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Role of cholecystokinin-A and cholecystokinin-B receptors in anxiety

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Summary. Evidence from several laboratories indicates that the anxiogenic effects of cholecystokinin (CCK) are mediated by CCKB receptors. However, it has been reported that CCKA receptors have been found in brain and CCKA antagonists have anxiolytic properties. The aim of this work was to study whether CCKA receptors are also involved in the modulation of anxiety. Anxiogenic effects were observed in the elevated plus maze in rats when pure CCKB receptor agonists (CCK-4 and CCK-8 non-sulfated) or CCK-8S, a CCKB/CCKA agonist, were injected into the lateral ventricle. In contrast, CCK-33, a CCKA agonist or CCK-(1–21) and CCK-(26–29) were ineffective. Furthermore, the anxiogenic effects of CCK-8S were prevented by blocking CCKB but not CCKA receptors. Finally, CCK-33 injected into the postero-medial nucleus accumbens failed to affect the anxiety level of the rats. These results indicate that CCKA receptors are not involved in anxiety, as measured by the paradigms used in this work.

Keywords: CCKA receptor – CCKB receptor – anxiety – elevated plus maze – CCK33

Introduction

Cholecystokinin (CCK), a family of neuropeptides whose members contain a different number of amino acid residues, seems to be involved in anxiety (van Megen et al., 1996). Two types of CCK receptors (CCKA and CCKB receptors) have been demonstrated which differ in their distribution and specificity for the individual members of the CCK family. Thus, the larger forms (i.e. CCK-33 and CCK-58) are ligands for the CCKA receptor whereas the smaller forms (i.e. CCK-4, pentagastrine (CCK-5), or CCK-8 non sulfated (CCK-8NS)), have specificity for the CCKB receptor (Bonetto et al., 1999; van Dijck et al., 1984; Knight et al., 1984; Innis and Snyder, 1980; Moran et al., 1986). However, CCK-8 sulfated (CCK-8S) the most abundant CCK form in the mammalian brain (Dockray, 1976) have a similar specificity for CCKA and CCKB receptors (van Dijck et al., 1984; Wank et al., 1994).

Experimental evidence from several laboratories indicates that the anxiogenic effects of CCK are mediated by CCKB receptors (Harro et al., 1993; van Megen et al., 1996; Ravard and Dourish, 1990), which are widely distributed in brain (Gaudreau et al., 1985; Pélaprat et al., 1987). However, CCKA receptors have been found also in some brain regions which seem to be involved in anxiety such as: nucleus tractus solitarius, area postrema, lateral hipothalamus, central periaqueductal gray, lateral septum and dorsal raphé, among others (Mercer and Beart, 1997; Moran et al., 1986). On the other hand, behavioral experiments have shown that CCKA antagonists have anxiolytic effects in several models (Ballaz et al., 1997; Chopin and Briley, 1993; Hendrie et al., 1993; Bickerdike et al., 1994; Revel et al., 1998; Ravard et al., 1990) and that under certain experimental conditions the activation of CCKA receptors results in anxiety (Daugé et al., 1989). Moreover, Otsuka Long-Evans Tokushima Fatty (OLETF) rats in which the CCKA receptor is missing show an increased anxiogenic behavior in comparison to normal rats (Yamamoto et al., 2000) indicating that CCKA receptors may have not only anxiogenic properties but an anxiolytic role also. The aim of this work was to study whether or not CCKA receptors are involved in the modulation of the CCK induced anxiety.

Material and methods

Male Wistar rats (180–200 g body weight) were used. The animals were kept on a normal 12 : 12 h light-dark cycle and had food and water ad libitum. Rats were used only once.

