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The effects of methyl β-carboline-3-carboxylate on social interaction and locomotor activity when microinjected into the nucleus raphé dorsalis of the rat

S.W. Hindley, A. Hobbs, I.A. Paterson & M.H.T. Roberts[1]

Department of Physiology, University College, Cardiff, CF1 1XL

1 Intraperitoneal and intracerebral injections of methyl β-carboline-3-carboxylate (βCCM) and intracerebral injections of RO 15-1788 were given to rats. The performance of the rats in the social interaction test was measured to determine if changes in social interaction induced by βCCM were mediated in part by the nucleus raphé dorsalis (NRD).

2 Intraperitoneal injections of βCCM, 2 and 4 mg kg^{-1}, reduced social interaction.

3 Intracerebral microinjections of βCCM (10–0.1 ng in 0.5 μl) into the NRD reduced social interaction. Injections outside the NRD did not have this effect.

4 Intracerebral microinjections of RO 15-1788 (1 ng in 0.5 μl) into the NRD had no effect when given alone, but blocked the reduction in social interaction caused by intracerebral or intraperitoneal injections of βCCM.

5 No effect was observed when R 15-1788 was microinjected into sites outside the NRD.

6 Changes in social interaction may reflect changes in anxiety. The NRD may be one of the important sites for the expression of the anxiogenic actions of βCCM.

Introduction

Benzodiazepines have anxiolytic effects in man and increase social interaction in the rat. File (1980) has presented evidence that the change in social interaction may reflect the anxiolytic effects of the drug. A number of β-carbolines which have high affinity for the benzodiazepine receptor have been shown to have actions opposite to those of the benzodiazepines (Braestrup *et al.*, 1983). Some of these β-carboline contragonists, including methyl β-carboline-3-carboxylate (βCCM), have been shown to have behavioural effects in rats which have been interpreted as being due to increased anxiety (File *et al.*, 1982; Corda *et al.*, 1983; Prado de Carvalho *et al.*, 1983). Recently one of these β-carbolines, FG 7142, has been shown to be anxiogenic in man (Dorow *et al.*, 1983). The benzodiazepine receptor antagonist RO 15-1788 [ethyl-8-fluoro-5, 6-dihydro-5-methyl-6-oxo-4H-imidazo (1,5a)(1,4) benzodiazepine-3-carboxylate (Bonetti *et al.*, 1982) has no effects on animal models used to measure anxiety when either low or high doses are given (File *et al.*, 1982; Bonetti *et al.*, 1982; Corda *et al.*, 1983; Prado de Carvalho *et al.*, 1983), though it causes a decrease in social interaction at intermediate doses (File *et al.*, 1982). RO 15-1788 blocks the effects of both the benzodiazepines and the β-carbolines in animal models of anxiety (Bonetti *et al.*, 1982; File *et al.*, 1982; Corda *et al.*, 1983; Prado de Carvalho *et al.*, 1983).

The anxiolytic benzodiazepines cause a decrease in 5-hydroxytryptamine (5-HT) turnover in the cerebral cortex of the rat (Wise *et al.*, 1972; Collinge *et al.*, 1982) and it has been shown that benzodiazepines specifically enhance γ-aminobutyric acid (GABA) responses which inhibit 5-hydroxytryptaminergic cells in the nucleus raphé dorsalis (NRD) (Gallagher, 1978). 5-Hydroxytryptaminergic fibres to the cerebral cortex originate mainly from NRD cells (Jacobs *et al.*, 1978). It has also been shown that microinjections of 30 pg of the benzodiazepine chlordiazepoxide into the NRD of the rat release punishment suppressed behaviour which suggests an anxiolytic action in an animal model of anxiety (Thiébot *et al.*, 1982). It may be that benzodiazepines exert their anxiolytic actions by acting upon a receptor which enhances the efficacy of GABA neurotransmission within the NRD; inhibiting 5-hydroxytryptaminergic cells which project to the forebrain.

