It is quite possible that the anxiogenic state produced by these doses of cholecystokinin may in turn confer a state of heightened awareness with post-trial administration. This heightened sentience could therefore be implicit in strengthening consolidatory processes involved. This experiment may have therein joined the large number of studies implying a role of cholecystokinin in memory consolidatory processes, but for an observation that these animals exhibiting significantly lengthened latencies were prone to almost total inactivity when placed within the apparatus. This freezing behaviour is indicative of a profound anxiety state and would concur strongly with anxiogenic-like effects upon the plus-maze of these same cholecystokinin doses. The administration of drugs immediately after the training session block was designed to avoid behavioural state effects upon the learning processes and provide a view of activities upon internal memory consolidative processes. However, a question arose as to whether this post-trial administration would be able to, in the place of pro-mnestic activity, actually augment the conditioning of fear surrounding the shock chamber experiences. This would cause the same behavioural characteristics within a passive avoidance paradigm as enhanced memory retention, (i.e. an increased crossing latency). Scopolamine as per expectation was able to produce a significant level of amnesia with the retention session. Significance of combinations of cholecystokinin forms is discussed primarily within chapter three, with regard to anxiety state on the elevated plus-maze. The combination effects appear to imply a duality regarding mechanisms of cholecystokinin anxiogenesis. These also confirm findings of Katsuura and Itoh (1986b) that CCK-8 is able to antagonise the

effects of CCK-4.

Subsequent to the observation of freezing behaviour within passive avoidance experiments, a minor, yet apparently novel, variation of the active avoidance paradigm was developed whereby, in addition to measurement of crossing latency, a measure of re-crossing latency was taken. Pro-mnestic activity would be demonstrated within the active avoidance paradigm retention trial as a decrease in latency (whereby an animal has learned to traverse compartments in order to avoid a shock). However, dose response profiles for each cholecystokinin species examined showed an amnestic activity within an active avoidance paradigm. This observation was previously made by Fekete *et al*., where CCK-8s and CCK-8us following peripheral administration (Fekete *et al*., 1982b), were able to considerably impair acquisition of active avoidance behaviour compared to the control. In addition, Fekete *et al*., (1982b), observed that intraperitoneal administration of CCK-8s and CCK-8us increased the latency of passive avoidance in each treatment group. These two seemingly incompatible observations were explained by a proposal of duality of cholecystokinin activity within the two paradigms. These observations, which I have repeated in this study, can be explained simply by an induction of freezing behaviour underlying increased latencies in both paradigms. Furthermore, the measure of recrossing latency within the active avoidance paradigm adds credence to this proposition. An animal exhibiting amnesia with regard to avoidance of the shock chamber, as is proposed with single measurement of crossing latency, will then upon placement in the non-shock compartment re-cross back into the shock compartment (given the strong lighting within the safe compartment). This is demonstrated significantly by scopolamine, but is lacking in the cholecystokinin administered groups. Effectively, the animals administered cholecystokinin neither cross nor recross within the chamber, which are two wholly contradictory behaviours, unless explained by induction of freezing behaviour. This observation is very important in that since 1981, a role for cholecystokinin within memory consolidation processing has been proposed. This supposition maintained throughout

almost twenty years of work, appears to be negated by this series of experiments. Table 5.1 summarises supporting research.

Drugs Tested	Route	Effect on Avoidance Retention Latency	Reference
CCK-8s & CCK-8us	ICV	Increased Active Latency	Bernard *et al*., 2000
CCK-8s & CCK-8us	SC	Increased Active Latency	Fekete *et al*., 1982b
CCK-8s	IP	Increased Active Latency	Camello *et al*., 2000
CCK-8s & CCK-8us	ICV	Increased Passive Latency	Yamamoto *et al*., 2000
CCK-8s & CCK-8us	IP	Increased Passive Latency	Rozengurt and Walsh, 2001
CCK-4, -5, -6, -7, -8	ICV & IP	Increased Passive Latency	Schweiger *et al*., 2000
CCK-4	IP	Increased Passive Latency	Aguilera *et al*., 2000

Table 5.1; Summary of previous research into cholecystokinin effects upon one-way avoidance paradigms

Intracerebroventricular administration of cholecystokinin-8 antisera is able to delay the extinction of active avoidance while ineffective on acquisition and maintenance (Fekete *et al*., 1982a). The purported delay in extinction is an antithesis of the activity of cholecystokinin ligands (Fekete *et al*., 1984) which is as expected. Cholecystokinin ligands are able to promote extinction probably via an increase in anxiety exhibited within the model. This increase again produces freezing, or a similar locomotor inhibition, thus interpreted prima facie as "promoting extinction".

The study of animals receiving chronic daily administration of CCK-8s post two way active avoidance training demonstrated that CCK-8s pre-trial, failed to depress avoidance responding as with those given "chronic saline" (Cohen *et al*., 1983). The absence of activity is undoubtedly due to development of cholecystokinin drug tolerance over chronic administration, but contrary to the conclusions of the authors, tolerance to the anxiogenic properties of cholecystokinin is more probable.

Fekete *et al*., (1984) overlooked their own finding that the effects of CCK-8 upon passive avoidance, were reversed by chlordiazepoxide pre-treatment. This strongly supports my hypothesis. Chlordiazepoxide is of course a well-defined benzodiazepine anxiolytic and

would undoubtedly reverse the anxiogenic properties of CCK.

Katsuura and Itoh (1986b) demonstrated that CCK-4 injected into the lateral cerebral ventricle prior to the retention test caused a shortened latency in a one-trial passive avoidance paradigm. This is not in contradiction to my own and the findings of Fekete *et al*., (1984) where central ventricular administration must surely be highly sensitive to the site of injection with regard to adjacent brain structures. Cholecystokinin with an almost ubiquitous distribution within the brain, would almost certainly activate an area adjacent to the site of injection. Subsequently, intracerebroventricular administration would appear to be a rather haphazard method by which to deliver cholecystokinin forms to the brain. The shortcomings of this methodology are further confirmed by Fekete *et al*., (1984) where microinjection of CCK-8s or CCK-8us into the nucleus accumbens facilitated the extinction of active avoidance behaviour and attenuated the retention of passive avoidance behaviour, while microinjection of these peptides into the central amygdaloid nucleus caused opposite effects on these behavioural tests.

The observations of Winnicka and Wisniewski (2000) that bilateral 6-OHDA lesions to the hippocampus significantly attenuate the "facilitatory effect" of CCK-8us and caerulein on retention of passive avoidance behaviour, may provide evidence of the central structures involved in development of the proposed conditioned fear.

Several studies have highlighted a possible role of cholecystokinin in restoring deficits conferred by experimentally induced amnesia. Kadar *et al*., (1984) observed that a 0.8 picomole dose of CCK-8s (within a range of doses tested), administered pre-retention trial was found to significantly restore passive avoidance retention. This is of course also explained by a freezing behaviour hypothesis.

Katsuura and Itoh (1986a) also studied the effects of electroconvulsive shock (ECS) upon passive avoidance latencies. CCK-8 injected prior to the training trials, following ECS and before the first retention test all "prevented" ECS derived amnesia. Yet again this can be

explained by cholecystokinin anxiogenesis, whereby development of conditioned fear may not by disrupted by ECS, and thus anxiogenic factors will again be involved in retention sessions. However, CCK-8 may provide some protection against ECS (administered pre-ECS) in addition to anxiogenic effects pre-retention trial and post-ECS. It is plausible however that CCK-8 if it indeed does gate endogenous anxiety responses within the brain, that it might exhibit some protective properties. Given that normal reactive anxiety is driven by perceived danger, cholecystokinin might exert a protective function within the brain. This is of course conjecture but perhaps worthy of investigation.

Several studies have implicated activation of cholecystokinin receptors within the mediation of meal-induced enhancement of memory retention in spatial memory tasks (Flood and Morley 1989). This form of memory retention is quite different to that of shuttle box shock avoidance and given that cholecystokinin is implicated in satiation, it is not surprising that it would exert effects upon appetitive tasks.

In strong support of my proposal is a study by Uvnas-Moberg *et al*., (1999) where examination of the endocrine and behavioural profile of two colonies of rats differing in their ability to acquire a conditioned avoidance response was performed. The poor avoidance performing animals, interestingly, were characterized by higher reactivity to sensory stimulation: higher startle amplitude and shorter startle latency, strongly implying a greater anxiety level.

The nature of freezing behaviour with subsequent exposure to the shock chamber may lie within the following proposition. It may be possible that cholecystokinin administered post-acquisition session produces an additional noxious state, given that it has been observed to produce an anxiety-like state (see chapters two and three), to that induced by shock. This state may augment conditioning of fear related to the environment in which shock was administered, rather like manipulations within conditioned place preference models. The addition of a further noxious element associated with the shock compartment may then

augment the intensity of the anxious state exhibited within the retention session exposure, resulting in this freezing-like behaviour. If cholecystokinin was to possess an activity upon memory consolidative processes directly it would undoubtedly be upon those associated with conditioning of fear and not of higher order associative function. Thus, the heightened anxiety state observed within retention tests is in all probability induced by enhanced memory acquisition associated with fear conditioning.

Conclusion

It is shown by various studies that the cholecystokinin forms examined may indeed exhibit ostensibly mnestic properties within avoidance paradigms. However, analysis of related variables has provided strong evidence of an anxiety-based factor underlying these observations. With simple analysis of an initial traversing latency in either passive or active avoidance paradigms, it would appear prima facie that these cholecystokinin forms either improve memory retention in the passive avoidance paradigm (by increasing latency) and exhibit amnestic properties within the active avoidance paradigm (again by increasing latency). These phenomena were adjudged incongruous to one another where considering activities upon memory retention as the underlying cause. The experimental findings herewith provide evidence for involvement of an anxiety-based factor in behaviour observed within the avoidance paradigm.

