Psychopharmacology (1995) 121 : 109–117

ORIGINAL INVESTIGATION

Gerard R. Dawson · Nadia M. J. Rupniak
Susan D. Iversen · Rachel Curnow · Spencer Tye
Kelly J. Stanhope · Mark D. Tricklebank

Lack of effect of CCK_B receptor antagonists in ethological and conditioned animal screens for anxiolytic drugs

Received: 18 July 1994 / Final version: 23 January 1995

Abstract The effects of the CCK_B receptor antagonists L-365,260, CI-988 and L-740,093, a new compound with improved bioavailability and CNS penetration, were assessed for anxiolytic-like effects in three rat anxiolytic screens sensitive to benzodiazepines, the elevated plus maze (EPM), conditioned suppression of drinking (CSD) and conditioned emotional response (CER) tests. In the EPM, L-740,093 (0.1–1.0 mg/kg), L-365,260 (0.00001–10.0 mg/kg), and CI-988 (0.01–1.0 mg/kg) did not increase the time spent on the open arms of the maze or the number of entries onto the open arms. In contrast, the benzodiazepine receptor partial agonist, bretazenil (0.3–10.0 mg/kg), significantly increased both the time spent on the open arms and the number of open arm entries. In the CSD and the CER tests, L-740,093 (0.1–1.0 mg/kg) L-365,260 (0.0001–0.1 mg/kg) and CI-988 (0.01–10.0 mg/kg) failed to increase suppression ratios compared to the vehicle-treated control rats, whereas, the benzodiazepine receptor partial agonist FG 8205 (10.0 mg/kg) (CSD) and bretazenil (0.3–3.0 mg/kg) (CER) both significantly increased suppression ratios compared to vehicle-treated control rats. In addition, L-365,260 (1.0–50.0 mg/kg), CI-988 (0.1–10.0 mg/kg) and diazepam (0.1–1.0 mg/kg) were assessed in a squirrel monkey conflict procedure. Although diazepam significantly increased suppressed lever pressing rates, L-365,260 and CI-988 were without effect. The present findings provide little support for the hypothesis that CCK_B receptor antagonists have anti-anxiety effects in animals.

Key words L-740,093 · L-365,260 · CI-988 · CCK_B antagonists · Anxiety · Rats · Squirrel monkeys

G. R. Dawson (✉) · N. M. J. Rupniak · S. D. Iversen
R. Curnow · S. Tye · K. J. Stanhope · M. D. Tricklebank
Merck, Sharp and Dohme Research Laboratories,
Neuroscience Research Centre,
Terlings Park, Eastwick Road, Harlow,
Essex. CM20 2QR, UK

All the experiments reported in this manuscript were conducted within British Government Home Office approved techniques, procedures and project licences

Introduction

A number of studies have shown that the neuropeptide cholecystokinin (CCK) may be involved in mediating panic- or anxiety-like symptoms in humans. For example, Bradwejn et al. (1990) reported that the C-terminal tetrapeptide fragment of CCK_8, CCK_4, given intravenously, elicited panic-like symptoms in patients with panic disorder. By contrast, the intravenous injection of CCK_8-S, which does not penetrate the blood-brain barrier, induced only severe gastrointestinal symptoms (De Montigny 1989). At present, two subtypes of CCK receptors have been identified, CCK_A and CCK_B. As CCK_4 is a selective CCK_B receptor agonist (Chang and Lotti, 1986), CCK_B receptors may play a role in mediating panic symptoms and thus CCK_B receptor antagonists may have therapeutic utility as anti-panic or anti-anxiety agents.

The preclinical development of CCK_B receptor antagonists has been hampered by the lack of a well defined animal model of panic. However, selective CCK_B receptor antagonists, such as CI-988 (Hughes et al 1990) and L-365,260 (Bock et al. 1989), appear to have anxiolytic-like effects in so-called "ethologically valid" rodent models of anxiety such as the rat and mouse elevated-plus maze, the rat social interaction and the mouse light/dark box test (Rataud et al. 1991; Singh et al. 1991). However, as Dooley and Klamt (1993) point out, CCK_B receptor antagonists induce dose-related anxiolytic-like effects only in rodent paradigms that depend upon 'naturally aversive' stimuli to induce anxiogenic-like behaviour. In

conflict or punishment paradigms in which electric shock is used as an anxiogenic stimulus, the effects of CCK_B receptor antagonists are much less robust. For example, using a modified operant punishment procedure in squirrel monkeys, Powell and Barrett (1991) showed that CI-988 (0.03–10.0 mg/kg) had an anxiolytic-like effect, but only at a single dose of 3.0 mg/kg. Singh et al (1991) found that although CI-988 was active over a wide dose-range in the elevated plus maze test (0.01–3.0 mg/kg), it was active at only one dose (0.01 mg/kg) in a shock motivated rat conflict test. Similarly, Dooley and Klamt (1993) report that mice given doses of either 0.0001 or 0.1 mg/kg CI-998 took more shocks than controls in a four-plate test, but intervening doses were without effect. In these studies the anxiolytic-like effect of CI-988 was modest compared to the appropriate positive control group given a benzodiazepine receptor agonist, such as chlordiazepoxide.

There are at least two possible explanations for the lack of a consistent effect of CCK_B receptor antagonists in shock-motivated tests. First, it is possible that the level of fear induced by electric shock is much higher than that induced by the 'natural' aversive stimuli present, for example, in the elevated plus maze test. If this is the case, then CCK_B receptor antagonists might be expected to be effective in tests that induce mild, but not strong, states of fear. This may have clinical implications, as a diagnosis of panic disorder requires that the patient reports the experience of intense anxiety. An alternative explanation is that whilst L-365,260 readily crosses the blood-brain barrier, it is not water soluble and its bioavailability crucially depends on the vehicle in which it is dissolved or suspended (Jackson et al. 1994). Similarly, although CI-988 is water soluble, it crosses the blood-brain barrier poorly (Patel et al. 1994). As a consequence, it may not be possible to achieve brain concentrations of L-365,260 or CI-988 to occupy a sufficient number of central CCK_B receptors to induce a robust anxiolytic-like effect in shock-motivated tests following systemic administration.

In the present study, we sought to evaluate whether either of these explanations, or a combination of them, accounted for the inconsistent effects of CCK_B receptor antagonists in shock motivated tests. We used three rodent screens sensitive to conventional anxiolytic drugs: (i) a rat elevated plus maze test (EPM); (ii) a rat conditioned-suppression-of-drinking (CSD) test; (iii) a rat conditioned-emotional-response (CER) test. In addition, L-365,260 and CI-988 were evaluated in a primate conflict procedure (PC). Finally, in order to address the pharmacokinetic and brain penetration problems of L-365,260 and CI-988, we examined the effects of L-740,093, a recently developed highly selective, water soluble CCK_B receptor antagonist which has improved bioavailability and also readily crosses the blood-brain barrier (Showell et al. 1994).