The aim of these experiments was to determine the importance of the NRD for the effects of βCCM on social interaction.

[1] Correspondence.

Methods

Experimental animal

Male Wistar rats from the University of Nottingham Animal Breeding Unit were used in all experiments. Five rats were housed in each cage and the rats were given free access to food and water. The rats were kept in a room with a 12 h light, 12 h dark cycle. The lights switched on at 07 h 00 min and off at 19 h 00 min.

Surgical procedures

Male Wistar rats, 200–250 g, were anaesthetized with sodium pentobarbitone, 60 mg kg^{-1}, and the head placed in a stereotaxic frame. The incisor bar was set at 5 mm above the interaural line and coordinates established according to the atlas of Pellegrino *et al.* (1979). The scalp was incised, a hole drilled and a 23 gauge stainless steel guide cannula inserted at an angle of 24° to the vertical so that the tip was 4 or 6 mm away from the NRD. The cannula was then fixed in place with steel screws and acrylic paste. The animals were housed 5 to a cage and were allowed 7 days recovery before testing.

Social interaction test

The apparatus used for the social interaction test was an open topped box 60 × 60 × 35 cm. The locomotor activity of rats in the box was measured using 2 'Automex' locomotor activity units (Columbus Instruments). The social interaction test was conducted between 19 h 00 min and 21 h 00 min under low light conditions. The observer scoring the behaviour did not know the drug treatment.

Normally the social interaction test is conducted using two animals dosed identically (File, 1980). However, with intracerebral microinjections it is unlikely that the injection sites of any two animals will be the same and this is only determined when the animals have been killed and the brain sectioned. To avoid the complications arising from this, all implanted animals were tested in conjunction with an untreated male Wistar rat of approximately the same weight as the experimental rat. Each test partner was used with only one implanted rat.

Two hours before testing, an implanted rat and its test partner were placed together in the testing box for 10 min. The rats were then removed and the box cleaned. This familiarisation process doubles the active social interaction of the rats in the subsequent exposure to the box without affecting their locomotor activity. This makes the test more sensitive to decreases in social interaction. Intraperitoneal injections of drugs were given either 15 or 5 min before the test. Microinjections were always given 5 min before testing. Microinjections of 0.5 μl of drug or vehicle (saline) were given over a 2 min period using a fine glass needle (70–90 μm outer diameter) (Azami *et al.*, 1980).

The implanted rat and its test partner were replaced in the testing box for 10 min. The active social interaction of the treated rat and the locomotor activity of the pair were scored. The activities scored as active social interaction were grooming, sniffing, close following, mounting and pushing of the partner and boxing or fighting with the partner.

Four days later the procedure was repeated and the implanted animal was given a microinjection of either vehicle or drug so that all implanted animals had received both a control injection and a drug injection. Following this second test the implanted animals received a microinjection of 0.5 μl pontamine sky blue and were killed for histological verification of the microinjection site.

To test the effects of βCCM administered intraperitoneally, naive male Wistar rats were used and were only tested once; they received 1 ml kg^{-1} of either the drug or vehicle (one drop of Tween 80 per ml of saline) 15 or 5 min before the test. Otherwise the testing procedure was as described above, with only one rat of a pair being given an injection.

Histology

Brains were fixed in 4% formyl saline for 48 h. Fifty μm sections were cut and the sections were stained with neutral red. The position of the dye spot was determined under a microscope by an observer who was ignorant of the behavioural response of the rat.

Statistical analysis

For experiments where each animal was tested under control and test conditions statistical comparisons were made using the paired t test on raw data. Otherwise analysis of variance was used.