Surgery

Rats were anesthetized with ketamine (100 mg/kg; i.p.). They were placed in a stereotaxic frame and an incision was made into the scalp to expose the bregma. A unilateral stainless steel cannula guide, made from a 18 gauge syringe needle was implanted into the lateral ventricle using coordinates (AP: −0.8, L: 1.4, V: 3.8) relative to bregma and skull. In some experiments, bilateral guide cannulae (26 gauge) were implanted 1 mm above postero-medial nucleus accumbens (AP: +1.2, L: ±1.5, V: 6.0). As a reference the stereotaxic atlas of Paxinos and Watson (1986) was used. Cannulae guides were kept in place with stainless steel screws and dental acrylic cement. After surgery, the animals received an intramuscular antibiotic injection (Benzetacil V-Fortificado; Fort Dodge Animal Health Labs) to prevent infection and were returned to the animal house for recovery. During the recovery period (8 days) the animals were handled daily (2–4 min) by the investigator responsible to perform the behavioral experiments.

Injection procedure

One day before the experiment the rats were kept in the experimental room without food but with water ad libitum. The day of the experiment the CCK peptide (9 fmol), dissolved in 3 μl of an artificial cerebrospinal fluid (CSF), was injected through the cannula guide into the lateral ventricle using a Hamilton microsyringe. The same volume of artificial CSF was injected to the control group. In all cases the experimenter was unaware of the composition of the solution injected. The intracerebral injections (9 fmol CCK peptide in 0.2 μl artificial CSF) were made with injection cannulae constructed as described by Peterson (1998) using 36 gauge stainless steel tubing connected to a Hamilton microsyringe by PE50 polyethylene tubing. The fully inserted injection cannulae protruded 1 mm beyond the guide cannula to reach the postero-medial nucleus accumbens. CCK peptides or artificial CSF were injected in a constant volume (0.1 μl/min) using an infusion pump (KdScientific). After the injection, the injection cannulae were kept in position for 30 s to allow for diffusion. Behavioral tests started 15 min after the CCK injection. In some experiments, L-365,260 and L-364,718 were injected into the lateral ventricle (900 fmol in 3 μl) 15 min before the administration of CCK-8S.

Behavioral experiments

All experiments were carried out in a special room equipped with video-recording facilities. The apparatuses used for the evaluation of the different behaviors were placed beneath the video camera and the corresponding behavior was video-recorded in the absence of any observer.

Locomotor activity. It was measured in an open field test (40 × 40 × 30 cm) divided in 16 squares (10 × 10 cm). The rat was placed in one of the corners and allowed to explore the arena for 5 min. The number of square crossings determined as the number of instances that at least a $^3/_4$ part of the rat body reached a following square was counted from the corresponding video-recordings.

Elevated plus maze test. A wooden maze built as described by Pellow et al. (1985) was used. The maze was elevated 50 cm from the floor and contained two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) with an open roof opposite each other. An open square (10 × 10 cm) was formed at the intersection of the arms. Rats were placed on the open square facing an open arm and the exploration of the maze was video-recorded for 5 min. The cumulative time spent on the open arms, as well as, the number of entries into the open and closed arms was evaluated from the corresponding videotapes. Entry into an arm was considered when the rats placed their four paws into the arm. In addition, other types of behaviors displayed in the maze such as the cumulative time spent on the central square, the number of fecal boluses, the elongation of the rat from the central square into any of the open arms and the latency from the beginning of the experiment to the first entry into any arm of the maze were also evaluated. At the end of the test the locomotor activity was evaluated as described above.

Four hole box test. The test was carried out as described by Derrien et al. (1993) but using a wooden square box (45 × 45 × 24 cm) with an open roof and painted in black. As described, the cage contained a small corridor of 5 cm in each of the corners that was extended by a tube (3.5 cm diameter × 8 cm length) placed 4 cm above the floor. Photoelectric cells were placed at the entrance of the tubes to register the number and duration of the hole visits. During the experiments the four hole box was illuminated (170 lux) from above. Rats were placed at the middle of the box and their behavior was recorded for 15 min. At the end of the test period the behavior of the rats on the elevated plus maze was studied as described above.