CHAPTER SIX - RECEPTOR BINDING STUDIES

Introduction

Cholecystokinin has a ubiquitous distribution throughout the mammalian brain (Rehfeld 1978; Williams *et al*., 1986). All known neuropeptide receptors conform to a G protein coupled superfamily, which is characterised by a heptahelical structure (seven transmembrane spanning regions). Studies by Snyder *et al*., (1981) were the first to characterise two distinct receptors for cholecystokinin. Their study demonstrated that "the pancreatic receptor does not react with cholecystokinin derivatives of fewer than eight amino acids; i.e. CCK-8 and larger, while the brain receptor was unable to recognize pentagastrin, the carboxyl-terminal five amino acids of CCK, but interacts with CCK-4, the carboxyl-terminal tetrapeptide of CCK". This study subsequently has been shown to exhibit several inaccuracies, but nevertheless was the first to show evidence of broad differences between receptor populations both peripherally and centrally. Van Dijk *et al*., (1984) showed that binding sites for the cholecystokinin sulphated octapeptide form (CCK-8s) are also distributed throughout the brain. Central receptor subtypes have since been differentiated, on the basis of differing affinities of a range of specific ligands, into CCK_1 and CCK_2 (Moran *et al*., 1986; Chang *et al*., 1989; Hill and Woodruff, 1990; Hunter *et al*., 1993). Formerly known as CCK-A and CCK-B, cholecystokinin receptors have undergone a recent revision in nomenclature to CCK_1 and CCK_2 respectively (Noble *et al*., 1999). CCK_1 receptors are located in highest concentrations in the pancreas, gall bladder and in discrete regions of the brain (Hill *et al*., 1987) whereas CCK_2 receptors exhibit an almost ubiquitous distribution throughout the brain. The sulphated cholecystokinin octapeptide, CCK-8s exhibits far greater affinity than the non-sulphated CCK-8us and the tetrapeptide CCK-4, for the CCK_1 receptor. The major forms of cholecystokinin, CCK-4, pentagastrin, CCK-8us and CCK-8s however, all exhibit similar affinities for CCK_2 receptors (Noble *et al*., 1999).

Neuropeptide receptor antagonists can be divided generally into two main classifications, either peptide or non-peptide. The first non-peptide antagonist to be discovered was asperlicin in 1986. This substance is a naturally occurring benzodiazepine isolated from the fungus *Aspergillus alliaceus*. Asperlicin was found to be a highly specific CCK_1 receptor antagonist void of the bioavailability problems usually encountered with peptide antagonists. Many non-peptide cholecystokinin antagonists have been since synthesised. Specific non-peptide CCK_1 antagonists such as L-364718 and MK-329 bind with a high affinity, whilst demonstrating negligible binding at the CCK_2 subtype (Hill *et al*., 1987). Specific synthetic non-peptide antagonists such as CI-988, PD-140376 and L-365260 also exhibit a high affinity for CCK_2 receptor subtypes; (Hill *et al*., 1992).

Evidence for CCK_2 Receptor Subtypes

Non-peptide cholecystokinin antagonists are chemically quite distinct from the endogenous peptide cholecystokinin forms. Until relatively recently, the mechanism by which these antagonists exert their effects remained an enigma. However, radioligand binding studies combined with mutational analysis of cholecystokinin receptors has revealed the existence of distinct binding domains for peptide agonists and non-peptide antagonists (Beinborn *et al*., 1993). These techniques have shown that most peptide agonists interact at sites within the extracellular region of the receptor, particularly at the NH_2 terminal strand region. Non-peptide antagonists, however, appear to interact with sites within the transmembrane region. Hence these binding sites are quite distinct and do not overlap. In a study by Schwartz *et al*., (1995), it was proposed that cholecystokinin receptors, like that of other neuropeptide receptors, follow an allosteric model. This model purports that specific ligands exhibit effects that shift a receptor between either an active or inactive conformational state. Thus, it was proposed tentatively that non-peptide antagonists exert their effects by shifting the equilibrium away from the active state. The mechanism by which this activation / inactivation is conferred is

probably via a coupling / de-coupling respectively of the G protein.

Evidence for presence of multiple binding sites attributed to the CCK_2 receptor in the brain arose from several competition experiments performed with selective linear or cyclic CCK-8 analogues (Durieux *et al*., 1986; Knapp *et al*., 1990; Rodriguez *et al*., 1990). Derrien *et al*., (1994) characterised binding of a peptide based CCK_2 agonist [^{3}H]pBC-264 in brain membranes of guinea pig, mouse, and rat. The receptor binding profile of [^{3}H]pBC-264 in competition with another specific CCK_2 agonist, BC-197, was observed to adhere significantly more closely to a two site model than to that of a one site model. This was not observed with unlabelled BC-264 at the concentrations studied, which adhered more closely to a one-site occupancy model. This suggested that BC-197 exhibited differing affinities for putative high and low affinity sites, whereas BC-264 exhibits a very similar affinity for both subsites which cannot be distinguished at these concentrations (Derrien *et al*., 1994).

Further evidence for this mechanism was provided by Harper *et al*., (1996) with analysis of competition profiles obtained by the CCK_2 "antagonist" L-365260 and [^{125}I]-BH-CCK-8s in guinea-pig gastric gland, and mouse and rat cerebral cortex preparations. Competition curves obtained for radiolabelled L-365260 in a mouse cortex assay demonstrated close adherence to a one-site model analysis, thus implying presence of a single population of binding sites. This confirmed an early study using radiolabelled pentagastrin and CCK-8 probes which bound with high affinity to the mouse cerebral cortical suggesting the absence of more than one binding site and the lack of cooperativity of cholecystokinin receptor binding (Clark *et al*., 1986). However, Harper *et al*., (1996) showed that within the rat cortex and in guinea-pig gastric gland assays, the mean slope of the competition curves was significantly less than one. This is consistent with analysis determining expression of two binding sites in this case; a high affinity L-365260 specific site and a low affinity site, both for which CCK-8s exhibits affinity.

Investigation of specific CCK_2 receptor ligands; CI-988, PD-140376, YM-022 and JB-93182,

in the rat cortex, adhere closely to a one site model (indicative of a homogeneous population of CCK_2 sites) where Hill slope estimates were not significantly different from unity (Harper *et al*., 1999). This study however found that the estimated affinity values for JB-93182 and YM-022 in rat cortex were significantly higher, and that for CI-988 was significantly lower, than those obtained in the mouse cortex when these radioligands were used. Rat cortex affinity data were subsequently interpreted using a two-site model where it was revealed that SR-27897 expressed approximately 9 fold, CI-988 approximately 13 fold and PD-140376 approximately 11 fold selectivity for the putative CCK_{2B} site whereas JB-93182 expressed approximately 23 fold and YM-022 approximately 4 fold selectivity for the CCK_{2A} site. Harper *et al*., (1999) proposed that where CCK_2 receptors are of two subtypes, PD-140376, used in this study is labelling only one of these subtypes. This might explain data described forthwith.

Studies of the CCK_2 subtype imply that the transmembrane domains of the receptor comprise a putative ligand-binding pocket. Amino acid substitutions within this proposed pocket altered the affinities and/or functional activities of L-365260, YM-022 and L-740093S (a partial agonist). Site-directed mutagenesis of residues within this pocket alters individual affinities but fails to modify ligand-gated inositol phosphate production. The cholecystokinin octapeptide profile is however unaltered by mutations within this CCK_2 pocket (Blaker *et al*., 2000).

Binding studies performed using rat brain CCK_2 receptor transfected CHO cells showed that two affinity states of the CCK_2 receptor are discriminated by constrained dipeptoids CCK_2 antagonists (Bellier *et al*., 1997). Some evidence that CCK_2 receptors may even exist in three different affinity states has also been provided (Huang *et al*., 1994). Brain CCK_2 receptors have been demonstrated via radioligand binding studies, to exist in three different affinity states. Two of these states, exhibiting a high or low affinity to CCK-8 were detected. A third state of very low affinity was detected using [^{3}H]-L-365260. The third state represents a much larger percentage of the receptor population than the higher affinity states (Chang *et al*., 1989). This and other studies suggest that non-peptide antagonists may bind with a greater affinity to

the G protein uncoupled conformations (low or very low affinity) whereas peptide agonists bind with a greater affinity to the high affinity form. Peptide agonists also bind to the G protein uncoupled receptor conformation but with a much lower affinity. These different affinity states may represent the putative subtypes of CCK_2 proposed to underlie behaviour in several models within this thesis.

CCK_1 Affinity States

Meanwhile, the idea of two distinct states of the peripheral CCK_1 receptor is well established. Iodine labelled TN-CCK-9 (cholecystokinin nonapeptide) bound to high (with a K_D 0.17 nM) and low (with a K_D 13 nM) affinity receptors in dispersed rat pancreatic acini. Its dissociation, in the presence of unlabelled TN-CCK-9, showed a rapid binding component, associated with low affinity receptors and a slow binding kinetic component, by default associated with high affinity receptors. These receptors were further characterised with one class exhibiting a high affinity and a low capacity for CCK, while the other class having a low affinity and a high capacity for cholecystokinin (Winand *et al*., 1991).

Studies of binding of ^{125}I-Bolton-Hunter-labeled cholecystokinin non-sulphated octapeptide (^{125}I-BH-CCK-8) to guinea pig pancreatic acini, revealed that non-sulphated agonist profiles were biphasic with 20% tracer bound to high affinity sites with relative potencies: gastrin-17-I ; pentagastrin; CCK-8s; and much lower CCK-4; 80% was bound to low-affinity sites with the following potencies: CCK-8s; then gastrin-17-I equivalent to pentagastrin far greater than CCK-4. Despite analysis of the dose-inhibition curve for CCK-8 adhering to a two binding site profile, comparison with the analysis in the presence of a high (0.1 micromolar) concentration of gastrin-17-I suggested three binding sites. The gastrin-17-I dose-inhibition curve actually fitted a three-site model significantly closer than a two-site model (Yu *et al*., 1990).

N-terminal truncations of the human CCK_1 receptor, lacking the first 37 amino acids displayed a pharmacological profile identical to that of the full receptor. A truncated form lacking the first 42 amino acids however, did not bind agonist CCK-9 with high affinity. This ligand however, could compete for binding at low affinity sites (Kennedy *et al*., 1995). It may be inferred from this study that this N-terminal site is essential for the high affinity interaction with cholecystokinin whereas low affinity binding occurs upstream. Site directed mutagenesis experiments conducted by Gigoux *et al*., (1998) in conjunction with three-dimensional modelling, provided evidence that methionine 195 of the CCK_1 receptor is an essential amino residue for interaction with the aromatic ring of the sulphated tyrosine of CCK-8s. This appears to interact with the sulphated tyrosine-7 (at the seventh position from the carboxyl terminal) of the cholecystokinin peptide and is crucial for cholecystokinin binding to the CCK_1 receptor. The unsulphated octapeptide form (CCK-8us) exhibits poor affinity for the CCK_1 receptor so much that the CCK_1 receptor were highly selective (800-fold) toward sulphated versus nonsulphated CCK, whereas low and very low affinity sites were poorly selective (10- and 18-fold). The site-directed mutagenetic exchange of Met-195 for a leucine residue however caused a minor (2.8-fold) decrease in the affinity of the high affinity sites for sulphated CCK-9 but a strong decrease (73%) of receptor density. However this exchange produced a 30-fold decrease on the affinity of the low and very low affinity sites for sulphated CCK-9, with no change in their number. The mutation also caused a 54-fold decrease of the potency of the receptor to induce inositol phosphate production. In addition, the mutant exhibited affinity and efficacy towards the unsulphated cholecystokinin form identically to the wild-type receptor. The authors postulate that Met-195 interacts with the aromatic ring of the sulphated tyrosine to correctly position the sulphated group of cholecystokinin in the binding site of the receptor. This interaction is essential for CCK-dependent transition of the CCK_1 receptor to a high affinity state.