Materials and methods

Animals

Three to 4 month-old Sprague-Dawley (225–275 g) rats were used in the EPM test. Hooded PVG rats (300–350 g) were used in the CSD and CER tests because pilot studies showed that they maintained a more stable instrumental rate on operant schedules of reinforcement during and between drug tests than Sprague- Dawley rats. In the CSD test the rats were water deprived for 22.5 h in each 24-h period. In the CER test the rats were maintained at 85% of their free-feeding weight by post-sessional feeding. Animals were maintained on a 12/12-h light-dark cycle in humidity and temperature controlled rooms and were obtained from Bantin and Kingman, Hull, UK. Four adult male squirrel monkeys (*Saimiri sciureus*; 800–1200 g), maintained at 85–90% of their free feeding weight appropriate for their age, served as subjects for the PC paradigm.

Drugs

L-740,093 [(3*R*)-*N*-[5-(3-azabicyclo[3.2.2]nonan-3-yl)-2,3-dihydro-1-methyl-2-oxo-1H-1,4-benzodiazepin-3-yl]-*N'*-[3-methylphenyl] urea hydrochloride](Showell et al. 1994), L-365,260 (3*R*)-*N*-[2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-*N'*-[3-methylphenyl]urea](Bock et al. 1989) 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 *N*-methyl-D-glucamine)(Hughes et al. 1990) and FG 8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3-(5-isopropyl-1,2,4-oxadiazol-3-yl)-4H-imidazol[1,5a][1,4]benzodiazepine)(Tricklebank et al. 1990) were synthesised by the Merck, Sharp and Dohme Neuroscience Research Centre's medicinal chemistry group. Bretazenil was kindly donated by Hoffmann La Roche and diazepam was obtained from Sigma, St Louis, USA. For experiments using rodents, all compounds were prepared freshly each day and injected in a volume of 1 mg/ml. L-740,093 and CI-988 were dissolved in 0.9% saline and L-365,260 was dissolved in labrafil (ALFA Chemicals, Berks, UK.). Bretazenil was dissolved in 100% polyethylene glycol (PEG 400). FG 8205 and diazepam were suspended in 0.5% methyl cellulose + 0.2% Tween 80. All compounds were administered IP 30 min before the beginning of each experiment. For experiments using squirrel monkeys, CI-988 was dissolved in sterile water and administered IM as described by Powell and Barrett (1991). L-365,260 was suspended in 90% Imwitor/10% Tween and diazepam in 0.5% methylcellulose prior to oral administration.

Apparatus and procedures

Elevated plus maze

The EPM was made from black Perspex and its floor was covered with black rubber matting. The maze was arranged in a "+" shape with two open arms facing each other. The other two arms were enclosed by 40 cm high walls. Each arm measured 10 × 50 cm and was raised 50 cm above the floor. Four fluorescent strip-lights were mounted on the ceiling, one above each arm, and illuminated the maze with plane-polarised light. At the beginning of a trial the rat was placed in the centre of the maze with its nose facing one of the open arms and allowed to freely explore for 5 min. The rats were observed via a video camera, fitted with a polarising lens, mounted directly above the centre platform of the maze. The camera was connected to a television monitor and a BBC microcomputer via a VP112 tracking unit (HVS, London) housed in an adjacent room. The walls and ceiling of the maze room were painted in matt black and a black rubber mat completely covered the floor. As a

consequence of the black maze and room, a white rat provided a high contrast image that could be tracked through the maze by the VP112 unit. The computer software calculated the time the animal spent in the open and closed arms, the total distance the rat travelled while in the maze, the distances travelled in the closed and open arms and the number of entries into closed and open arms.

Before testing of the CCK_B receptor antagonists began, the sensitivity of the test was assesed by establishing a dose-response relationship for the low efficacy benzodiazepine receptor partial agonist, bretazenil. Thus, 60 animals were assigned to one of five groups and given either vehicle or 0.3, 1.0, 3.0, 10.0 mg/kg bretazenil. For the L-740,093 experiment, 60 animals were assigned to one of five treatment groups ($n = 12$) receiving Vehicle; 0.1, 0.3, or 1.0 mg/kg L-740,093 or 3.0 mg/kg diazepam. The effects L-365,260 were evaluated over a wide dose range in two experiments. In the first L-365,260 experiment two doses of CI-988 (0.01 and 0.1 mg/kg) were also evaluated and 108 animals were assigned to nine treatment groups: vehicle; 0.00001, 0.0001, 0.001, 0.01 or 0.1 mg/kg L-365,260 or 0.01 or 0.1 mg/kg CI-988; or 3.0 mg/kg bretazenil. In the second experiment, 60 animals were assigned to five groups ($n = 12$): vehicle, 1.0, 3.0, or 10.0 mg/kg of L-365,260; or 3.0 mg/kg bretazenil. Each experiment was carried out over 2 days, with six animals from each group tested on each day.

Conditioned-suppression-of-licking

Twelve standard operant boxes were fitted with grid floors through which scrambled electric shock (0.4 mA) could be delivered to the animal's feet. A food magazine was placed 130 mm above the grid floor in the middle of the front wall through which the animals could gain access to a metal drinking spout recessed 5 mm behind the front wall. This arrangement required the animal to stand with its front paws on the wall in order to lick the drinking spout which was connected to a water reservoir via a peristaltic pump. When the rat licked the spout a circuit was made between the tongue, the front wall of the operant chamber and a lickometer connected to an Archimedes A5000 computer running the real time language Arachnid (Paul Fray Ltd. Cambridge). The operation of the peristaltic pump was also controlled by the computer and as a consequence, water (0.1 ml) could be delivered to the spout after a random interval between reinforcers. A houselight (2.4 W) was positioned in the middle of the front wall 250 mm above the grid floor, the illumination of which served as a conditioned stimulus (CS) during conditioning sessions.

On day 1, thirsty rats were placed in the operant chamber for 30 min and licking the metal spout was reinforced with 0.1 ml water according to a random interval (RI) 5 s schedule. This procedure rapidly established licking and on the second, third and fourth successive days the schedule was increased to RI 60 s. On the fifth day, the conditioning day, the session length was increased to 60 min. At three 15 min intervals the houselight was illuminated for 60 s, and 1 s before the houselight was switched off a 0.4-mA shock was delivered to the feet of the animals and terminated at the same time as the light was switched off. Thus, the rats received three light-shock pairings, and as a consequence when the light was presented for the third time their licking-rates were suppressed. The degree of suppression was quantified by expressing it as a ratio: Suppression ratio = CS rate/(CS rate + Pre CS rate), where the "CS rate" is the number of licks during the light presentation and the 'Pre CS rate' is the rate during the minute immediately before the light presentation. Rats with suppression ratios for the third light presentation $> = 0.15$ were excluded from drug testing. Thus only rats that had learnt the light-shock relationship, as indicated by a suppression ratio < 0.15 on the third trial, were tested. On day 6 the rats were given a further 30-min RI 60-s session and on day 7 were rested. On day 8, the test day, the procedure was identical to that on the conditioning day, with the exception that electric shocks were not delivered during the light presentations.