In order to evaluate the behavior of the rats, each animal was subjected always to the elevated plus maze first and immediately after to the open-field test. When the four hole box paradigm was used, this test preceded the successive evaluation of the behavior in the elevated plus maze and in the open-field tests.

Histological examination

The placement of the cannulae was verified on coronal sections (40 μm) made with a kryostat and stained with cresyl violet. Only rats with correct cannulae placement were included in this study.

Statistical evaluation

The results were expressed as mean ± SEM. When more than three groups were compared against each other, their were evaluated by one-way ANOVA analysis followed when needed by the Tukey's test for multiple comparisons. Mann-Whitney "U"-test was employed to compare a single experimental group against its control. A level of $p < 0.05$ for statistical significance was set in all cases. GraphPad Prism (Version 3.0) software was used for all calculations.

Material

CCK-8S, CCK-4, CCK-(26–29) and CCK-(1–21) were purchased to Sigma Chemical Co (St Louis Mo, USA). CCK-8NS was obtained from Peninsula Laboratories (California, USA). CCK-33 came from Peptide Institute (Osaka, Japan). L-365,260 and L-364,718 were a gift from Merck Sharp and Dohme (Essex, UK).

Results

Elevated plus maze test

In order to study the involvement of CCKA and CCKB receptors in anxiety several CCK forms with different receptor specificity were injected into the lateral ventricle of the rat and their anxiogenic properties were assessed in the elevated plus maze. As shown in Fig. 1A CCK-4, CCK-8NS and CCK-8S decreased the

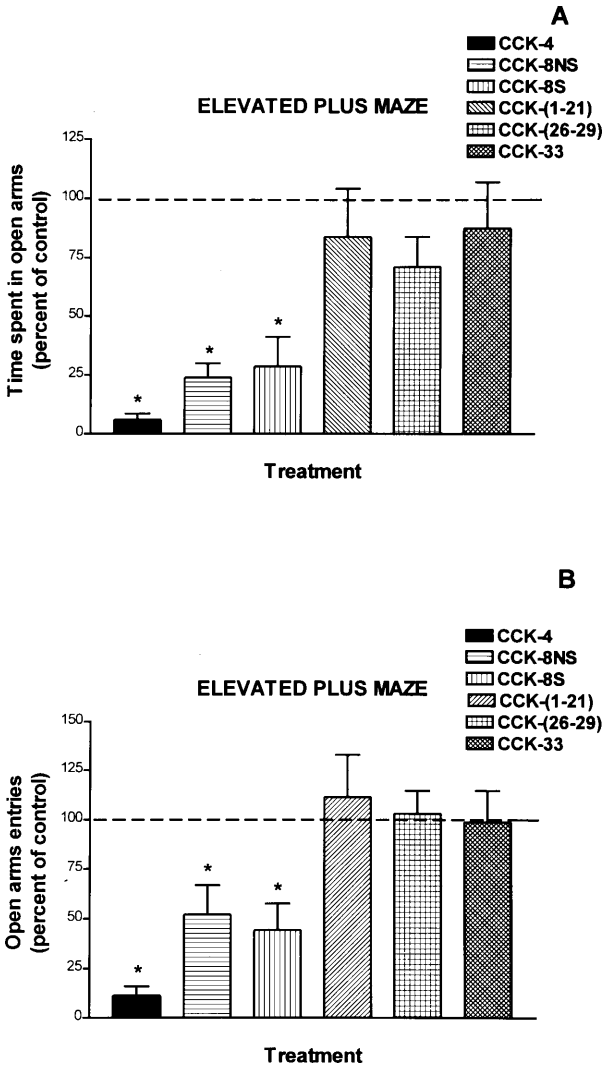


Fig. 1. Effects of different CCK-forms on the exploratory behavior of rats in the elevated plus maze test. The exploratory behavior of different groups of rats was evaluated for 5 min in the elevated plus maze test 15 min after the intraventricular injection of each CCK form and compared with its respective control group. Results are means ± SEM of 12–16 rats in each control and experimental group. Statistical significant differences (*$p < 0.05$) were found when the CCK-4; CCK-8NS and CCK-8S groups were compared to their respective control groups (Mann-Whitney "U" test). **A** shows the time spent by the rats on the open arms of the maze expressed as a percent of their respective control. **B** shows the number of entries into the open arms of the maze as a percent of the respective control values. For methodological and statistical details see Material and methods