Interactions between Sites

These high and low affinity receptor populations are quite probably efficacious via discrete second messenger cascades. An indicator of this may lie indirectly within a study by Suman-Chauhan *et al*., (1996) using the highly CCK_2 specific radioligand [^{3}H]PD-140376 labelling cholecystokinin receptors in guinea-pig brain and gastric glands. In the presence of the guanyl nucleotide 5'-guanylimidodiphosphate (GppNHp), cholecystokinin agonist affinity in binding competition in gastric glands but not in the brain was significantly inhibited. This study implied that despite the cholecystokinin labelling receptor sites in the brain and gastric glands (CCK_2/gastrin subtype) being of the same molecular structure (Wank 1995), receptors in the two respective tissues appear to differ in the nature of post-receptor coupling. It may thus be possible that two proposed subtypes within the brain are indeed efficacious via two different second messenger cascades given the case in point. Furthermore, post-receptor coupling appears to effect affinity for the radioligand.

Thus there is a mounting body of evidence for multiple binding sites present within both CCK_1 and CCK_2 receptors. This was hoped to be demonstrated using the ligands studied within these experiments

During the course of these studies the methodology was refined using several different protocols. Variations included addition of peptidase inhibitors, phenyl methyl sulphonyl fluoride, amastatin, actinonen and interchanging buffer solutions for Krebs-Ringer phosphate (as both a wash and an incubation buffer). The aforementioned revised method was used in all experiments after determination of that which allows optimal binding efficiency of the radioligand.

Methods and Materials

Animals

Sprague-Dawley rats weighing between 300-370g were housed in identical conditions to those used in behavioural experiments.

Chemical Preparation

cholecystokinin peptide was stored under desiccation at -70^{o}C. cholecystokinin peptide solutions were prepared freshly each day approximately two hours prior to testing. Powdered peptide was dissolved in a minimal volume of DMSO with subsequent addition of buffer solution. Specific concentrations were prepared through serial dilution with thorough vortex mixing at each stage. Radioligand solutions were prepared through direct dilution from a stock solution.

Tissue Acquisition: Rats were sacrificed by direct decapitation using a Stoetling small animal guillotine and brains were quickly removed. Brain dissection with removal of the cerebellum and brain stem from the cerebral cortices provided the whole brain minus cerebellum, which was used in biochemical studies.

Tissue preparation: Protocols are adapted from Hunter *et al*., (1993). Brain structures are immediately placed in a test tube containing a 50mM Tris-HCl buffer A containing 5mM $MgCl_2$ at pH 6.9 stored at 4^{o}C (pH adjusted by addition of Hydrochloric acid) and kept on ice throughout the entire preparatory procedure. Tissue was diluted under 20 volumes of tissue wet weight (g) / volume (ml), therefore, 7 g of tissue suspended in 140ml of buffer. Brain structures are homogenised using a Kinomatica Polytron for two 10-second periods (setting 6). The resulting homogenate is shaken using the vortex unit and then placed in a Beckman ultracentrifuge J25-I at 40,000g for 15 minutes at 4^{o}C. Immediately after centrifugation samples are removed and placed on ice. The Pellet is re-suspended in excess buffer after

pouring and pipetting away the supernatant. The remaining pellet is washed with 10 volumes of buffer A (original wet weight / volume). The pellet is resuspended in buffer by homogenisation as per previous instruction. The homogenate again undergoes centrifugation under identical conditions to those abovementioned. The final pellet in re-suspended in 10 volumes (original wet weight), of a buffer (Buffer B = 10mM HEPES, 130mM NaCl, 4.7mM KCl, 5mM $MgCl_2$, 1mM EGTA, 0.025% Bacitracin, and Phenyl-Methyl-Sulphonyl Fluoride, PMSF (1mM) at pH 7.2 and 22^{o}C) and is homogenised and shaken as by the abovementioned method.

Incubation: Incubation tubes are divided and labelled. Solutions documented within subsequent individual sections are added in the order, buffer B; then unlabelled competitive component; then tissue homogenate; lastly the radiolabelled component is added. Solutions are incubated for 45 minutes at 25^{o}C in a temperature maintained water bath and lightly shaken.

Filtration: Filter papers (Whatman GF-B) are soaked in ice cold buffer A and stored on ice at 4^{o}C. Within the last 5 minutes of the incubation period the wet filter papers were placed onto the vacuum filter and vacuum dried of excess buffer for a 10 second period. At the end of the incubation period sequentially labelled tubes were emptied into corresponding wells, tubes are washed once with 4ml of ice cold buffer (4^{o}C) with the contents emptied into the well. Tubes were placed upside down in each corresponding well. Upon completion of this procedure for each of the tubes, sequential washing begins again with 4ml of 4^{o}C buffer twice more (making a total of three washes). The vacuum filtration is maintained after the last wash for fifteen seconds and each filter paper is carefully removed with tweezers and placed in a corresponding scintillation vial. This procedure is completed for subsequent batches of incubated samples.

Scintillation Counting: Each vial, containing filter paper is filled with 8ml of Beckman HP Scintillation fluid and shaken using both hand shaking in order to submerge the filter paper, and vortex apparatus to aid absorption. After a period of twenty-four hours, vials are placed in

a Beckman scintillation counter.

Protein Assay

Modified from Lowry *et al*., (1951). Reaction Mixture minus protein consists of solution A which is prepared as per the following: 15ml of 2% Na_2CO_3 in 0.1M NaOH with addition of 0.15ml of 2% Na-K Tartrate. After waiting for a few minutes 0.15ml of $CuSO_4$ is added. Bovine Serum Albumin standard solutions of 1mg/ml concentration were added to the reaction mixture in volumes; 10µl, 30µl, and 50µl in order to provide a standard spectrophotometry curve. The protein sample diluted 1 in 10 is added (30µl) to the reaction mixture. 10 minutes later 0.5ml of Folin-Ciocaltens Phenol (FCP) reagent is added to each tube. Each BSA standard and protein sample are performed in triplicate. After a period of 90 minutes spectrophotometric measurement of absorbance (=500nm (visible spectrum); W lamp (tungsten filament) was carried out for each tube. In order to achieve a reliable measure of absorbance differences, each absorbance measure is subtracted from absorbance of a blank solution (Solution A and FCP without protein). The protein sample is plotted on a slope of BSA concentration versus absorbance.

Data Analysis

Binding analysis was performed using GraphPad Prism version 3.00 for windows, (GraphPad Software, California USA). This software allows specific non-linear curve fit regression, linear regression and model fitting analysis.

Results and Data

Experiment 6.1

In rat cerebral cortex membranes, [^{3}H]-PD-140376 demonstrated saturable binding to apparently a single population of receptors. This is shown by close adherence to a one-site model analysis with Prism 3.00 software analysis. This software is able to analyse nonlinear regression curves. Therefore linear regression analysis via schild and scatchard charts is not deemed necessary (see Avoid Scatchard, Lineweaver-Burke and similar transforms in Graphpad manual. Copyright (c) 1994-1999 by GraphPad Software). Total binding is shown in figure 6.1.1 within a range of concentrations from 0.1 to 8.0nM. With competition from pentagastrin at a concentration of 200μM, specific binding is determined. Chart 6.1.2 shows specific binding data for [^{3}H]-PD-140376 by subtraction of total binding from the linear regression plot for pentagastrin competition data. Figure 6.1.2 shows that [^{3}H]-PD-140376 has a K_D value of 1.087nM (0.2061 S.E.M.) and a B_{MAX} of 862.8 counts per minute (53.98 S.E.M.). This non-linear regression analysis did not deviate significantly from a one site binding model analysis but deviated to a significant degree from that of a multiple site model analysis.

Experiment 6.2

A concentration range of the peptoidal CCK_2 antagonist CI-988 was examined in competition with [^{3}H]-PD-140376 at 4.0nM. Analysis confirmed that binding adheres closely to a one site binding competition model compared to two-site competition analysis. An EC_{50} of 0.747 nM CI-988 was determined. This experiment was deemed necessary to compare binding of peptide (pentagastrin) and non-peptide (CI-988) binding in competition with [^{3}H]-PD140376. Both appear therefore to exhibit affinity for the same CCK_2 binding site at the concentrations tested.

Experiment 6.3

[^{3}H]-CCK-8s at concentration of 1.5nM in competition versus unlabelled CCK-4, CCK-8us or CCK-8s. All three cholecystokinin forms versus radiolabelled CCK-8s appeared to adhere to one site competition model analysis, except for certain anomalies with regard to very high concentrations of competing ligands. At concentration in a range of 0.1 to 1.0mM upwards, radioligand binding seemed to increase or unlabelled competition appeared to decrease. This is represented statistically by either a loss of significance compared to lower concentrations within the exponential phase, which in itself is not truly conclusive. With CCK-4 however, high doses of 10 and 20mM are significantly greater than counts measured with the plateau phase at 0.01 to 1.0mM; $F_{11,36}$=40.732, $P<0.05$ and $P<0.01$. The possible significance of this finding is discussed later in this chapter.