Results

Intraperitoneal injections of βCCM

Intraperitoneal injections of βCCM, 2 and 4 mg kg^{-1}, 15 min before testing, significantly reduced social interaction (SI) by 50% ($F = 2.959$, 3 and 31 d.f. For 2 mg kg^{-1}, $t = 2.446$, $P < 0.05$; for 4 mg kg^{-1}, $t = 2.355$, $P < 0.05$; see Figure 1). βCCM, 1 mg kg^{-1}, had no significant effect ($t = 0.853$). The scores of the vehicle-treated rats did not differ from the scores of untreated rats (75 ± 15.5 s, $n = 6$). βCCM tended to decrease the locomotor activity (LA) of these rats but at no dose level was this a significant reduction

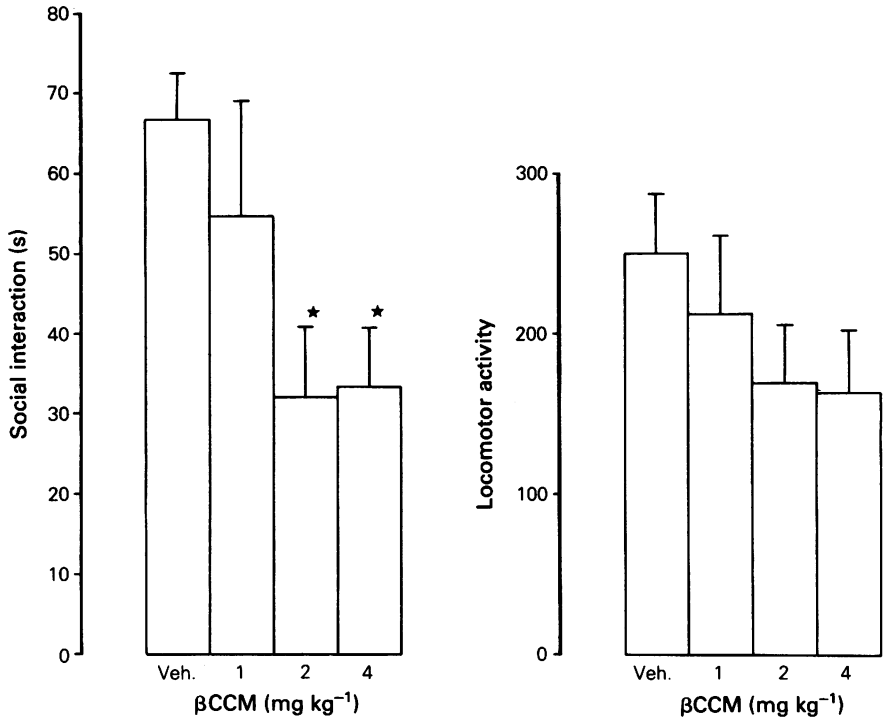

Figure 1 The social interaction and locomotor activity scores of groups of animals given intraperitoneal injections of vehicle (1 drop of Tween 80 per ml of saline, $n = 8$) or methyl-β-carboline-3-carboxylate (βCCM) 1, 2 or 4 mg kg^{-1} ($n = 9$). Injections were given 15 min before testing. The columns represent means and the vertical lines s.e.means. * Indicates a significant difference from vehicle treated rats ($P < 0.05$, analysis of variance).

(analysis of variance, $F = 0.969$, 3 and 31 d.f.). Although βCCM is a convulsant (Jones & Oakley, 1981) none of the rats receiving βCCM in these studies showed any signs of convulsions or tremor.

βCCM, 2 mg kg^{-1} i.p. had the same effect upon social interaction and locomotor activity when administered 15 min before testing (SI = 32.0 ± 8.8s, LA = 169 ± 36, $n = 9$) or 5 min before testing (SI = 35.6 ± 7.4 s, LA = 187 ± 29, $n = 8$).

Table 1 Changes in social interaction and locomotor activity of rats given either saline or methyl β-carboline-3-carboxylate (βCCM) on successive tests 4 days apart

	Change in social interaction	*Change in locomotor activity*
Saline (1 ml kg^{-1}, i.p., $n = 9$)	− 2.6 ± 13.9	− 5 ± 112
βCCM (2 mg kg^{-1}, i.p., $n = 7$)	− 11.5 ± 14.5	− 11 ± 178

The results show means ± s.d. There is no significant change in social interaction or locomotor activity when saline or βCCM are given twice at intervals of 4 days (paired t test).