time spent by the rat on the open arms of the maze in comparison with their respective control group, being CCK-4 the most effective form. In contrast, neither CCK-33, CCK-(26–29) nor CCK-(1–21) had any effect on this parameter. No significant effects were also observed on the number of entries into the open + closed arms of the maze (total arm entries; data not shown). However, in agreement with the effects of the different CCK forms on the time spent on the open arms CCK-4, CCK-8NS and CCK-8S decreased significantly the number of entries of the rats into the open arms of the maze in comparison with their respective control group (Fig. 1B). No effects on the cumulative time spent in the central square of the maze, on the number of fecal boluses, on the number of elongations from the central square into the open arms or the latency to the first entrance to any arm of the maze were observed with any of the CCK forms studied (data not shown).

To further study the involvement of CCKA receptors in anxiety, the effects of the intraventricular injections of the CCKA (L-364,718) (Chang and Lotti, 1986) and CCKB (L-365,260) (Lotti and Chang, 1989) receptor antagonists were studied on the anxiogenic effects of CCK-8S, a mixed CCKA/CCKB receptor agonist (van Dijck et al., 1984; Wank et al., 1994). As shown in Fig. 2A only L-365,260 prevented the CCK-8S-induced decrease on the percentage of time spent by the rat on the open arms of the maze. Similar results were found on the CCK-8S-induced decrease of entries into the open arms of the maze (Fig. 2B). No effects were noticed on the number of open arm entries (not shown) and on the percentage of time spent on the open arms of the maze when either L-365,260 or L-364,718 were injected alone into the lateral ventricle of the rat (Fig. 2C).