Figure 6.1.1

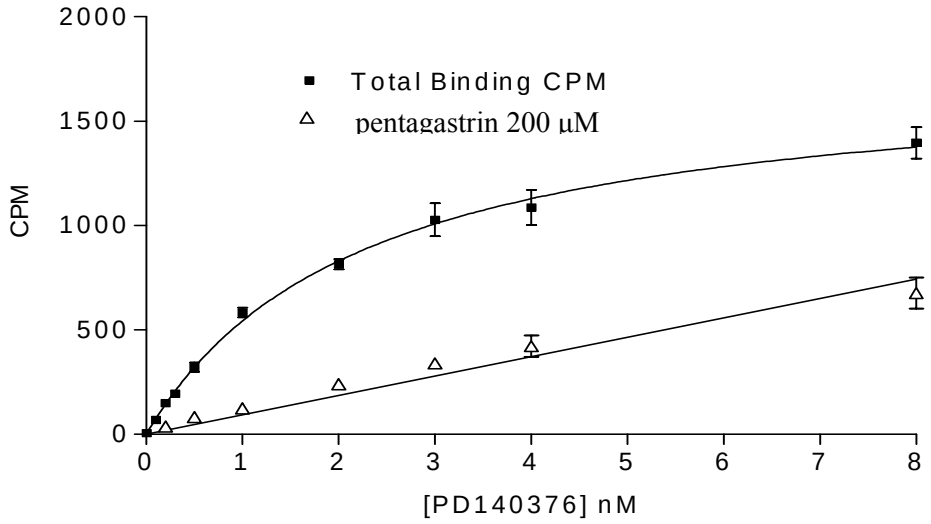


Figure 6.1.2

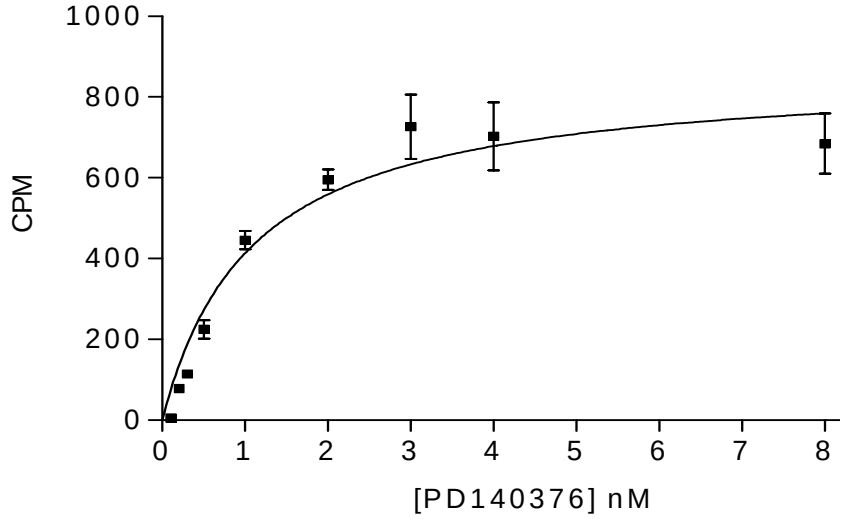


FIG 6.1.1 shows total binding data for [^{3}H]-PD-140376 over a $\log_{10}$ concentration range and in competition with 200µM Pentagastrin with ± S.E.M. A non-linear line of regression in representation of these data is also shown.

FIG 6.1.2 shows specific binding data for [^{3}H]-PD-140376 over a $\log_{10}$ concentration range by subtraction of total binding from the linear regression plot for pentagastrin competition data with ± S.E.M. A non-linear line of regression in representation of these data is also shown.

Figure 6.2

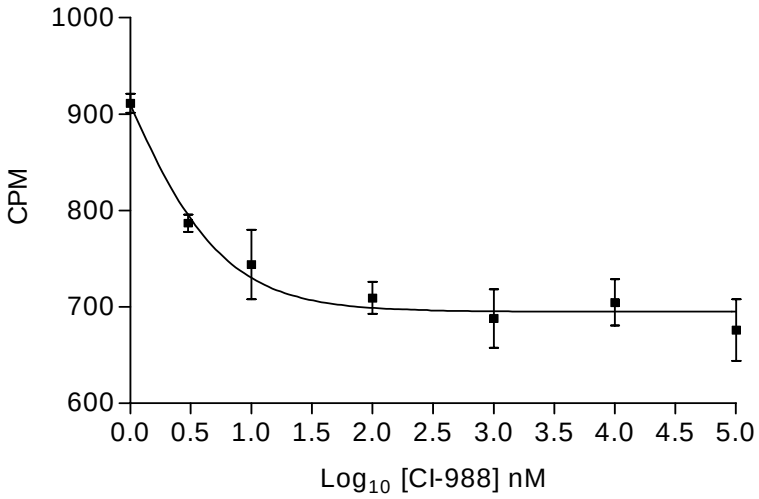


FIG 6.2 shows binding data for competition of 2nM [^{3}H]-PD-140376 with CI-988 within a $\log_{10}$ concentration range (CI-988) 0.5 to 5.0 nM with ± S.E.M. A non-linear line of regression in representation of these data is also shown.

Figure 6.4

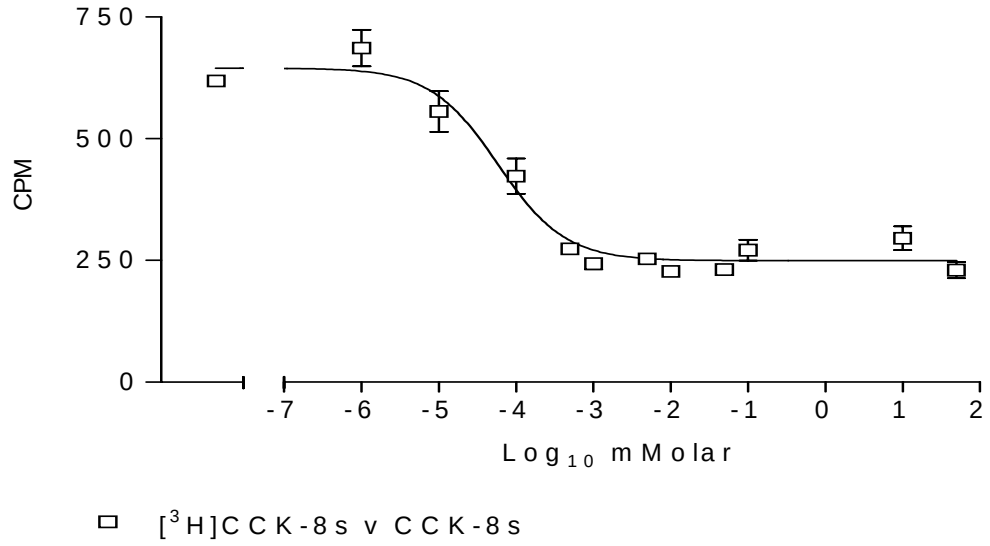


Figure 6.5

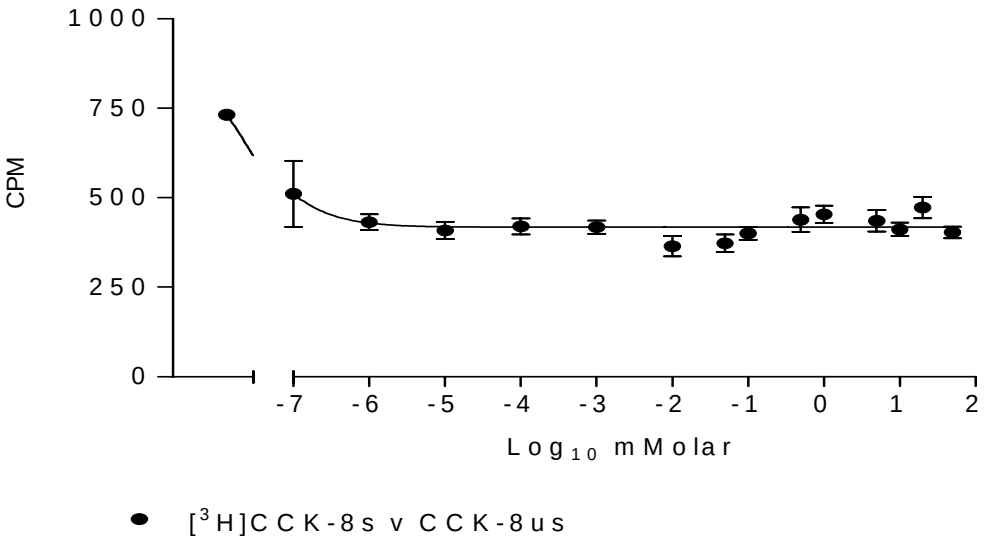


FIG 6.3 shows binding data for [^{3}H]-CCK-8s in competition with unlabelled CCK-8s over a $\log_{10}$ concentration range with ± S.E.M. A non-linear line of regression in representation of these data is also shown.

FIG 6.4 shows binding data for [^{3}H]-CCK-8s in competition with unlabelled CCK-8us over a $\log_{10}$ concentration range with ± S.E.M. A non-linear line of regression in representation of these data is also shown.

Figure 6.6

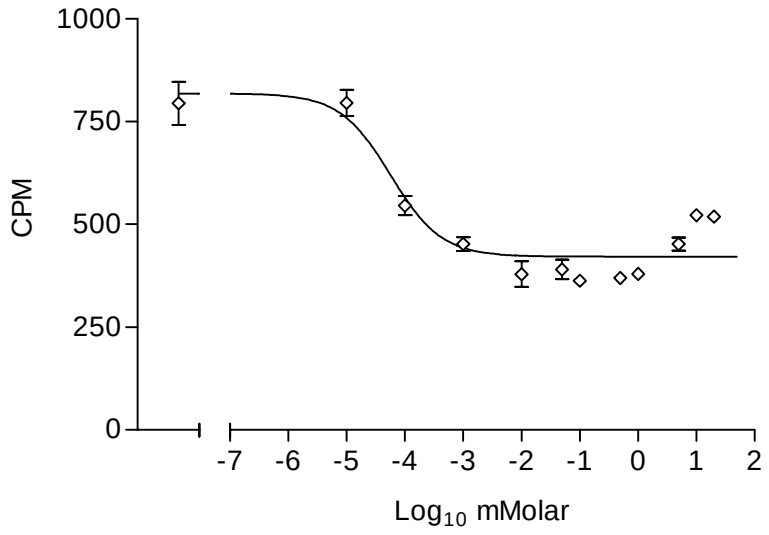


Figure 6.5 shows binding data for [^{3}H]-CCK-8s in competition with unlabelled CCK-4 over a $\log_{10}$ concentration range with ± S.E.M. A non-linear line of regression in representation of these data is also shown.