The sensitivity of this screen to benzodiazepine receptor agonists was established with FG 8205. The training criterion was achieved by 53 out of 60 rats which were randomly allocated to five groups ($n = 10$ or 11) and given either vehicle 0.3, 1.0, 3.0 or 10.0 mg/kg FG 8205. In the L-740,093 experiment, 55 of the 60 animals trained had suppression ratios of less than 0.15 for the final light/shock presentation and they were assigned to one of five treatment groups ($n = 11$): vehicle; 0.1, 0.3 or 1.0 mg/kg L-740,093 or 10.0 mg/kg FG 8205. In the L-365,260 experiment, 48 of the 60 animals trained met the < 0.15 suppression ratio criterion and they were assigned to six treatment groups ($n = 8$): vehicle, 0.0001, 0.001, 0.01, or 0.1 mg/kg L-365,260 or 10.0 mg/kg FG 8205. In the CI-988 experiment, 53 animals had ratios < 0.15 by the end of training. They were assigned to six groups ($n = 8$ or 9): vehicle, 0.01, 0.1, 1.0 or 10.0 mg/kg CI-988 or 10.0 mg/kg FG 8205.

Conditioned emotional response test

Sixty rats were trained in eight standard operant chambers housed in sound and light resistant boxes fitted with ventilating fans. Each chamber was fitted with a retractable lever. The lever was positioned on the front wall 70 mm above the grid floor and 20 mm to the right of a food trough positioned in the middle of the front wall 10 mm above a grid floor. Scrambled electric shock could be delivered to the animal's feet via the grid floor and 45 mg food pellets (Bioserv, Sandown Scientific, Esher, UK) could be delivered into the food trough from a pellet dispenser. A houselight was also positioned in the middle of the front wall 250 mm above the grid floor. On the first 2 days of training rats were placed in the operant chambers for 30 min and food pellets were delivered on average every 60 s. During the next 2 days the rats were placed in the chamber with the lever extended into the box. Each press of the lever delivered a food pellet and the session ended after 30 min or when 30 food pellets had been delivered. During the next two weeks an RI 60 s schedule was introduced and the session length was extended to 60 min. On this schedule, food pellets are available on average every 60 s and were delivered contiguously with a lever press. Initially the RI parameter was set to 7 s and gradually increased (15 s, 30 s), over the 2-week period to 60 s. The animals continued on this schedule for a further 2 weeks, during which lever pressing rates stabilised and did not vary by more than 10% from day to day.

In order to establish tolerance to the lever pressing rate-reducing effects of benzodiazepine receptor agonists, all the rats were given 10.0 mg/kg diazepam 30 min before the beginning of a session for 4 consecutive days. During the first of these sessions lever pressing rates were reduced to approximately 20% of the baseline lever pressing rate, but by the fourth session they had returned to approximately pre-diazepam lever pressing rates. Forty-eight rats with lever pressing rates closest to their pre-diazepam rates were selected from the 60 trained rats and served as subjects for the drug-testing sessions. Following the induction of tolerance to the rate-decreasing effects of diazepam, conditioned-suppression training began. Once between minutes 15 and 25, and minutes 35 and 45 into the session the houselight was switched on for 60 s, and 1 s before it was switched off a 0.4-mA scrambled electric shock was delivered to the rat's feet. The electric shock and the light then terminated together. During the 'light-on' period food pellets were delivered as normal. This procedure readily established conditioned suppression of lever pressing during the period when the light was on. Suppression ratios were calculated as described for the CSD procedure using the lever pressing rates 1 min immediately before and during illumination of the light (CS).

When conditioned suppression had been established (normally no more than two sessions were required), the probability of a shock occurring after a light presentation was reduced from 1.0 to 0.1, a level that, in general, maintained suppression ratios at less than 0.15. The day before each test day, the rats with suppression ratios less than 0.15 were randomly assigned to their respective drug groups and an analysis of variance was performed on the suppression ratios and mean lever pressing rates to ensure that there were no a priori significant differences between the drug groups.

The sensitivity of the test to benzodiazepine receptor agonists was established using bretazenil. The training criteria was met by 43 of the 48 rats trained and they were assigned to five groups (n = 8 or 9): vehicle, 0.1, 0.3, 1.0 or 3.0 mg/kg of bretazenil. In the L-740,093 experiment, 39 animals met the criterion and were assigned to one of five treatment groups (n = 7 or 8): vehicle, 0.1, 0.3, or 1.0 mg/kg L-740,093 or 3.0 mg/kg diazepam. The effects of L-365,260 were assessed in two experiments: in the first, 48 animals were assigned to five treatment groups (n = 8): vehicle, 0.01, 0.1 or 1.0 mg/kg L-365,260 or 3.0 mg/kg bretazenil; in the second 39 animals were assigned to five treatment groups (n = 7 or 8): vehicle, 0.3, 1.0 or 3.0 mg/kg L-365,260 or 3.0 mg/kg bretazenil. The effects of CI-988 were also evaluated in two experiments: In the first, 40 animals were assigned to five groups (n = 8): vehicle, 0.1, 0.3, or 1.0 mg/kg CI-988 or 3.0 mg/kg bretazenil; In the second, 42 animals were assigned to five groups (n = 8 or 9): vehicle, 1.0, 3.0 or 10.0 mg/kg CI-988 or 3.0 mg/kg bretazenil.

Squirrel monkey conflict procedure

Each monkey was restrained at the waist in a Perspex chair and placed in an operant box controlled by a BBC Master Computer running the real time control language, Spider (Paul Fray Ltd. Cambridge). The monkey faced a retractable lever, a red and a white stimulus lamp and a food hopper into which banana flavoured rewards (Bioserv, Sandown Scientific, Esher, UK) were delivered. The distal portion of the monkey's shaved tail was held in a Perspex stock and electric shock delivered via two brass electrodes resting on the tail (370 V (peak) AC, 100 Hz, 500 ms, 0.1–5 mA).

The paradigm was based on that described by Weissman et al. (1984). Each session comprised five alternating cycles of unpunished and punished responding. Unpunished components were signalled by the illumination of the white lamp. During these components every 30th lever press resulted in the delivery of a banana pellet (FR30). After 3 min had elapsed the white light was extinguished and a 30-s timeout period commenced during which lever pressing was not rewarded. At the end of this time the red lamp was illuminated and every 30th lever press now resulted in the simultaneous delivery of a food pellet accompanied by a brief electric shock to the tail. The shock intensity was titrated for each individual monkey to the minimum level necessary to maintain consistent suppression of lever pressing. Animals were able completely to avoid electric shocks by withholding lever presses when the red lamp was illuminated. After 3 min the red light was extinguished and a further 30-s timeout ensued before the cycle was repeated.