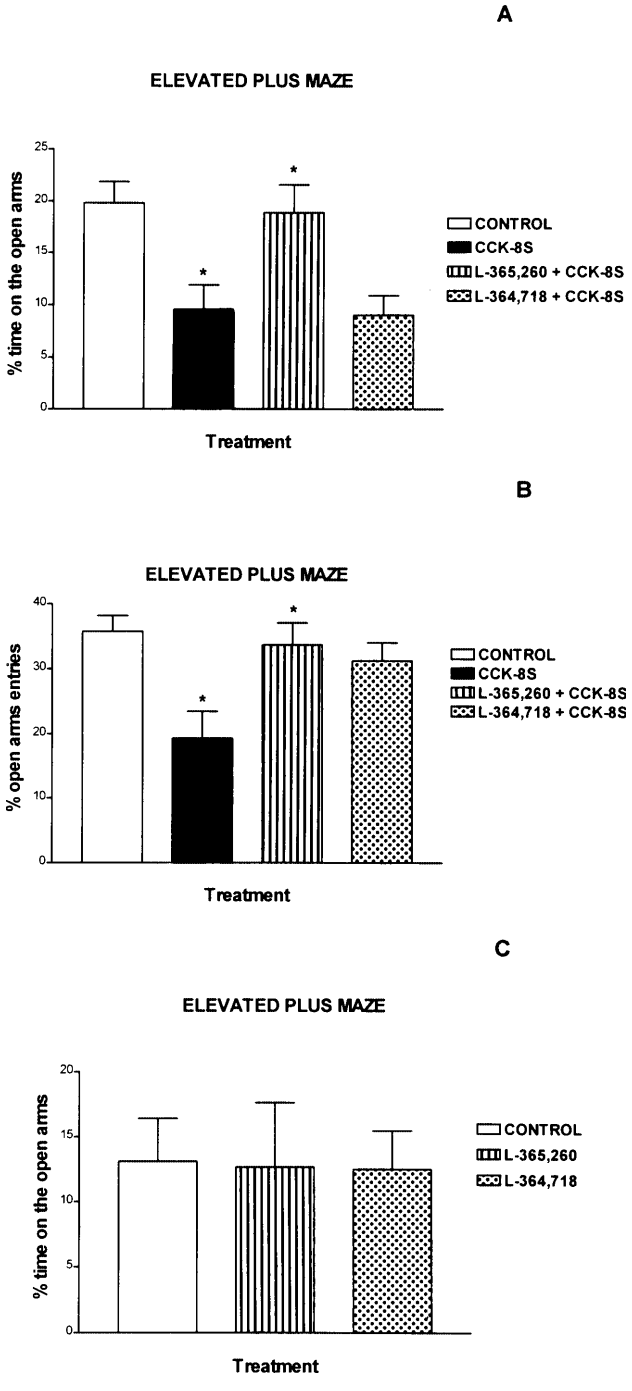
Four hole box + elevated plus maze test

Since it has been reported that the microinjection of CCK-8S into the postero-medial nucleus accumbens decreases the rat exploratory behavior in the four hole box and the elevated plus maze paradigms, and that this effect is prevented by the previous blockade of the CCKA receptor with L-364,718 (Daugé et al., 1989) we injected CCK-33 into the same region and studied the exploratory activity of the rats in the elevated plus maze test only or immediately after been evaluated in the four hole box paradigm. In the four hole test, CCK-33 did not to modify both the number of hole visits (Fig. 3A) and the time spent in the visits (Fig. 3B). On the other hand, CCK-33 failed also to affect the exploratory behavior in the elevated plus maze test in rats previously submitted or not to the four hole box test (Fig. 3C). The histological evaluation indicated, that in the animals used in this experiment,

all the tips of the injection cannulae were located within the postero-medial nucleus accumbens (Fig. 4).

Locomotor activity

None of the CCK forms studied nor L-365,260 and L-364,718 produced any significant change in the number of square crossings in comparison to their respective controls (not shown).