Discussion

Within rat cerebral membranes, [^{3}H]-PD-140376 demonstrated saturable binding to apparently a single population of receptors. This is not however in contradiction of hypotheses for two subtypes of CCK_2 receptors. As postulated by Harper *et al*., (1999) if CCK_2 receptors are of two subtypes, PD-140376, used in this study is labelling only one of these subtypes. Determination of specific binding with competition from pentagastrin shows that a resulting non-linear regression analysis did not deviate significantly from a one site binding model analysis but deviated to a significant degree from that of a multiple site model analysis. Furthermore, binding analysis of a concentration range of the peptoidal CCK_2 antagonist CI-988 confirmed close adherence to a one site binding competition model compared to two-site competition analysis. This experiment was deemed necessary to compare binding of peptide (pentagastrin) and non-peptide (CI-988) binding in competition with [^{3}H]-PD-140376. Both appear therefore to exhibit affinity for the same CCK_2 binding site at the concentrations tested. These experiments were conducted some time before publication of the aforementioned article by Harper *et al*., (1999). In the light of this very comprehensive study it was decided that no further experiments were to be conducted using PD-140376. At the time of these experiments only this and the CCK_2 antagonist L-365260 were available for commercially. These ligands were both shown to adhere to single site binding models and were obviously not of use in determination of potential CCK_2 receptor subtypes / subsites. Binding data for [^{3}H]-CCK-8s, being an endogenous form of cholecystokinin, was subsequently analysed within multiple binding site models in competition with unlabelled CCK-4, CCK-8us or CCK-8s. All three cholecystokinin forms in competition with radiolabelled CCK-8s appeared to adhere to one site competition model analysis, with the exception of certain anomalies with regard to very high concentrations of competing ligands. At concentrations in a range of 0.1 to 1.0mM upwards, radioligand binding seemed to increase, or unlabelled competition appeared to decrease, resulting in larger radioactivity counts observed. This is represented statistically by either a loss of significance compared to lower concentrations within the exponential phase, which in itself is not truly conclusive. With CCK-4 however, high doses are significantly

greater than counts measured within the plateau phase. This finding is peculiar, seemingly only to this receptor group. It might be possible that cholecystokinin at very high doses exhibits affinity for a very low affinity receptor site. Activation of this site may then confer either increased affinity at the higher affinity site, or at the very low affinity site itself. It is more likely however that binding at a very low affinity site would not change binding at this site itself in particular. It has been postulated previously that CCK_1 receptors may exhibit a high affinity active site and a lower affinity G-protein decoupling sites. This lower affinity site is postulated to act as a de-activating site, which decouples the second messenger system (G protein) from the receptor and decreases affinity of the high affinity site to that of very low affinity. This would confirm findings by Talkad *et al*., (1994) that the CCK_1 receptor exists within three affinity states; high, low and very low affinity. It has been also confirmed that at least two, a high and low affinity state, exist for CCK_2 receptors (Huang *et al*., 1994). The lack of evidence for a dual site binding within the whole brain minus cerebellum is also at odds with presence of a distinct population of CCK_1 receptors for which CCK-8s exhibits a fairly equivalent level of affinity to that of CCK_2. This observation may be due to a radioligand concentration, which confers binding only at the highest affinity site. This would certainly produce a binding profile in competition adhering to a one-site model. The possibility of using higher radioligand concentrations were prohibited by both high cost and relatively low CCK-8s concentrations of samples available commercially. These data do not discount a hypothesis for multiple binding sites given a body of evidence precluding to such. These are merely unable to categorically provide evidence by several aforementioned limiting factors.

Activation of the non-peptide site may then produce in addition to a proposed decoupling of the G-protein second messenger, an alteration in affinities at the peptide site. Schwartz *et al*., (1995) proposed that an allosteric mechanism alters the conformation of the receptor structure for other neuropeptide receptors. This mechanism may therefore account for the anomalies observed at very high concentrations of cholecystokinin ligands whereby binding competition

with the radioligand CCK-8s is attenuated. This concentration may thus have some kind of activity in inhibiting binding at the non-peptide site or may be at a level where affinity towards the deactivated site is observed. This could then produce further binding of the radioligand, which exhibits a greater affinity at the receptor than the CCK-8us and CCK-4 forms, thus radioligand binding will outweigh the competitive ligand effects at decreasing radioactivity counts.

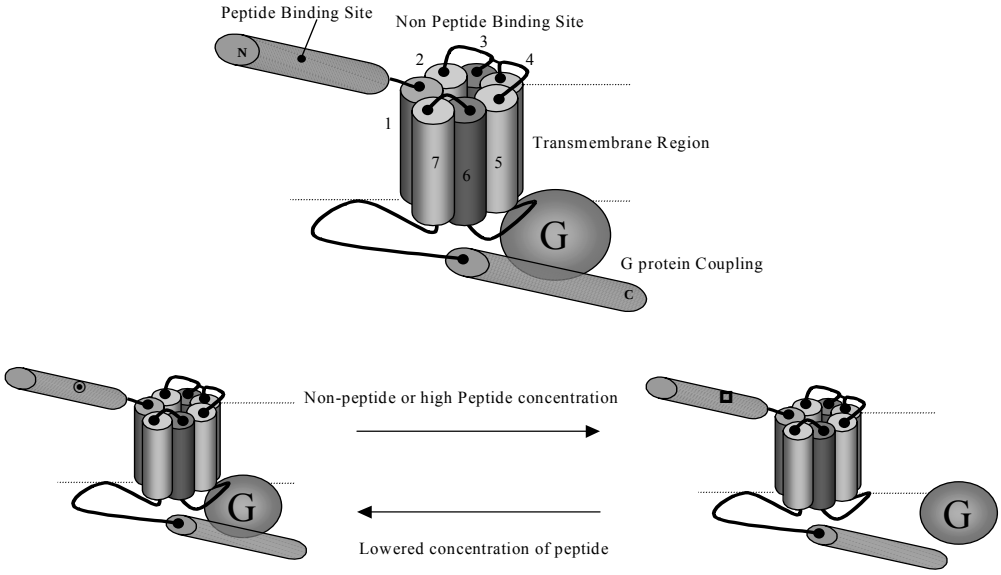


Figure 6.6 represents a proposed model of physiological effects of binding at the "non peptide" (CCK-8 low affinity) site. The G-protein coupled form (left of the equilibrium) is an active conformation with a high affinity peptide site (for CCK-8). The G-protein de-coupled form (right of the equilibrium) is an inactive conformation possibly with a structurally altered peptide specific binding site (conferring very low affinity to CCK-8). This model would account for presence of two affinity populations of receptor: active; high affinity for CCK-8 at peptide site, low affinity for CCK-8 at non-peptide site: inactive; very low affinity for CCK-8 at altered peptide site; cholecystokinin occupancy at non-peptide site required for maintenance of the de-activated state.

The putative mechanism represented in figure 6.6 should eventually yield data for four binding sites; two in the high affinity state (peptide and non-peptide specific) and two in the low affinity decoupled state (the same peptide and non-peptide specific). The existence of only

three binding states, as postulated by Huang *et al*., (1994) and Chang *et al*., (1989) is possibly not correct. This and similar studies are probably measuring two sites in the activated receptor with CCK-8s binding with highest affinity at the peptide specific followed by the non-peptide specific site, and then measuring the peptide site at the decoupled (deactivated) receptor. Therefore further study may provide ligands that would be able to bind also at the non-peptide decoupled receptor sites. One of the several cholecystokinin derivatives, the tetrapeptide form has been adapted by bismethylation of its carboxyl terminal in order to increase peptidase-resistance. Any further modifications of the terminal moieties decreased the CCK_2 receptor binding affinity (Weng *et al*., 1996). The receptor binding profile of this compound however, was examined within studies assuming a one-site binding model, any alteration of this proposed dual site binding has not obviously been characterised. With the assumption that endogenous CCK-4 is able to bind within this dual site model to both sites, as it might be assumed of endogenous cholecystokinin ligands, the bismethylated form was therefore not utilised in this series of experiments.

CHAPTER SEVEN - CONCLUSIONS

Animal Models of Anxiety

The administration of chlordiazepoxide showed consistent anxiolytic-like activity in both acoustic startle and elevated plus-maze models via either decreasing acoustic startle amplitudes or increasing time spent in the open arms respectively. Cholecystokinin forms; CCK-4, CCK-8us and CCK-8s, within an extremely narrow dose range reversed chlordiazepoxide activity within both models. Given the wealth of evidence of clinical activity of CCK-4 in panicogenesis, it may be proposed that cholecystokinin forms produce anxiogenic-like behaviour in rats within these models. Studies of both startle and plus-maze models showed that activation at the CCK_2 receptor is critical in cholecystokinin peptide induced anxiety-like behaviour. The specific antagonists, CI-988 and LY-288513, were able to attenuate the effects of those anxiogenic cholecystokinin forms.

Interestingly, despite significant anxiogenic-like activity within the plus-maze model, CCK-8us alone appears to be largely inactive in the acoustic startle paradigm. Furthermore, although CCK-8us decreased anxiety-like behaviour induced by CCK-4 in the plus-maze at specific doses, no activity was observed regarding CCK-8us affecting CCK-4 increased acoustic startle amplitudes. It would thus appear, prima facie, from this evidence that CCK-8us is almost entirely inactive within the acoustic startle model. This however is not entirely accurate, as is discussed later.

The chronological sequence of animal testing was firstly using the plus-maze, in late 1997, followed by acoustic startle testing, in late 1998, using the same peptide supply for CCK-4 and CCK-8us, and a fresh CCK-8s supply. These chemicals, specifically CCK-4 and -8us were stored under identical conditions, which would imply that both are free of significant degradation, in order for one to be efficacious and the other not to be. However, chemical differences may confer a greater degradation for CCK-8us, thus rendering it inactive in the

acoustic startle model. This however is disregarded with regard to positive CCK-8us activity in the passive and active avoidance and in social isolation experiments that were performed later still. This would therefore imply that CCK-8us is genuinely inactive in acoustic startle. That may imply that plus-maze anxiogenic-like activity control is through a separate mechanism to that of acoustic startle. Alternatively, given that there is a slight increase in amplitude observed for CCK-8us, although far from significant, suggests that the acoustic startle model may be less sensitive to measurement of anxiogenic-like behaviour than the plus-maze. Therefore, it may be inferred that relative intensity of the anxiety state generated by cholecystokinin forms is in the order, CCK-8s > CCK-4 > CCK-8us (CCK-8s having the strongest effect). This would also be shown to some extent in the plus-maze where CCK-8s only is able to inhibit chlordiazepoxide induced increases in open arm entries. The other forms despite effecting measures of time spent in the open arms are not efficacious with regard to open arm entries, thus implying that CCK-8s induces a greater intensity of anxiety than CCK-4 and -8us. However given that CCK-8s exhibits affinity for the CCK_1 receptors, the CCK_1 gated factor of inhibition of locomotor activity must be acknowledged within these models.