Monkeys were dosed once per week with test compounds, diazepam (0.1, 0.3 and 1.0 mg/kg, PO), CI-988 (1.0, 3.0 and 10.0 mg/kg) or L-365,260 (1.0, 10.0 and 50.0 mg/kg), 60 min prior to behavioural testing and the total number of responses made during punished and unpunished cycles were recorded. Each monkey received every treatment according to a quasi-Latin square design.

Statistical analysis

The BMDP programs 7D and 2V provided one- and two-way analyses of variance respectively. Post hoc Newman Keuls tests were used to determine group differences ($P < 0.05$).

Results

Elevated plus maze

Figure 1 shows the mean time spent on the open arms of the maze (expressed as a percentage of the total time in the maze, %TIME) and the mean number of open arm entries (ENT) for bretazenil (a), L-740,093 (b), L-365,260 (c) and L-365,260 and CI-988 (d). All the doses of bretazenil administered significantly increased the time the animals spent on the open arms of the maze [$F(4, 54) = 8.49$, $P < 0.0001$]. Similarly, all but

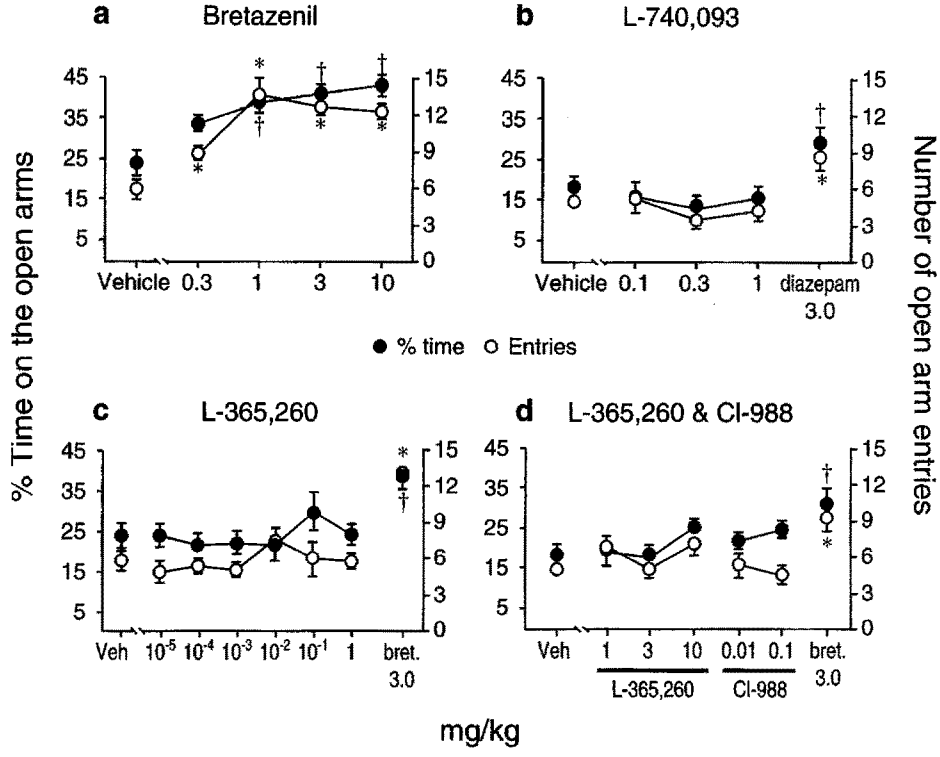

Fig. 1 Effect of CCK_B receptor antagonists in the elevated plus maze test. Results show the mean time spent on the open arms, expressed as a percentage of the total time in the maze and the mean number of open arm entries for each treatment group (± SEM, n=11–12) following a 30 min pretreatment (IP) of vehicle or compound. * Indicates a significant difference in the time spent on the open arms, and a † a significant difference in the number of arm entries compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$)

the 0.3 mg/kg dose of bretazenil significantly increased the number of entries onto the open arms of the maze. None of the doses of L-740,093, L-365,260, or CI-988 administered increased the mean time spent on the open arms or increased the mean number of entries to the open arms. In contrast, the positive control groups given diazepam (L-740,093 experiment) or bretazenil (L-365,260 experiments) spent significantly more time in, and made more entries to, the open arms when compared to the vehicle-treated control group (L-740,093; %TIME: treatment [$F(4, 53) = 3.89$, $P < 0.01$)]; ENT: treatment [$F(4, 53) = 5.33$, $P < 0.01$]). In the first L-365,260 experiment (0.0001–1.0 mg/kg L-365,260,) there was no significant effect of day [$F(1, 71) = 1.46$, $P = 0.23$] nor a treatment × day interaction [$F(5, 71) = 1.92$, $P = 0.10$] and as a consequence the data were collapsed across the day factor and analysed on treatment only: %TIME: [$F(7, 84) = 3.47$, $P < 0.01$)]; ENT: [$F(7, 84) = 8.94$, $P < 0.01$]. In the second L-365,260 experiment (1.0–10.0 mg/kg L-365,260; 0.01 and 1.0 mg/kg CI-988) two animals in the vehicle group and one from the 10.0 mg/kg L-365,260 group fell from the maze during testing and were excluded from the data analysis; %TIME: [$F(6, 74) = 5.47$, $P < 0.01$]; ENT: [$F(6, 74) = 6.75$, $P < 0.01$].

Conditioned suppression of drinking

Figure 2 illustrates the effects of FG 8205 (a), L- 740,093 (b), L-365,260 (c) and CI-988 (d) on mean suppression ratios and on mean Pre-CS and CS lick rates. FG 8205 dose-dependently increased suppression ratios [$F(4, 47) = 19.46$, $P < 0.001$] with doses of 3.0 and 10.0 mg/kg FG 8205 significantly increasing suppression ratios above those of the vehicle-treated controls. In the L-740,093, L-365,260 and CI-988 experiment, analyses of variance revealed main effects of treatment, [$F(4, 50) = 8.62$, $P < 0.001$], [$F(5, 42) = 19.34$, $P < 0.001$] and [$F(5, 47) = 8.66$, $P < 0.001$], respectively. Although FG 8205 induced a significant increase in mean suppression ratios compared to the vehicle-treated control group in all three experiments, none of the doses of L-740,093, L-365,260 or CI-988 administered had any significant effect on suppression ratios. None of the CCK_B receptor antagonists administered affected Pre-CS lick rates (analyses of variance by treatment: L-740,093, [$F(4, 50) = 1.40$, $P = 0.25$]; L-365,260, [$F(5, 42) = 0.13$, $P = 0.36$]; CI-988, [$F(5,47) = 1.26$, $P = 0.30$]), indicating that the compounds did not exert non-specific behavioural effects.