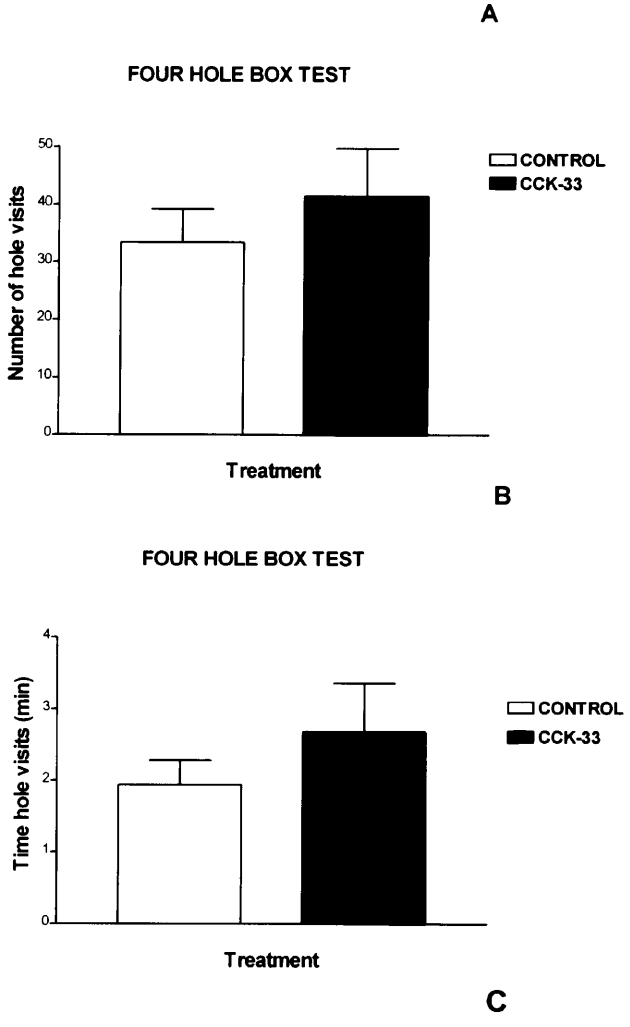
Discussion

In order to ascertain whether CCKA receptors have a role in the anxiogenic properties of CCK, we studied under the same experimental conditions the effects of different CCK forms which differ in their receptor selectivity. We studied also, for the first time, the potential anxiogenic properties of CCK-(26–29) and CCK-(1–21), which is involved in lipolysis (Richter and Schwandt, 1989). In order to keep the specificity of the different CCK forms used and to avoid side-effects, we used in this study a low (9 fmol), but anxiogenic dose of each peptide (Daugé et al., 1989). The elevated plus maze test was used because of its sensitivity to detect the anxiogenic effects of CCK (Biro et al., 1997; Daugé et al., 1989; Rex et al., 1994; Harro and Vasar, 1991).

Our results (Fig. 1A and 1B), in agreement with the work of other laboratories (Biro et al., 1997; Daugé et al., 1989; Rex et al., 1994; Vasar et al., 1992), indicate that CCK-4, CCK-8NS and CCK-8S which recognize CCKB receptors (van Dijck et al.; Innis and Snyder, 1980; Knight et al., 1984; van Megen et al., 1996) have an anxiogenic profile since all of them decreased the exploratory activity of the rats in the elevated plus maze test. Furthermore, in agreement also with Rex et

Fig. 2. Effects of L-365,260 and L-364,718 on the CCK-8S-induced decreased exploration of rats in the elevated plus maze test. **A** L-365,260 but not L-364,718 prevented the decrease in the time spent by the rats on the open arms of the maze induced by the intraventricular administration of CCK-8S. Results are expressed as a percentage of the total time of the test. Values are means ± SEM; n = 20 for the control and 13–25 for each of the CCK treated groups. One way ANOVA: $F(3,69) = 6.084$; $p < 0.05$. Tukey's post-hoc test showed significant differences (*$p < 0.05$) when the CCK-8S alone group was compared to the control group or the L-365,260 + CCK-8S group was compared to the CCK-8S alone group. **B** L-365,260 and L-364,718 reduced the decrease of the entries into the open arms of the maze induced by the intraventricular administration of CCK-8S, expressed as the percentage of the entries into the open arms relative to the entries into the open + closed arms of the maze (total entries), but only the effects of L-365,260 were statistically significant. Values are means ± SEM; n = 20 for the control and 13–25 for each of the CCK treated groups. One-way ANOVA $F(3,69) = 5.209$; $p < 0.05$. Tukey's post-hoc test showed significant differences (*$p < 0.05$) when the CCK-8S alone group was compared to the control group and L-365,260 + CCK-8S group was compared to the CCK-8S alone group. **C** No statistical significant differences on the time spent by the rats on the open arms of the maze were observed (One way ANOVA: $F(2,17) = 0.0075$; N. S. when L-365,260 or L-364,718 were administered alone. Values are means ± SEM; n = 6–7. In all cases (2A, 2B, 2C) CCK antagonists (900 fmol) were injected intraventricularly. CCK antagonists were administered 15 min before the injection of CCK-8S (9 fmol)

al. (1994), CCK-4 was the most anxiogenic CCK form. In contrast, the intraventricular administration of CCK-33 which activates CCKA receptors (Innis and Snyder, 1980; Sandberg et al., 1988; Wisiniewska, 1998) failed to modify any of these parameters (Fig. 1A and B) indicating a lack of anxiogenic effects and suggesting that CCKA-receptors are not involved in anxiety as it has been suggested by other workers (Ballaz et al., 1997; Bickerdike et al., 1994; Chopin and Briley, 1993; Daugé et al., 1989; Hendrie et al., 1993; Ravard et al., 1990; Revel et al., 1998; Yamamoto et al., 2000). The possibility that CCK-33 had not reached its receptors is unlikely since anxiety was induced for all CCKB agonists injected into the lateral ventricle. The lack of effects of CCK-(26–29) and CCK-(1–21) (Fig. 1A and B) in contrast to the anxiogenic effects of CCK-4 and CCK-8, either