Co-administration of cholecystokinin forms yielded similar data in both models with regard to CCK-4 and CCK-8s combinations. This particular combination negated the anxiogenic-like activity of either alone and did not produce any activity with combinations at half doses in both models. As discussed previously, a CCK-8us appears to have no activity within the acoustic startle paradigm. This is highlighted whereby, in the plus-maze combinations of CCK-4 with CCK-8us negated anxiogenic-like activity of either alone, but in the acoustic startle paradigm, CCK-8us did not alter activity of CCK-4. However, a further complication arises where CCK-8us is able to attenuate the anxiogenic-like activity of CCK-8s in the startle paradigm. This implies that CCK-8us is certainly chemically active within the paradigm, and supports both hypotheses of; either differing anxiety intensity induced by each cholecystokinin form, or of distinct systems controlling anxiogenic-like behaviour on the plus-maze and in the

acoustic startle paradigm. The latter hypothesis does however strongly support existence of multiple cholecystokininergic configurations underlying different anxiogenic pathologies. Ostensibly, it might not be surprising that behaviour within the plus-maze, which may duplicate clinical phobia to some degree, and naïve acoustic startle, which is probably akin to symptoms within generalised anxiety disorder, are controlled via different mechanisms.

An interesting finding showed that in the acoustic startle paradigm, CCK_2 antagonists, LY-288513 and CI-988 both exhibited an inverse bell shaped dose response curve with regard to anxiogenic-like behaviour. These findings are both complementary to those of CI-988 and contrary to those of LY-288513 in the plus-maze. A dose of $0.02mg.kg^{-1}$ of CI-988 produces anxiogenic-like startle behaviour and also inhibits chlordiazepoxide-induced increases in open arm exploration, also indicative of an anxiogenic response. However, a dose of $0.001mg.kg^{-1}$ LY-288513 both produces increases in startle and increases in open arm exploration. Hence the former is indicative of anxiogenesis and the latter of anxiolysis. This would again imply that the two models are measuring distinct forms of anxiety, which are indeed gated through different cholecystokininergic mechanisms. Plus-maze anxiogenesis is proposed to be controlled by equilibrium between activities of CCK-4 and CCK-8 specific receptors, thought to be putative CCK_2 subpopulations. According to evidence herein, acoustic startle anxiogenesis would be controlled by an imbalance between CCK-4 and only CCK-8s, with CCK-8us possessing ability to inhibit the activity or antagonise CCK-8s specific receptors. Evidence of contrary data using LY-288513 has indicated that anxiety-like behaviour in the plus-maze and in the acoustic startle models are not complementary to each other. A poignant question to be raised would thus be whether anxiety itself is measured at the critical dose of $0.001mg.kg^{-1}$, or whether this is some other behavioural characteristic. At higher doses of LY-288513 within the plus-maze, anxiolytic-like activity is observed and in acoustic startle, activity is normalised. Interestingly, CI-988 produces an increase in startle amplitude at the dose, $0.002mg.kg^{-1}$ at which no effect is observed within the plus-maze. This provides further

evidence of the dissimilarity of underlying mechanisms controlling either plus-maze behaviour or acoustic startle. While CI-988 induced differences in dose response studies can be explained perhaps by chlordiazepoxide co-administration in the plus-maze test, LY-288513 produces inversed behavioural profiles at the same doses in both models and in the vehicle solely. Interestingly, for LY-288513, a trend towards inhibition of total number of arm entries is observed at 0.01mg.kg^{-1}. This would imply, given the increases in startle at this dose, that a form of freezing behaviour is apparent in the plus-maze, thus inhibiting exploration. However, this cannot be attributed to the lower dose, so the enigma remains. The similarity between behaviour exhibited within the plus-maze and acoustic startle is therefore not as simple as at first sight. Where anxiety would be expected a priori to be a singular entity prevalent in both models, it is unquestionably clear that this anxiety-like behaviour is quite distinct in both models. Whether this can be applied to clinical conditions that these models attempt to represent is problematical. It might be predicted that an agoraphobic individual would experience increased propensity for increased startle behaviour, however, this cannot be directly applied to rodent behaviour, which is undoubtedly attuned to very different environmental variables and risk factors. An example of which may be in examination of the aversive nature of the plus-maze, whereby a rodent, for fear of predation or falling, will not explore the areas of potentially perilous open arms, with an option of relatively safe closed arm areas. Within this potentially predatory environment, startle would serve as an escape mechanism. Furthermore, it may be possible that anxiety itself is not actually measured within the plus-maze environment. Despite, a state of unrealistic risk assessment underlying clinical phobias, rodent open arm avoidance behaviour on the plus-maze may be as a result of a wholly realistic risk assessment. It cannot be assumed that unrealistic risk assessment in humans, known as phobia, is a state analogous to seemingly normal behaviour in rodents. Previous studies have not attempted to show whether benzodiazepines increase motivation to explore within the plus-maze and that cholecystokinin decreases this. The plus-maze has for the most part been validated pharmacologically with the observations that particular drugs that are

clinically active as anxiolytics are also active within the maze. This possibly far-fetched supposition, that the plus-maze actually measures anxious behaviour and not just pharmacological effects, has not been tested in great detail. Acoustic startle however may be a more realistic measure of anxious behaviour in that this is a simple reaction to a rapid stimulus, which is altered in intensity by a change in anxiety level.

With an attempt to equate these findings to the human condition, it may be speculated that activation of one cholecystokinin or other neurotransmitter subsystem may confer certain behavioural changes indicative of particular anxiety disorders, whereas activation of another may produce changes related to a different set of anxiety-like symptoms. This may partly explain the range of different anxiety disorders in the clinical condition, although this is highly speculative.

An interesting finding that LY-288513, with anxiolytic-like properties on naïve exposure to the plus-maze is no longer active when administered on subsequent exposures. This in itself demonstrates activity identical to that of chlordiazepoxide. However, administration of scopolamine following the initial plus-maze exposure was able to restore chlordiazepoxide activity but not that of LY-288513. This evidence may allow an insight into the nature of neural circuitry controlling these phenomena. This would imply that benzodiazepine neuronal circuitry lies either upstream, or in parallel to cholecystokinin circuitry underlying anxiety. This sequence of activation is however contradicted with evidence that CI-988 was not able to inhibit activity of the benzodiazepine inverse agonist FG-7142. This implies that FG-7142 induced anxiogenesis is either independent or is downstream from structures gating cholecystokinin-induced anxiogenesis. This apparent contradiction would imply that indeed the underlying neural circuitry, by which either cholecystokinin ligands or benzodiazepines achieve their effects upon anxiety, is distinct and separate. These projections possibly would intercept at an end structure through which anxiety itself is gated. Furthermore, evidence implies that differential activation of distinct cholecystokinin receptor subpopulations

controlling anxiogenesis, and attenuation of some of the resulting anxiogenic pathologies by benzodiazepines, may underlie anxiety. Evidence of plus-maze-induced increases in acoustic startle amplitudes is lacking. This increase is only distinctly demonstrated after the second exposure. This finding would imply that other factors such as repeated testing and handling, in addition to plus-maze exposure, might underlie resulting behaviour or at least add a further stress component. However, it may equally be assumed that changes in startle amplitudes are not sensitive to single plus-maze exposure and that only the anxiety level induced by cumulative exposures are of an intensity producing significant increases. This is probably more likely than acoustic startle exhibiting sensitivity only to a distinct type of anxiety exhibited in the second exposure.

Animal Models of Learning and Memory

Experiments within this thesis have attempted to provide a clear explanation for seemingly contradictory data implicating cholecystokinin in either amnestic or promnestic activity in memory acquisition. The data and discussion therein has strongly refuted claims of activity for cholecystokinin in learning and memory of associative, non-appetitive tasks.

The examination of differences in receptor binding between animals exhibiting differeing behaviour within the models examined was not examined in this thesis. However I have suggested this, in hindsight, as a possible avenue of research and this is being carried out currently by other researchers.

Complications

A recent paper by Rehfeld (2000) drew attention to a lack of evidence as to whether CCK-4 itself is an endogenous peptide. There is insufficient evidence according to the author that CCK-4 is synthesised endogenously, and may merely be an artefact of in-vivo degradation of, probably CCK-5. Rehfeld proposed that CCK-4 is an exogenous test substance. Whether this

is exact is perhaps mostly irrelevant in this study. Although CCK-4 may indeed be only an exogenous compound, the fact remains that clearly CCK-4 and CCK-8 achieve their anxiogenic-like profile via distinct and separate mechanisms, possibly via different CCK_2 receptor subpopulations. In the case that CCK-4 is not an endogenous factor in anxiety, surely this distinct mechanism described must have a role within anxiety. It may be possible that CCK-4 and CCK-8 differ only in their permeability to the blood brain barrier, where one may activate peripheral receptors and the other central receptors. In this case, there must still be a purpose for these different populations. It may also be quite possible that CCK-4, being as proposed a degradation product of CCK-5 would exert effects via the same receptor populations. Therefore, the possibility that the effects of CCK-4 can be transposed to those of CCK-5 should be examined in future study. The possibility that any form of cholecystokinin injected peripherally may be degraded to another active by product may also require further investigation.

Given the numerous neurotransmitter classes thought to control different aspects of both clinical and experimental anxiety, pathology underlying the array of distinct clinical anxiety disorders cannot be assumed to involve a simple elevation of specific neurotransmitter activity. This diversity of clinical anxiety disorders in itself implies several neurotransmitter systems, distinct regions within these, and interactions between receptor subtypes as involved in a strikingly complex underlying mechanism. Early hypotheses stating particular overly simplistic mechanisms underlying not only a single, but also an entire range of anxiety disorders, are certainly to be discounted. Study of the brain and behaviour, until very recently, has been prone to this over simplistic view of this highly complex structure. Neuroscience has been plagued with reductionist ideas, in the sense that the complexity of the brain relating to similarly complex behaviours has not been appreciated sufficiently. With millions of individual neurones and in turn billions of intricate interconnections, still simplistic ideas surrounding neurotransmitter function, related to behaviour, are alluded to. This reductionist

approach is indeed useful when considering distinct elements involved in a specific pathology, but should not be viewed in isolation. Furthermore, where various studies are found to contradict one another, detailed examination of experimental differences must occur. To merely state that a particular research group were incorrect in their findings is a wholly ineffectual standpoint. Failure to examine causes of these variations allows further misuse of valuable resources in carrying out, with hindsight, meaningless experiments.

In conclusion experimental data provided within this thesis has hopefully addressed some of the points of issue within the field studied. With certainty, many questions have been raised which it is hoped will be elucidated upon in future study.

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Zhou, Y., Arifuzzaman, A. J., Josselyn, S. A., Frankland, P. W., Bradwejn, J., and Vaccarino, F. J. (1996) Effects of intraperitoneal administration of cholecystokinin agonist and antagonist on acoustical startle response, an anxiety model in rats. Soc. Neuroscience Abstract 22, 1153.

APPENDIX A.1.1: CHOLECYSTOKININ RECEPTOR AGONISTS USED WITHIN THIS THESIS.