Conditioned emotional response

Figure 3 shows the effects of bretazenil (a), L-740,093 (b), L-365,260 (c) and (d) and CI-988 (e) and (f) on mean suppression ratios and on mean Pre-CS and CS lever pressing rates. Bretazenil had a dose-proportional effect [$F(4, 38) = 3.87$, $P < 0.01$], with doses of 0.3, 1.0 and 3.0 mg/kg significantly increasing suppression ratios compared to vehicle-treated control rats. In the L-740,093 experiment none of the doses administered significantly increased mean suppression ratios, whereas diazepam induced a small, but significant increase in the mean suppression ratio when compared to the vehicle-treated control group (one-way analysis for treatment: [$F(4, 34) = 3.43$, $P < 0.02$]). The effect of

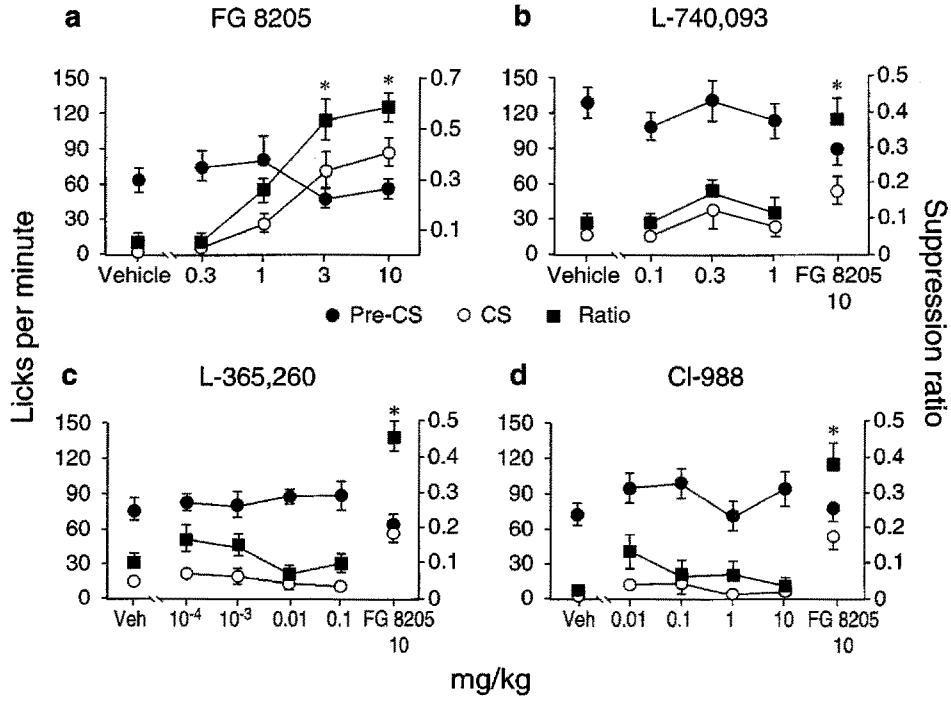

Fig. 2 Effect of CCK_B receptor antagonists in the conditioned suppression of drinking test. The results show the mean number of licks (± SEM, $n = 8$–10) 1 min before (*Pre CS*) and 1 min during (*CS*) the illumination of a light that 48 h earlier predicted the delivery of a mild electric shock, and the suppression ratio for each treatment group. * indicates a significant difference in the suppression ratio compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$)

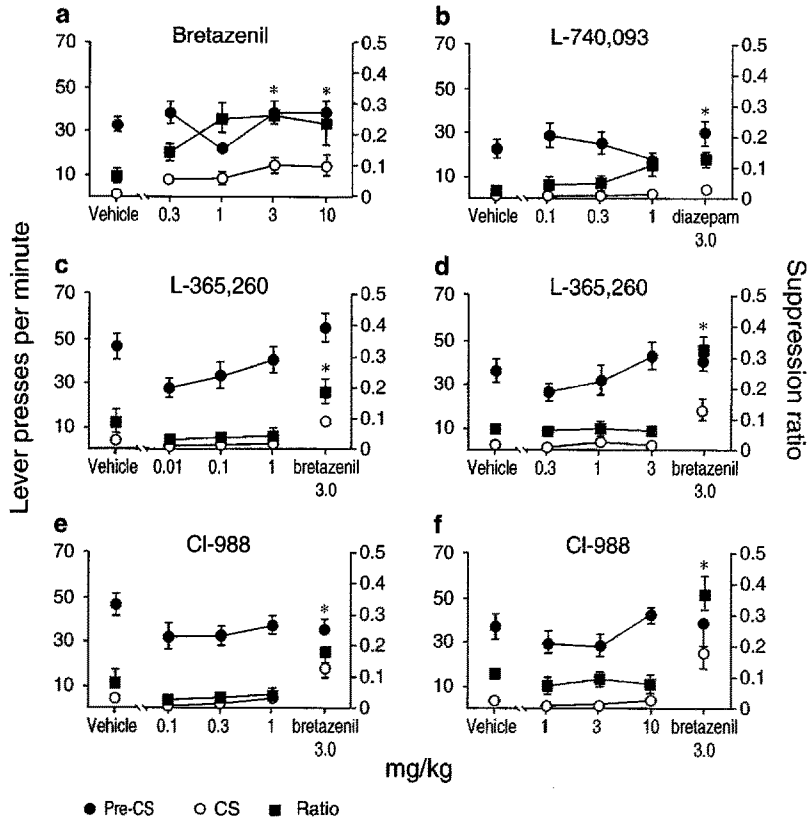

Fig. 3 Effect of CCK_B receptor antagonists in the conditioned emotional response test. The mean number of lever presses (± SEM, $n = 8$–10) 1 min before (*Pre CS*), and 1 min during (*CS*), the illumination of a light that predicted with a probability of 0.1 the delivery of a mild electric shock and the suppression ratio, are shown for each treatment group (Diazepam and L-365,260, PO; CI-988, IM). * Indicates a significant difference in the suppression ratio compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$)

L-365,260 was evaluated in two experiments. In the first experiment, L-365,260 was without effect at all the doses tested (0.01–1.0 mg/kg), although bretazenil did increase the mean suppression ratio compared to the vehicle-treated control group (one way analysis for treatment: [$F(4, 43)=5.05$, $P < 0.002$]). In the second L-365,260 experiment bretazenil again significantly increased the mean suppression ratio compared to the vehicle-treated control group, but the doses of L-365,260 (0.3–3.0 mg/kg) given again had no significant effects on suppression ratios (one-way analysis for treatment: [$F(4, 34) = 11.83$, $P < 0.001$]).