To determine the variation in social interaction scores and locomotor activity which resulted from repeated testing, and to see if there was any attenuation or enhancement of the effects of βCCM (2 mg kg^{-1} i.p. 5 min before test), 2 groups of rats were given an injection of either saline or drug in two successive tests four days apart (see Table 1). Neither social interaction nor locomotor activity changed over the two tests when either saline or βCCM was given.

Microinjection of βCCM

A total of 50 rats received microinjections of βCCM. The location of these microinjection sites is shown in Figure 2. Twenty three sites lay within NRD. Microinjections of βCCM into all but three of these resulted in a fall in social interaction of more than 28 s (28 s is twice the standard deviation of the variation in social interaction scores between two tests, see Table 1). The other three had no effect on social interaction. Only one of the 27 microinjections outside the NRD caused a fall in social interaction, 9 resulted in an increase in

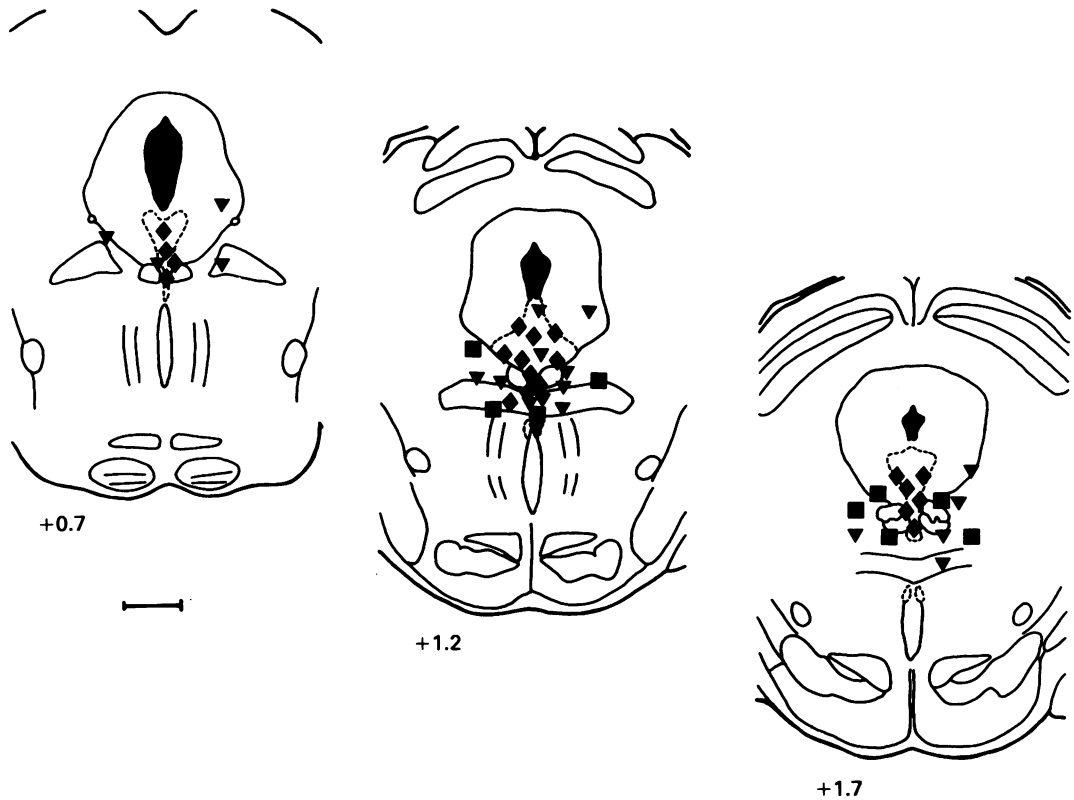

Figure 2 A diagram of the positions of microinjection sites and the effects of microinjections of methyl β-carboline-3-carboxylate (βCCM) on social interaction scores. The diagram shows 3 coronal sections of the rat brain at 0.7, 1.2 and 1.7 mm anterior to the interaural line. (▼) Represent sites where βCCM had no effect, (◆) represent sites where βCCM reduced social interaction by more than 28 s and (■) where βCCM increased social interaction by more than 28 s. Twenty eight seconds was chosen as the cut off as this is twice the standard deviation of the variation in social interaction scores between two tests (see Table 1). The calibration bar represents 1 mm. The sections of the brain are taken from Paxinos & Watson (1982).