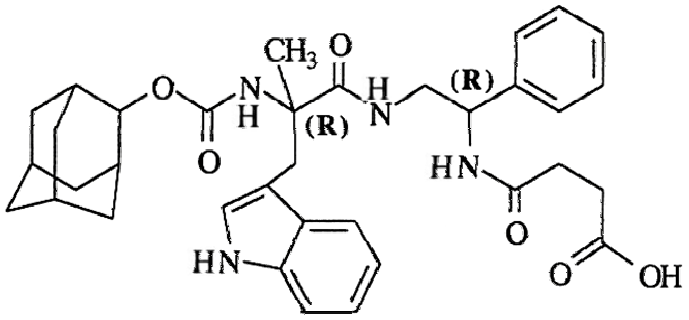
CCK-8s: Cholecystokinin Sulphated Octapeptide; [Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2]

CCK-8us: Cholecystokinin Sulphated Octapeptide; [Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2]

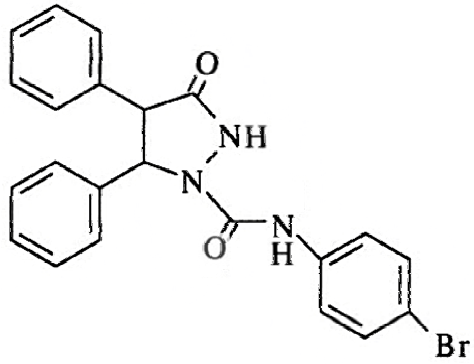
CCK-4: Cholecystokinin Tetrapeptide; [Trp-Met-Asp-Phe-NH_2]

APPENDIX A.1.2: CCK_2 RECEPTOR ANTAGONISTS USED WITHIN THIS THESIS

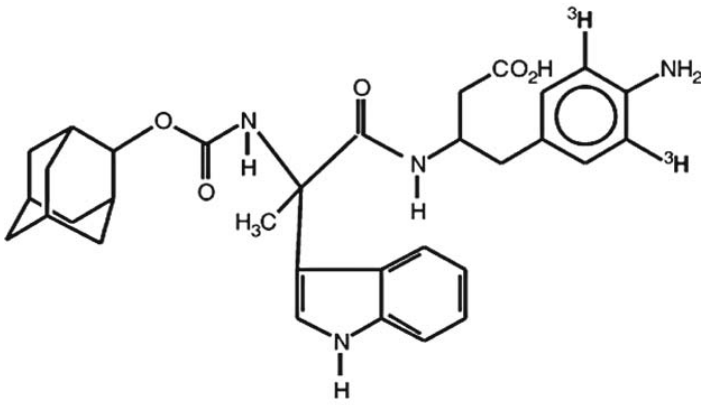
CI-988 (PD-134,308)



LY-288,513



[^{3H}]-PD-140,376



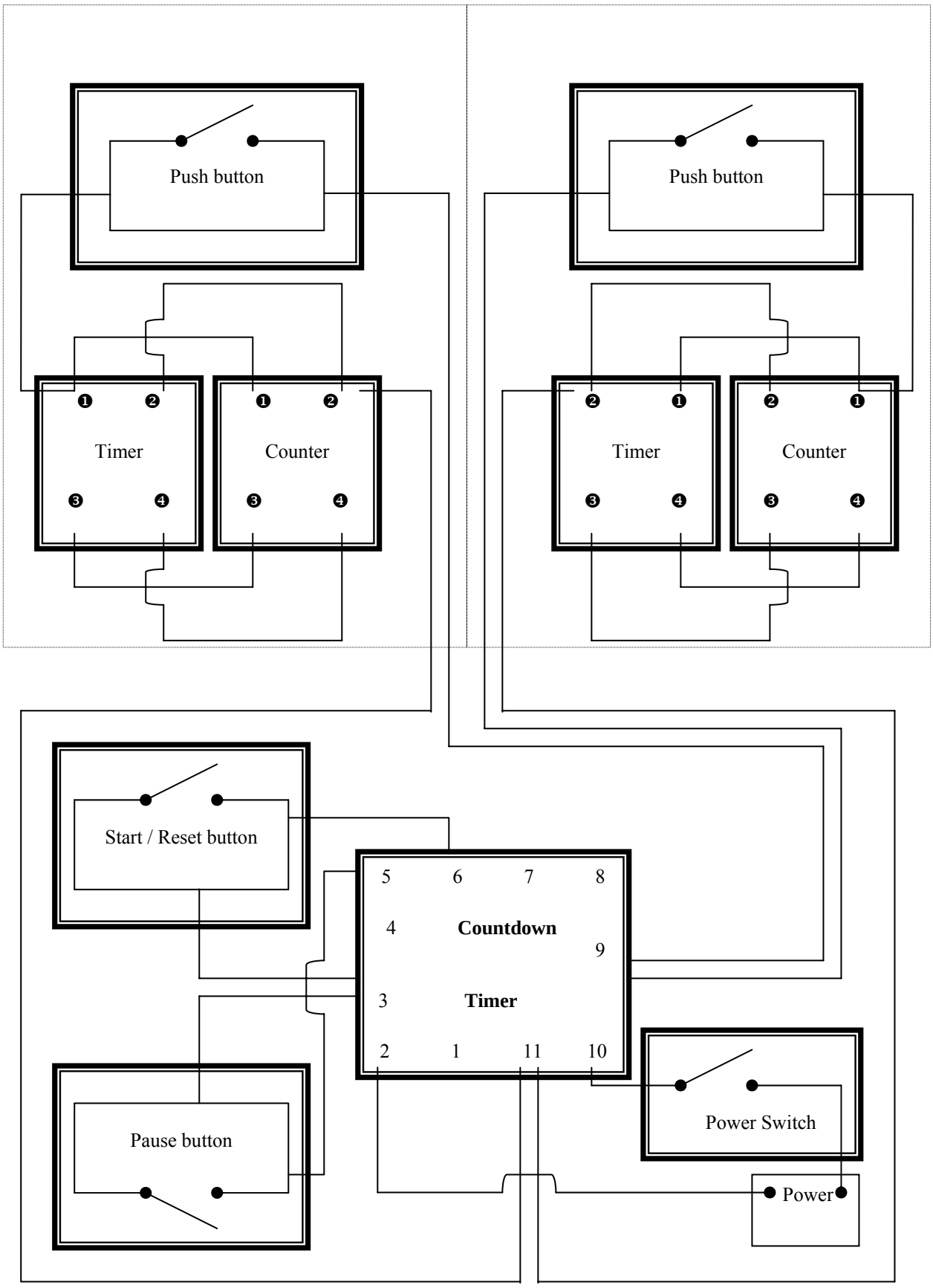
APPENDIX B.1: WIRING DIAGRAM FOR ELEVATED PLUS-MAZE COUNTER TIMER DEVICE

Elevated Plus-maze Data Collection Device

The counter / timer unit was used for collection of elevated plus-maze data. This was a rectangular box with two top mounted buttons of one touch on and one touch off operation. One such button was operated when the animal entered the open arms (button marked O) and the other button was operated when the animal entered the closed arm (button marked C). The box also consisted of two sets of timers (OMRON H7ET self powered RESET button only); two sets of counters (OMRON H7EC self powered RESET button only); and a centrally positioned countdown timer (OMRON H3CA), which was set for a specific period of time. After this period had elapsed the counter and timer units ceased operation. Each timer and counter unit was switched on or off via the top mounted buttons and counter /timer units were dedicated to either open arm buttons or closed arm buttons.

When the countdown timer inactivated the counter /timer units each thus displayed entry numbers or time spent in either open or closed arms of the maze.

The wiring diagram for the control box is represented overleaf.


Push button
Push button
Timer
Counter
Timer
Counter
Start / Reset button
5
6
7
8
4
Countdown
9
3
Timer
2
1
11
10
Pause button
Power Switch
Power

APPENDIX C.1: PROGRAM LISTINGS

Random Number Generator Programs

These programs served to randomise drug treatment groups for consecutive animals. First using Microsoft Quick basic, Revision 2 was refined in order to provide a range of drug group number other than that starting a zero. This was deemed necessary when particular treatment groups were allocated their maximum number of animals and random number generation was required within a narrower range. The program was rewritten for Microsoft Visual Basic in order to provide an easily executable program.

1. Random Number Generator v.1 by C.J Greengrass, (1997) for Quick Basic 4.5

```
10 REM PRESS F5 TO RUN
20 REM SELECT File AND Exit TO QUIT
30 CLS 0: COLOR 15, 0, 1
40 PRINT "NUMBER RANDOMISER V.1 By C.J.Greengrass (c) 1997"
50 INPUT "Enter the Range Minimum"; a
60 INPUT "Enter the Range Maximum"; b
70 INPUT "How Many Random Numbers <70"; n
80 BEEP
90 RANDOMIZE TIMER
100 FOR y% = 1 TO n
110 D% = INT(RND * b)
120 c% = INT(RND * 6) + 9
130 COLOR c%, 0, 0
140 IF D% <= a THEN D% = D% + a
150 PRINT D%,
```

```
160 NEXT y%

170 COLOR 15, 1, 1

180 LINE INPUT "again Y/N    ", y$

190 IF y$ = "y" THEN GOTO 40 ELSE GOTO 200:

200 PRINT "Cheerio": END
```

2. Random Number Generator v.2 by C.J Greengrass, (1997) for Quick Basic 4.5

```
10  REM PRESS F5 TO RUN

20 REM SELECT File AND Exit TO QUIT

30 CLS 0

40 COLOR 15, 0, 1

50 PRINT "NUMBER RANDOMISER V.2 By C.J.Greengrass (c) 1997"

60 INPUT "Enter the Range Minimum"; a

70 INPUT "Enter the Range Maximum"; b

80 INPUT "How Many Random Numbers <70"; n

90 RANDOMIZE TIMER

100 FOR y% = 1 TO n

110 D% = INT(RND * b)

120 c% = INT(RND * 6) + 9

130 COLOR c%, 0, 0

140 IF D% <= a THEN D% = D% + a

150 PRINT D%,

160 NEXT y%

170 COLOR 15, 1, 1

180 LINE INPUT "again Y/N    ", y$
```

```
190 IF y$ = "y" THEN GOTO 3 ELSE GOTO 50:

200 PRINT "Cheerio": END
```

3. Random Number Generator by C.J Greengrass, (1999) for Visual Basic 6.0

```
Dim a As Integer

Dim b As Integer

Dim X As Object       'create Word object

Dim n As Integer

Dim y1 As Integer

Dim d1 As Integer

Dim c1 As Integer

Private Sub cmdEnd_Click()

End

End Sub


Private Sub cmdStart_Click()

Set X = CreateObject("Excel.Application") 'open excel

X.Visible = True 'make it visible

X.workbooks.Add    'open a new workbook

a = txtMin.Text     ' choose a as min

b = txtMax.Text

n = txtnumber.Text


Beep

Randomize
```

```
For y1 = 1 To n

d1 = Int((b - a + 1) * Rnd + a)


X.Range("A" & y1).Select

X.ActiveCell.FormulaR1C1 = d1

Next y1

End Sub
```

APPENDIX C-2 SAN DIEGO INSTRUMENTS SOFTWARE PROGRAM LISTING AND OPERATING INSTRUCTIONS

The software prompts for variables required within the protocol, on-screen notifications, without input facility, are shown in lower case; on-screen notifications, with input prompting, are shown in as capitalised; variables and key operations are shown in bold text and are underlined; and annotation is in parenthesis. (↵ represents the return (enter) key)

C.2.1 SR-Lab Software Program Listing PSR2.EXE

C.2.1.1: The following dedicated computer software program dictated a sequence of twenty-five 100-millisecond bursts of 120dB white noise with an intermittent background noise level of 70dB. There was an eight second interval between 120dB bursts.