The effect of CI-988 on conditioned suppression was also evaluated in two experiments. In the first, bretazenil significantly increased mean suppression ratios compared to the vehicle-treated control group, but CI-988 (0.1–1.0 mg/kg) was without effect (one-way analysis for treatment: [$F(4, 35) = 13.52$, $P < 0.001$]). In the second experiment a similar pattern of results were observed. Bretazenil again significantly increased the mean suppression ratio compared to the vehicle-treated control group and CI-988 (1.0–10.0 mg/kg) was again without effect (one-way analysis for treatment: [$F(4, 37) = 13.72$, $P < 0.001$]).

Primate Conflict Procedure

On control days when monkeys received no drug treatment, the number of lever presses made during punished cycles was approximately 5% of that during unpunished cycles. Administration of diazepam (0.1–1.0 mg/kg) caused a dose-proportional increase in punished responding up to 70% of unpunished control levels (one-way analysis for treatment: [$F(4, 12) = 6.67$, $P < 0.05$]). In contrast, lever pressing during unpunished cycles was not altered by treatment with diazepam in this dose range [one-way analysis for treatment: $F(4, 12) = 0.77$, $P > 0.05$; Fig. 4a]. Unlike the release of punished lever pressing observed after treatment with diazepam, administration of CI-988 (1.0–10.0 mg/kg) or L-365,260 (1.0–50.0 mg/kg) did not alter lever pressing during either punished [$F(4, 12)$ 0.48, $P > 0.05$] or unpunished [$F(4, 12)$ 0.99, $P > 0.05$] components (Fig. 4b and c).

Discussion

L-740,093 is a highly selective, water soluble CCK_B receptor antagonist which has high bioavailability and readily crosses the blood-brain barrier (Showell et al. 1994). However, in the present study, L-740,093 (0.1–1.0 mg/kg) failed to induce an anxiolytic-like effect in either ethological (EPM) or classical operant (CSD and CER) animal screens for anxiolytic agents. It has been reported previously that CI-988 has dose-proportional anxiolytic-like effects in ethological paradigms (Rataud et al. 1991; Singh et al. 1991) and non-dose-proportional effects in paradigms in which

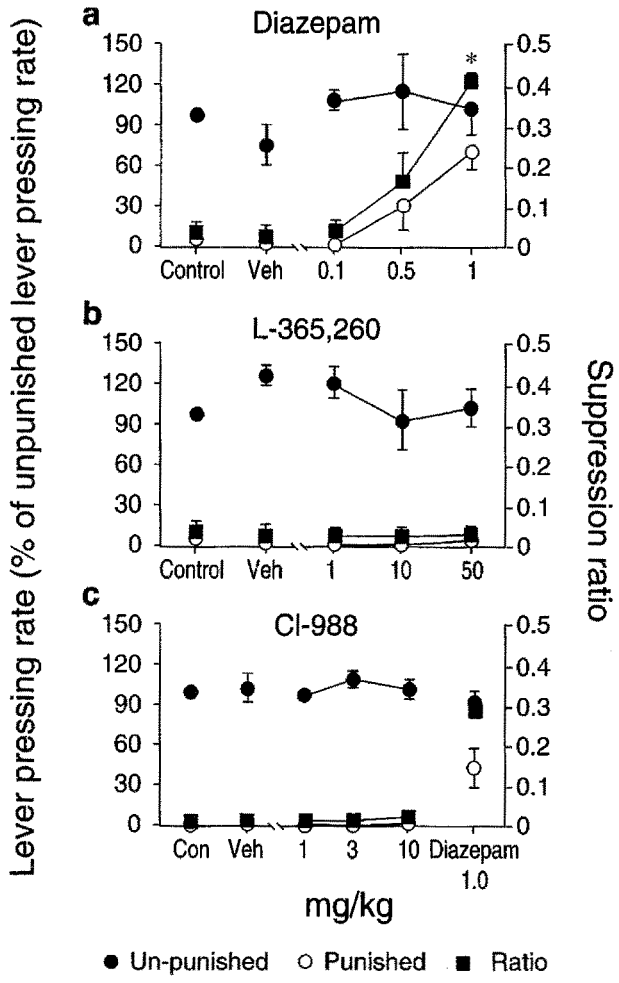

Fig. 4 Effect of CCK_B receptor antagonists in the primate punishment test. The mean number of lever presses (± SEM) expressed as a percentage of the control (non-injected) unpunished lever pressing rate during unpunished and punished components and the suppression ratio, are shown for each treatment group. * Indicates a significant difference in the suppression ratio compared to the vehicle-treated control group (Newman-Keuls tests, $P < 0.05$ assumed)

electric shock is used as a reinforcer (Powell and Barrett 1991: Dooley and Klamt, 1993), but there was no evidence of a similar pattern of results in the present experiments. The CCK_B receptor antagonists CI-988 and L-365,260 were also without effect in the animal tests described above and in a primate anxiolytic screen.

The efficacy of anxiolytic agents in a given animal behavioural test is likely to vary according to the level of fear or anxiety induced by the aversive stimulus used. This is illustrated by the varied activities of compounds used in the present study across a range of anxiolytic paradigms. Thus, with the full benzodiazepine receptor agonist, diazepam, and partial receptor agonist, FG 8205, similar doses of each of the compounds were active in all of the rodent tests here described, whereas the partial receptor agonist, bretazenil, which has lower intrinsic activity at the GABA-A/benzodiazepine receptor than FG 8205 (Tricklebank et al. 1990), was active only in the elevated plus maze and CER tests. This phenomenon is similarly illustrated in the report by Nevins and Anthony (1994), who showed that a number of $5\text{-}HT_3$ receptor antagonists were active in a conditioned fear paradigm only when low intensity shock was used, whereas diazepam was active regardless of shock intensity.

Unlike bretazenil, however, the CCK_B receptor antagonist, L-365,260, did not exhibit significant anxiolytic-like activity in the CSD, CER or elevated plus maze tests. One interpretation of this finding is that the anxiolytic efficacy of CCK_B receptor antagonists may be very low. Clearly, this may be for reasons other than a lack of involvement of the CCK_B receptor in modulating behavioural responses to novelty and fear. Thus, although an antagonist of high affinity and selectivity for the CCK_B receptor in vitro (Chang and Lotti 1986), L-365,260 is very poorly water soluble and, despite being readily able to penetrate the CNS, the failure to see behavioural effects with the compound could reflect the use of a formulation (labrafil) from which the compound may have precipitated on IP injection in a poorly absorbable form. However, pharmacokinetic studies suggest that this is unlikely (Hargreaves and Lin 1992). Moreover, this question was obviated in the present experiments by the use of the water soluble and brain penetrating CCK_B receptor antagonist, L-740,093, at doses that are sufficient to occupy more than 90% of brain CCK_B receptors for the duration of the behavioural assays (Showell et al. 1994; M. Graham, personal communication): this compound was also inactive in all of the rodent anxiolytic tests. CI-988 was similarly without effect, although this may simply reflect the poor ability of the compound to penetrate the CNS (Patel et al. 1994). On this basis, there is clearly no evidence that CCK_B receptor antagonists have any significant activity in conventional anxiolytic screens sensitive to benzodiazepines, regardless of whether they are ethological in nature or derived from the principles of behavioural conditioning.