social interaction, and 17 microinjections had no effect (Table 2).

Table 2 A contingency table of the effects of microinjections of methyl β-carboline-3-carboxylate (βCCM) on social interaction

	Decrease in social interaction	*No charge*	*Increase in social interaction*
In NRD	20	3	0
Outside NRD	1	17	9

A decrease or increase in social interaction was defined as a change of more than 28 s in social interaction from control levels: 28 s is ± 2 s.d. of the mean difference between two tests. The effect of microinjection into the nucleus raphé dorsalis (NRD) was significantly different from the effect of injections outside the NRD ($\chi^2 = 35.9$, $P < 0.001$).

Microinjections of βCCM 10, 1, and 0.1 ng into the NRD resulted in significant decreases of about 50% in social interaction (Figure 3a). βCCM, 0.01 ng, microinjected into the NRD resulted in a non-significant decrease of 32% in social interaction. None of these doses of βCCM had any effect on locomotor activity (Figure 3b). When these animals were given microinjections of 0.5 μl saline their social interaction scores did not differ from the social interaction scores of 6 untreated rats. Microinjections of βCCM outside the NRD had no net effect on social interaction or locomotor activity (Figure 4).

Microinjection of RO 15-1788

A total of 42 rats were used in this study. Microinjection of RO 15-1788 1 ng into the NRD had no effect on either social interaction or locomotor activity (see Table 3A). However, the same dose of RO 15-1788