Session Definition

From the main menu;

SELECT **F2** (Definition Menu)

SELECT **F5** (Sessions Definition**)**:

Session Name: **"asrsess1"**

Background Noise Level?: **420**

(A noise level of 420 corresponds to 70dB as a background noise level)

Number of Record Samples?: **250**

Sample Intervals (Milliseconds)?: **1**

(Between stimulus bursts 250 samples of animal movement data are recorded separated by one millisecond)

Inter-trial Interval #1 (Seconds)?: **8**

(The interval between stimulus noise bursts)

Inter-trial Interval #2 (Seconds)?: ↵

Trial #1: "asrtrl1" ↵

Sequence Repetitions?: **25** ↵

(Number of bursts of noise stimulus during the trial)

Acclimatisation Period (Minutes)?: **3** ↵

(Period during which the animal is placed in the apparatus prior to the first noise burst. This period is necessary in order to allow acclimatisation to the apparatus. Hence, any change in the level of responding measured will be in response predominantly to the noise burst stimuli.

IS THIS CORRECT Y/N?: **Y** ↵

Trial Definition

From the main menu;

SELECT **F2** ➔ (Definition Menu**)**

SELECT **F1** ➔ (Build a TRIAL definition**)**

TRIAL NAME: **"asrtrl1"** ↵

SELECT **F1** ➔ NOISE LEVEL?: **720** ↵

(A noise level of 720 corresponds to 120dB)

SELECT **F4** ➔ Record Data

(This begins immediate data recording of the animal's reflexive response to the noise)

SELECT **F3** ➔ WAIT TIME (MS)? **10** ↵

(Length of stimulus)

SELECT **F2** ➔ Background

(Revert to background noise level of 70dB)

PRESS Escape

DO YOU WANT TO SAVE THE TRIAL Y/N?: **Y**

Execution Protocol

From the main menu;

SELECT **F1** ➔ (*Execution Menu*)

SELECT **F1** ➔ SESSION NAME?: **"asrsess1"**

CHAMBERS 1-8: SELECT **F1** (indicating chamber 1 only)

Identification codes: (*User specified*)

RESULT FILENAME: (*User specified*)

Display Waveforms Y/N? **n**

Are your Subjects ready Y/N? **y**

(Session Proceeds)

C.2.1.2: The following dedicated computer software program dictated a sequence of twenty-five 100-millisecond bursts of 70dB white noise with an intermittent background noise level of 70dB. There was an eight second interval between 120dB bursts. The stimulus and background noise are identical in volume amplitude. This is a pseudo-startle paradigm in order to measure spontaneous locomotor activity within the apparatus. The session definition and execution protocol listings are identical except in trial name and session name respectively.

Trial Definition

From the main menu;

SELECT **F2** ➔ (Definition Menu)

SELECT **F1** ➔ (Build a TRIAL definition):

TRIAL NAME: **"trial2"** ↵

SELECT **F1** ➔ NOISE LEVEL?: **420** ↵

(A noise level of 420 corresponds to 70dB)

SELECT **F4** ➔ Record Data

(This begins immediate data recording of the animal's reflexive response to the noise)

SELECT **F3** ➔ Wait Time (Ms)? **10** ↵

(Length of stimulus)

SELECT **F2** ➔ Background

(Revert to background noise level also of 70dB)

SELECT Escape

DO YOU WANT TO SAVE THE TRIAL Y/N?: **Y**

C.3.1: The following dedicated computer software program dictated a 30-second shock sequence while utilising SR-LAB and Gemini II apparatus in the shock induced fear potentiated startle paradigm. The acoustic startle program listing C.2.1 was used although on two consecutive trials, pre and post-shock. This program listing uses the GEMINI II apparatus and the GEMII.exe program

From the main menu;

Session Definition

SELECT **F2** ➔ (Definition Menu)

SELECT F4 ➔ "Build a SESSION definition":

SESSION NAME: **"shkfpsr1"** ↵

START GATE OPEN OR CLOSED? O/C: **C** Gate Starting Position: CLOSED

ENTER NEXT ITI IN SECONDS: 0 Inter Trial Interval 1: 0

Enter next ITI in seconds: ↵

ENTER NEXT TRIAL NAME: **"shkfpsr1"** ↵

ENTER NEXT TRIAL NAME: ↵

Sequence Repetitions: **1**

Acclimation Period Following Button Press in Seconds: **0**

Trial Definition

SELECT **F2** ➔ (Definition Menu**)**

SELECT **F1** ➔ (Build a TRIAL definition)

TRIAL NAME: **"shkfpsr1"** ↵

BEGINNING of Start Block.

SELECT **M** (M*ark Side*)

SELECT **H** **(**House Lights) **M (**Light on marked side) **1** **(**Light on**) U** (Untimed)

SELECT **G** (*Gate*) **C** (*Closed*) **U** (*Untimed*)

SELECT **W** (Wait for) **B (**button press)

SELECT **S** (*shock*) **O** (*on*) **M** (M*arked side*) **1** (*on*) **T** (*timed*)

Milliseconds to maintain Marked Shock On: **30000** ↵

SELECT **W** (Wait for) **I** (interval)

ENTER WAIT TIME IN MILLISECONDS: **30000** ↵

SELECT **J (**Jump to Start of Finish Block)

END of Start Block.

BEGINNING of Finish Block.

END of Finish Block.

C.3.2. Gemini II: One Way Step-Through Passive Avoidance using the PA2.exe program.

Acquisition Trial

SELECT ALT/C.

Primary Data site? X:\

Total Enclosures Connected? 1

Use Shock (s), Airstim (A) or Both (B)?: **S**

Duration of Shock (seconds)? 1

Query User Questions (Y or N)? N

Top level menu message? Trial name and message

(C)LASSIC or (T)RIALS TO CRITERION? T

Stop Session if can't get Trials to criterion (y/n)? **n**

At Main Menu:

SELECT 1 TO Set Up Header For Test

FILENAME? "**xxxxxxxxx**" (*8 characters*)

Number of Trials? 8

Trials to Criterion? 3

Enclosures used to test? 1

Adaptation period (seconds)? 10

Maximum Trial length (seconds)? 60

SELECT 2 TO START TEST SESSION

Retention Trial

SELECT ALT/C.

Primary Data site? X:\

Total Enclosures Connected? 1

Use Shock (s), Airstim (A) or Both (B)?: **S**

Duration of Shock (seconds)? 0

Query User Questions (Y or N)? N

Top level menu message? "Trial name and message"

(C)LASSIC or (T)RIALS TO CRITERION? C

SELECT 1 TO Set Up Header For Test

Filename (8 characters)? "xxxxxxxxx"

Enclosures used to test? 1

Is this a training session (Shock on) Y/N? N (*for first session only*)

Adaptation period (seconds)? 10

Maximum Trial length (seconds)? 300

START TEST SESSION

C.2.4. Gemini II: One Way Step-Through Active Avoidance for GEMII.EXE program

Training Trial Definition

Initiation Trial

SELECT **F2** ➔ (Definition Menu)

SELECT **F1** ➔ Build a TRIAL definition:

TRIAL NAME: **"shkfpsr1"** ↵

BEGINNING of Start Block**.**

Mark Side.UnMarked House Lights On.

Wait 10000 mSecs.

Marked Shock On.

Gate Open.

END of Start Block.

Until Timeout, DO Condition Block #1 IF

Subject Crosses,

Otherwise, DO Default Block.

Timeout at 40 seconds.

BEGINNING of Condition Block #1.

Gate Closed.

Jump to Start of Finish Block.

END of Condition Block #1.

BEGINNING of Default Block.

END of Default Block.

BEGINNING of Finish Block.

END of Finish Block.

Avoidance Trial

SELECT **F2** ➔ (Definition Menu)

SELECT **F1** ➔ Build a TRIAL definition:

TRIAL NAME: **"shkfpsr2"** ↵

BEGINNING of Start Block.

Both House Lights On.

Wait for Button Press.

Mark Side.

Gate Open.

Clear Interval Timer A.

Start Interval Timer A.

END of Start Block.

Until Timeout, DO Condition Block #1 IF

Subject Crosses,

Otherwise,

DO Condition Block #2 IF

Interval Timer A >= 10000 Milliseconds,

Otherwise, DO Default Block.

Timeout at 45 seconds.

BEGINNING of Condition Block #1.

Jump to Start of Finish Block.

END of Condition Block #1.

BEGINNING of Condition Block #2.

Marked Shock On.

END of Condition Block #2.

BEGINNING of Default Block.

END of Default Block.

BEGINNING of Finish Block.

Marked Shock Off.

Gate Closed.

END of Finish Block.

Session Definition

Gate Starting Position: CLOSED

Inter Trial Interval 1: 30

Inter Trial Interval 2: 30

Inter Trial Interval 3: 30

Inter Trial Interval 4: 30

Inter Trial Interval 5: 30

Inter Trial Interval 6: 30

Inter Trial Interval 7: 30

Inter Trial Interval 8: 30

Inter Trial Interval 9: 30

Inter Trial Interval 10: 30

Trial 1: SHOCKER1

Trial 2: SHOCKER2

Trial 3: SHOCKER2

Trial 4: SHOCKER2

Trial 5: SHOCKER2

Trial 6: SHOCKER2

Trial 7: SHOCKER2

Trial 8: SHOCKER2

Trial 9: SHOCKER2

Trial 10: SHOCKER2

Trial 11: SHOCKER2

Sequence Repetitions: 1

Acclimation Period: 0

Retention Trial

Due to problems involving programming this protocol. Retention sessions were manually operated and timing was recorded using electronic timers and screen observation.