Nevertheless, it has been previously reported that CI-988 and L-365,260 have dose-proportional effects in ethological animal models of anxiety (Rataud et al. 1991; Singh et al. 1991) and non-dose-proportional effects in animal models in which electric shock is used as a reinforcer (Powell and Barrett, 1991; Singh et al 1991; Dooley and Klamt, 1993). The lack of effect of CCK_B receptor antagonists in the present experiments is not, therefore, readily explained. It is possible that methodological differences may account for the presence, in previous study, and the absence, in the present studies, of an anxiolytic-like effect by CCK_B receptor antagonists. The rodent shock-motivated tests used in the present study employed conditioned emotional response procedures rather than the conditioned punishment procedure described by Singh et al. (1991). Thus, in the present study response suppression was induced by a signal for response-independent shock, whereas the signal in the Singh et al experiment

indicated a period during which responses would be punished (response contingent shock). However, in conditioned punishment procedures a signal for response contingent shock does not usually induce anxiety-like behaviour in well-trained animals, because the delivery of shock is avoidable (Kamin et al. 1963). As "response disinhibition" is one of the behavioural effects of benzodiazepines (Gray 1982), it is possible that the increase in punished responding observed in punished paradigms might be a reflection of response disinhibition rather than a reduction in the magnitude of the fear response during the punished component. If this were the case then it might be expected that CCK_B receptor antagonists would also disinhibit responses in operant procedures such as the differential reinforcement of low rates of responding schedule (DRL) in which the withholding of a response for longer than a specified period (response inhibition) is required for reinforcement. However, neither CI-988 nor L-365,260 had such an effect in a DRL 20s schedule, whereas bretazenil significantly reduced the animals' ability to withhold a response (Dawson et al. unpublished data).

The magnitude of the anxiolytic-like effect of CI-988 in the punished procedure described by Singh et al. (1991) was small (mean increase in punished responding ~ 25%) compared to the effect of chlordiazepoxide (~137%) and occurred only at one dose (0.01 mg/kg). In the Dooley and Klamt (1993) experiment the magnitude of the effect of CI-988 in the mouse four-plate test was again small compared to diazepam, and not dose dependent. Furthermore, between 20 and 40 animals were required at each dose in order to achieve statistical significance. Taken together, these results, and the failure to see anxiolytic-like effects of CCK_B receptor antagonists in the present rodent shock-motivated experiments, suggests that the anxiolytic-like effects of CCK_B receptor antagonists are slight and may only be observed in very limited circumstances. Moreover, the lack of effect of L-365,260 and CI-988 in the present primate conflicit procedure was not surprising, given that their effects in the Powell and Barrett (1991) experiment were confined to a narrow dose range and again the effect was not dose proportional. Furthermore, of the five monkeys included in the Powell and Barret experiment, two were tested with alternating punished/unpunished components, whereas the remaining three were tested with one unpunished and one punished component. Since the animals were tested under different conditions, it would not have been valid statistically to analyse the combined data. Consequently, combination of the data from the two groups of animals for illustrative purposes can give no statistical support to the small increase in punished responding at one dose being meaningful. It is worth noting, however, that in the primate punishment procedure used in the present experiments, a fixed ratio schedule maintained lever pressing, whereas a fixed interval schedule maintained lever pressing in the Powell and Barrett experiment. As drug-induced changes in lever pressing rates can vary depending on schedule maintaining the lever pressing rate, Dews and De Weese (1977) it may be that fixed interval schedules are more sensitive to the rate increasing effects of CCK_B antagonists than fixed ratio schedules. This differential sensitivity may account for the small increase in punished responding seen in the Powell and Barrett experiment.

Although CCK_B receptor antagonists appear to have anxiolytic-like effects in certain ethological screens, the effective dose range varies markedly between paradigms. Thus, for CI-988 active dose ranges vary from 1.0 µg/kg in the mouse light/dark box and 0.01 µg/kg in the marmoset threat test (Costall et al. 1991), to 0.1 mg/kg in the mouse the light/dark box (Singh et al. 1991). In contrast, Hendrie et al. (1993) reported that L-365,260 was without effect in this test. In the elevated plus maze the anxiolytic-like effects of CCK_B receptor antagonists are also inconsistent. Singh et al. (1991) reported that in the rat elevated plus maze the minimum dose of CI-988 that significantly increased the time on the open arms and the number of open arm entries was 0.01 mg/kg (PO). In the mouse elevated plus maze, Rataud et al. (1991) reported that 0.01 mg/kg (IP) of L-365,260 increased the time spent on the open arms and 0.1 mg/kg (IP) significantly increased the number of open arm entries. By contrast, Harro and Vasar (1991) found no effect of L-365,260 in the rat elevated plus, which is in agreement with the lack of effect of L-365,260 observed in the present study.

Thus, even in ethologically based anxiolytic tests, CCK_B receptor antagonists have inconsistent effects which vary markedly in terms of both potency and efficacy between laboratories. Inconsistent effects have also been seen in our own laboratory: on one occasion an anxiolytic-like effect of L-365,260 in the CSD test was detected and reported (Dourish et al. 1991) but as shown in the present work, these findings could not be reliably replicated. One conclusion from the overall results might be that the CCK_B receptor does not have a robust influence on fear-motivated behaviour in rodents or subhuman primates. Such a conclusion is in marked contrast to the induction of panic attacks by the rapid intravenous infusion of CCK_B receptor agonists in man and in some species of primate (De Montigny, 1989; Bradwejn et al. 1990; Palmour et al. 1992), but is consistent with the failure of the rapid intravenous or intracerebral infusion of CCK_4 to disrupt the operant response rate of freely moving rats trained to press a lever for food rewards, a procedure sensitive to the benzodiazepine receptor inverse agonist, FG 7142 (Bayley and Dawson, 1993). Similarly, intravenous administration of pentagastrin failed to induce the behavioural or cardiovascular disturbances previously reported for β–carbolines in rhesus monkeys (Rupniak et al. 1993).