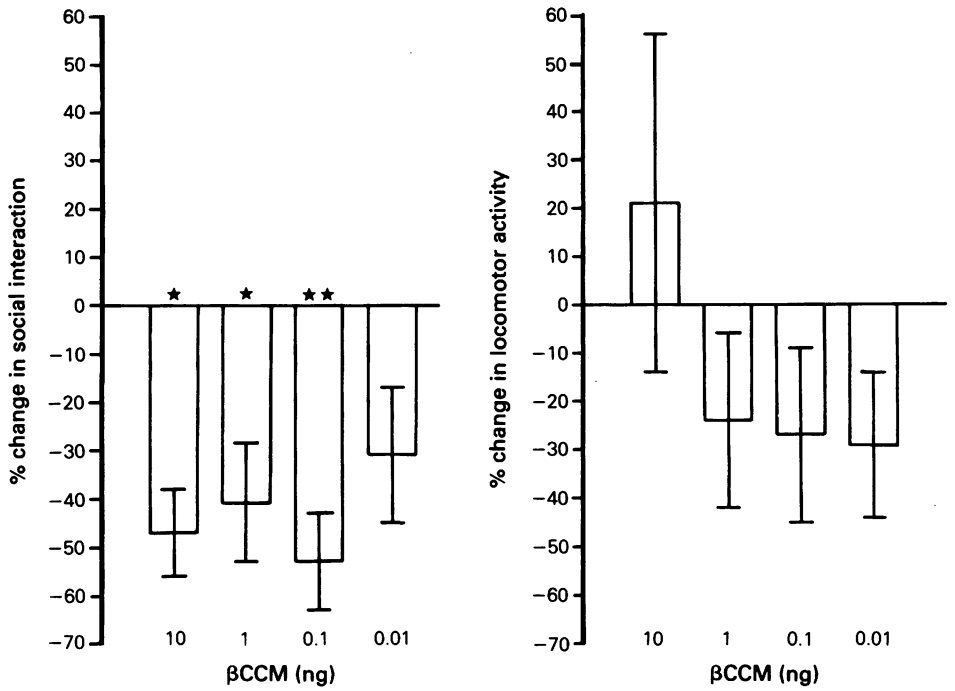

Figure 3 The percentage change in social interaction and locomotor activity scores for groups of animals given intracerebral microinjections into the nucleus raphé dorsalis (NRD) of methyl β-carboline-3-carboxylate (βCCM), 10 ($n = 4$), 1 ($n = 6$), 0.1 ($n = 5$) and 0.01 ($n = 7$) ng. The columns represent means and the vertical lines s.e.means. The control levels of social interaction when saline was microinjected were 74.5 ± 7.3 and for locomotor activity 198 ± 20. * $P < 0.05$, ** $P < 0.01$, paired t test. Statistical analysis was conducted on the data before normalisation.

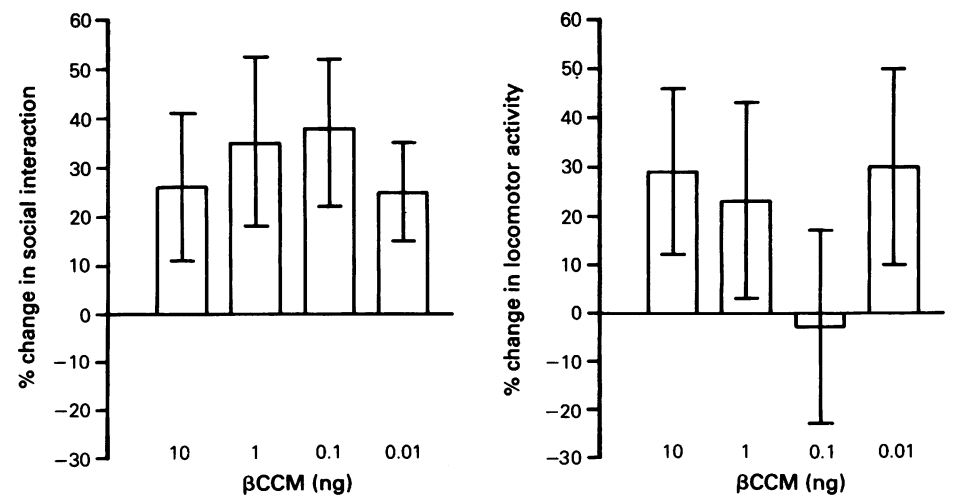

Figure 4 The percentage change in social interaction and locomotor activity scores for groups of animals given intracerebral microinjections close to the nucleus raphé dorsalis (NRD) of methyl β-carboline-3-carboxylate (βCCM), 10 ($n = 6$), 1 ($n = 5$), 0.1 ($n = 6$) and 0.01 ($n = 7$) ng. The columns represent means and the vertical lines s.e.means. The control levels of social interaction when saline was microinjected were 76.3 ± 6.4 and for locomotor activity 215 ± 35. There was no significant change in locomotor activity or social interaction.

Table 3 Effects of RO 15-1788 on social interaction and locomotor activity

	Microinjection	*Social interaction*	*Locomotor activity*
A			
	Saline	53.8 ± 8.5	198 ± 37
	RO 15-1788	57.5 ± 11.8	287 ± 49
B			
	βCCM	39.3 ± 8.5	171 ± 51
	βCCM + RO 15-1788	82.6 ± 5.7*	205 ± 57

Results shows means ± s.e.mean. (A) the effect of saline and 1 ng RO 15-1788 ($n = 6$) microinjected into the nucleus raphé dorsalis. (B) The effect of 0.1 ng methyl β-carboline-3-carboxylate (βCCM) and 0.1 ng βCCM + 1 ng RO 15-1788 ($n = 7$). RO 15-1788 had no effect on social interaction or locomotor activity when given on its own but blocked the reduction of social interaction caused by microinjected βCCM. *$P < 0.001$, paired t test.

significantly attenuated the effects of βCCM (0.1 ng) microinjected into the NRD (see Table 3B). Microinjections of RO 15-1788 outside the NRD had no effect on social interaction or locomotor activity ($n = 12$).