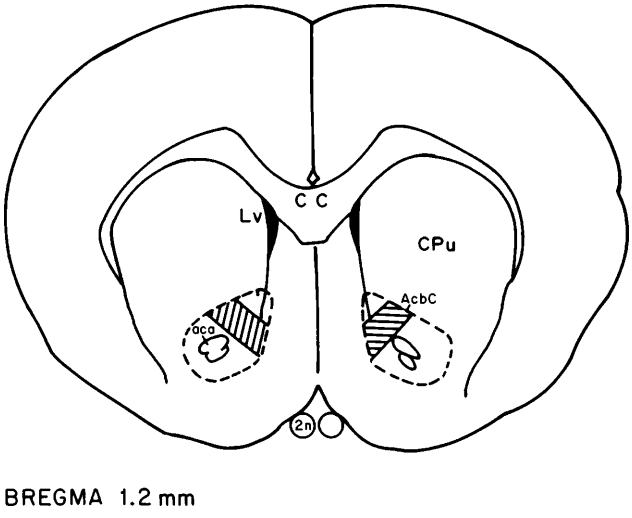


Fig. 4. Schematic localization of the injection sites within the nucleus accumbens. Tips of the injection cannulae were all located within the hatched area indicated in the drawing. Distance from bregma was taken from Paxinos and Watson (1986). *aca*, anterior commissure; *AcbC*, nucleus accumbens; *cc*, corpus callosum; *Cpu*, caudatus-putamen; *LV*, lateral ventricle

Fig. 3. Effects of the intra-accumbens administration of CCK-33 on the exploratory activity of rats in the four hole box and in the elevated plus maze tests. CCK-33 (9 fmol) was injected into the postero-medial nucleus accumbens and 15 min afterwards the behavior of the rats was evaluated for 15 min in the four hole box test or for 5 min in the elevated plus maze. In some experiments one group of rats after being evaluated in the four hole box paradigm (15 min) was tested immediately in the elevated plus maze test (5 min). No statistical significant effects were observed ($p > 0.05$; Mann-Whitney "U"-test) either on the hole visits (**A**) or their duration (**B**). Values are means ± SEM; n = 9–10. Likewise no statistical significant effects were observed on the time spent by the rats on the open arms of the maze when they were evaluated in the elevated plus maze only (No FHBT) or immediately after being tested in the four hole box paradigm (FHBT) (**C**). Values are means ± SEM, n = 19 for the control and 9 for each of the CCK-33 treated groups. One-way ANOVA: F(2,34) = 0.5557 N. S.

sulfated or not (Fig. 1A and B) together with the anxiogenic properties of pentagastrine (Singh et al., 1991) underlines the importance of the last five C-terminal amino acid residues of the small CCK forms in determining their anxiogenic properties (Rehfeld, 1992). None of the CCK forms tested in this study affected the locomotor activity of the rats since neither changes on the total number of entries into the open + closed arms of the maze, which is considered a measure of the locomotor state of the animals (Pellow et al., 1985), nor changes in the number of square crossings in the locomotor activity test were observed in our experiments (data not shown). As a consequence of the lack of effects on the locomotor activity of the different CCK forms studied, the exploratory activity observed in the elevated plus maze might be only dependent upon the emotional state of the animals treated. In line with the lack of anxiogenic effects of CCK-33, the specific CCKB antagonist, L-365,260 (Lotti and Chang, 1989) but not the CCKA antagonist, L-364,718 (Chang and Lotti, 1986) prevented the decrease in the exploratory activity elicited by the previous administration of CCK-8S, a mixed CCKB/CCKA agonist (Fig. 2A and B) (van Dijck et al., 1984; Wank et al., 1994). No effects on locomotion (not shown) and on the anxiety level of the rats were observed after the intraventricular treatment with L-365,260 or L-364,718 (Fig. 2C).