Thus, animal behavioural tests of anxiety do not give much impetus to the use of CCK_B receptor antagonists for the treatment of human panic and/or anxiety disorders where the intensity of anxious behaviour must be great compared to that induced by exposing, for example, an animal to the open arms of an elevated plus maze. It remains to be seen whether the ability of the acute administration of L-365,260 to block CCK_B induced panic in man (Traub et al. 1993) is a good indicator of the therapeutic potential of CCK_B receptor antagonists in panic disorder. Although preliminary findings with repeated dosing of L-365,260 are not encouraging (Kramer et al. 1994), the formulation difficulties associated with the compound prevented a definitive evaluation of the therapeutic utility of CCK_B receptor antagonists. A more rigorous appraisal of the anxiolytic efficacy of these agents in man is awaited, using more suitable compounds. The outcome of such studies may help to clarify the predictive validity of the wide range of anxiolytic screens currently employed in animals.

References

Bayley P, Dawson GR (1993) The effect of i.v. administration of CCK-4 on lever pressing rates of rats on an operant random interval schedule. Br J Pharmacol 108:244P

Bock MG, DiPardo RM, Evans BE, Rittle KE, Whitter WL, Veber DF, Anderson PS, Freidinger RM (1989) Benzodiazepine gastrin and brain cholecystokinin receptor ligands: L-365,260. J Med Chem 32:13–16

Bradwejn J, Koszycki D, Meterissian G (1990) Cholecystokinin-tetrapeptide induces panic attack in patients with panic disorder. Can J Psychiatry 35:83–85

Chang RSL, Lotti VJ (1986) Biochemical and pharmacological characterisation of an extremely potent and selective non-peptide cholecystokinin antagonist. Proc Natl Acad Sci USA 83:4923–4926

Costall B, Domeney AM, Hughes J, Kelly ME, Naylor RJ, Woodruff GN (1991) Anxiolytic effects of CCK-B antagonists. Neuropeptides 19:65–73

De Montigny C (1989) Cholecystokinin-tetrapeptide induces panic attacks in healthy volunteers. Arch Gen Psychiatry 46:511–17

Dews PB, DeWeese (1977) Schedules of reinforcement. In: Iversen LL, Iversen SD, Snyder SH (eds) Handbook of psychopharmacology (7). Plenum Press N.Y. pp 107–150

Dooley DJ, Klamt I (1993) Differential profile of the CCK_Breceptor antagonist CI-988 and diazepam in the four-plate test. Psychopharmacology 112:452–454

Dourish CD, Rycroft W, Dawson GR, Tattersall FD, Iversen SD (1990) Anxiolytic effects of the CCK antagonists devazepide and L-365,260 in a conditioned suppression of drinking model. Eur J Neurosci Suppl 3:38

Gray JA (1982) The neuropsychology of anxiety: an enquiry into the functions of the septo-hippocampal system. Oxford University Press, Oxford

Hargreaves R, Lin J (1992) Blood-brain transfer of the CCK antagonists L-365,260 and devazepide. In: Multiple cholecystokinin receptors in the CNS. Dourish CT, Cooper SJ, Iversen SD, Iversen LL, (eds) Oxford University Press, Oxford

Harro J, Vasar E (1991) Cholecystokinin-induced anxiety: How is it reflected in studies on exploratory behaviour. Neurosci Biochem Rev 15:473–477

Hendrie CA, Neill JC, Shepherd JK, Dourish CT The effects of CCKA and CCKB antagonists on activity in the black/white exploration model of anxiety in mice. Physiol Behav 1993 Oct; 54(4):689–693

Hughes J, Boden P, Costall B, Domeney A, Kelly E, Horwell D, Hunter JC, Pinnock RD, Woodruff GN (1990) Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. Proc Natl Acad Sci USA 87:6728–6732

Jackson A, Tattersall D, Bentley G, Rycroft W, Bourson A, Hargreaves R, Tricklebank M, Iversen SD (1994) An investigation into the discriminative stimulus and reinforcing properties of the CCK_B-receptor antagonist, L-365,260, in rats. Neuropeptides 26:343–353

Kamin L, Brimer C, Black AH (1963) Conditioned suppression as a monitor of fear of the CS in the course of avoidance learning. J Comp Physiol Psychol 56:497–501

Kramer MS, Cutler N, Ballenger J, Patterson W, Mendels J, Chenault A, Shrivasta R, Matzura-Wolfe D, Lines C, Reines S (1995) A placebo controlled trial of L-365,260, a CCK_B antagonist, in panic disorder. Kramer et al. Biol Psychiat 37:462–466

Nevins ME, Anthony EW (1994) Antagonists at the serotonin-$_3$ receptor can reduced the fear-potentiated startle response in the rat: evidence for different types of anxiety. J Pharmacol Exp Ther 268:248–254

Palmour RM, Ervin FR, Bradwejn J, Howbert J (1991) Anxiogenic and cardiovascular effects of CCK-4 in monkeys are blocked by the CCK-B anatgonist LY262691. Soc Neurosci Abstr 17:637.1

Patel S, Chapman KL, Heald A, Smith AJ, Freedman SB (1994) Measurement of central nervous system activity of systemically administered CCK_B receptor antagonists by ex vivo binding. Eur J Pharmacol 253:237–244

Powell KR, Barrett JE (1991) Evaluation of the effects of PD 1343-8 (CI-988), a CCK_B antagonist, on the punished responding of squirrel monkeys. Neuropeptides 10:75–78

Rataud J, Darche F, Piot O, Stutzmann JM, Bohme GA, Blanchard JC (1991) "Anxiolytic" effect of CCK-antagonists on plus-maze behavior in mice. Brain Res 548:315–317

Rupniak NMJ, Schaffer L, Siegl P, Iversen SD (1993) Failure of intravenous pentagastrin challenge to induce panic-like effects in rhesus monkeys. Neuropeptides 25:115–119

Showell GA, Bourrain S, Neduveil JG, Fletcher SR, Baker R, Watt AP, Fletcher AE, Freedman SB, Kemp JA, Marshall GR, Patel S, Smith AJ, Matassa VG (1994) L-740,093: high affinity and potent, water soluble 5-amino-1,4-benzodiazepine CCK_B/gastrin receptor antagonist containing a cationic solubilising group. J Med Chem 37:719–721

Singh L, Field MJ, Hughes J, Menzies R, Oles RJ, Vass AV, Woodruff GN (1991) The behavioural properties of CI-988, a selective cholecystokinin$_B$receptor antagonist. Br J Pharmacol 104:239–245

Traub M, Lines C, Ambrose J (1993) CCK and anxiety in normal volunteers. Br J Clin Pharmacol 36:504P

Tricklebank MD, Honore T, Iversen SD, Kemp Knight JA, Marshall GA, Rupniak NMJ, Singh L, Tye S, Watjen F, Wong EHF (1990) The pharmacological properties of the imidazobenzodiazepine, FG 8205, a novel partial agonist at the benzodiazepine receptor. Br J Pharmacol 101:753–761

Weissman BA, Barrett JE, Brady LS, Witkin JM, Mendelson WB, Paul SM, Skolnick P (1984) Behavioural and neurochemical studies on the anticonflict actions of buspirone. Drug Dev Res 4:93