The lack of anxiogenic effects after the stimulation of CCKA receptors with CCK-33 found in our experiments, although in agreement with results of other workers, which suggest that only CCKB receptors are involved in anxiety (Biro et al., 1997; Daugé et al., 1989; Rex et al., 1994; Vasar et al., 1992), is in conflict with evidence which indicates that the stimulation of CCKA receptors have an anxiogenic role (Ballaz et al., 1997; Bickerdike et al., 1994; Chopin and Briley, 1993; Daugé et al., 1989; Hendrie et al., 1993; Ravard et al., 1990; Revel et al., 1998). The reason for this differences is difficult to explain, but a number of unknown factors may be involved. Thus, practically all the reported evidence, which suggests that CCKA receptors are involved in anxiety has been obtained in experiments in which micromolar doses of CCKA antagonists were injected systemically. However, since CCKA antagonists at high concentrations block CCKA and CCKB receptors (Chang and Lotti, 1986) is then plausible that their anxiolytic effects were due to a CCKB blocking effect in some brain areas or even within the circumventricular organs which lack blood brain barrier and are involved in anxiety (Shekhar and Keim, 1997) rather than an action on CCKA receptors.

Since high levels of CCKA receptors are restricted only to a few brain regions (Mercer and Beart, 1997; Moran et al., 1986) and CCK-33 may has been broken down or inactivated in the cerebro-spinal fluid before reaching its target, this peptide was injected directly into the postero-medial nucleus accumbens and the anxiety state of the animals was evaluated in the four hole box (Derrien et al., 1993) and the elevated plus maze anxiety paradigms. CCKA receptors have been found in this nucleus (Mercer and Beart, 1997) and anxiogenic effects have been reported after the selective injection of femto mol quantities of CCK-8S into the postero-medial nucleus accumbens (Daugé, 1989). Moreover, it was also found that such effects were prevented by the previous i.p. administration of the CCKA antagonist L-364,718 suggesting that CCKA receptors are mediating the anxiogenic effects of CCK-8S (Daugé et al., 1989). In contrast with these results, in our experiments the injection of CCK-33 into the postero-medial nucleus accumbens was not anxiogenic since no changes in the exploratory behavior of the rats were found in the elevated plus maze test alone (Fig. 3C), the four hole box test (Fig. 3A and 3B) or the elevated plus maze test when carried out immediately after the four hole box test (Fig. 3C). The reason for the above differences is unknown, but methodological variations may be involved since in the experiments of Daugé et al. (1989) a different CCKA agonist was used, higher doses of CCK antagonists were given systemically and the rats in their experiments were used twice. It is possible however, that an hitherto unknown subtype of CCKA receptor with no affinity for CCK-33, but with affinity for CCK-8S might be present in the nucleus accumbens.

In conclusion, the results of the present paper do not support the involvement of CCKA receptors in the anxiogenic properties of CCK, as measured behaviorally, in the elevated plus maze or the four hole box paradigms. However, it is still possible that CCKA receptors may have a role in the modulation of some autonomic manifestations of anxiety since a great density of CCKA receptors have been found within regions, such as the nucleus of the solitary tract, the dorsal nucleus of vagus and the nucleus ambiguous (Moran et al., 1986), which control several autonomic functions and it has been shown that the injection of CCK-8S and CCK-33 results in the production

of cardiovascular changes (Härfstrand et al., 1986; Wisiniewska, 1998).

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