Seventeen rats were given microinjections of 1 ng RO 15-1788 and intraperitoneal injections of βCCM. Six were given 4 mg kg^{-1} βCCM 15 min before testing and 11 were given 2 mg kg^{-1} βCCM 5 min before testing. The results from these two experiments did not differ and the data have been combined. The positions of the microinjection sites are shown in Figure 5.

Microinjections of RO 15-1788, 1 ng, into the NRD significantly blocked the decrease in social interaction caused by intraperitoneal injections of βCCM (see Figure 6). However, microinjection of RO 15-1788 1 ng, into brain regions close to the NRD had no effect on the decrease in social interaction caused by intraperitoneal injections of βCCM. RO 15-1788 had no effect on locomotor activity (Table 4).

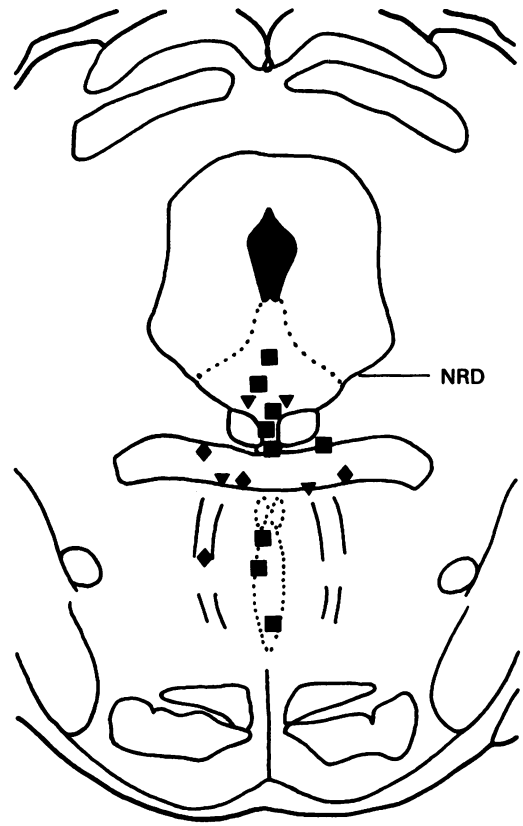

Figure 5 Diagram of the injection sites for intracerebral microinjections of 1 ng RO 15-1788 given with intraperitoneal injections of methyl β-carboline-3-carboxylate (βCCM) and the effects of these microinjections on social interaction. (▼) Represent sites where RO 15-1788 had no effect on social interaction, (◆) where RO 15-1788 decreased social interaction by more than 28 s, and (■) where RO 15-1788 increased social interaction by more than 28 s. Twenty eight seconds is twice the standard deviation of the variation in social interaction between two tests. NRD = nucleus raphé dorsalis.

Table 4 Locomotor activity scores of animals which received microinjections into the nucleus raphé dorsalis (NRD) and outside the NRD (outside)

Position	*Saline + βCCM*	*RO 15-1788 + βCCM*
NRD ($n = 7$)	248 ± 38	293 ± 51
Outside ($n = 10$)	229 ± 46	216 ± 24

Results show means ± s.e.mean. Animals received a microinjection of saline and intraperitoneal methyl β-carboline-3-carboxylate (βCCM 2 or 4 mg kg^{-1}) and on another occasion a microinjection of RO 15-1788 (1 ng) and i.p. βCCM. RO 15-1788 had no effect on locomotor activity.

Discussion

Intraperitoneal injections of βCCM caused a fall in social interaction scores. The effects on locomotor activity were less clear. It may be that βCCM was causing a fall in locomotor activity but the effect was being masked by the fact that the locomotor activity scores were from a treated and an untreated rat. Certainly the closely related compound ethyl β-car-

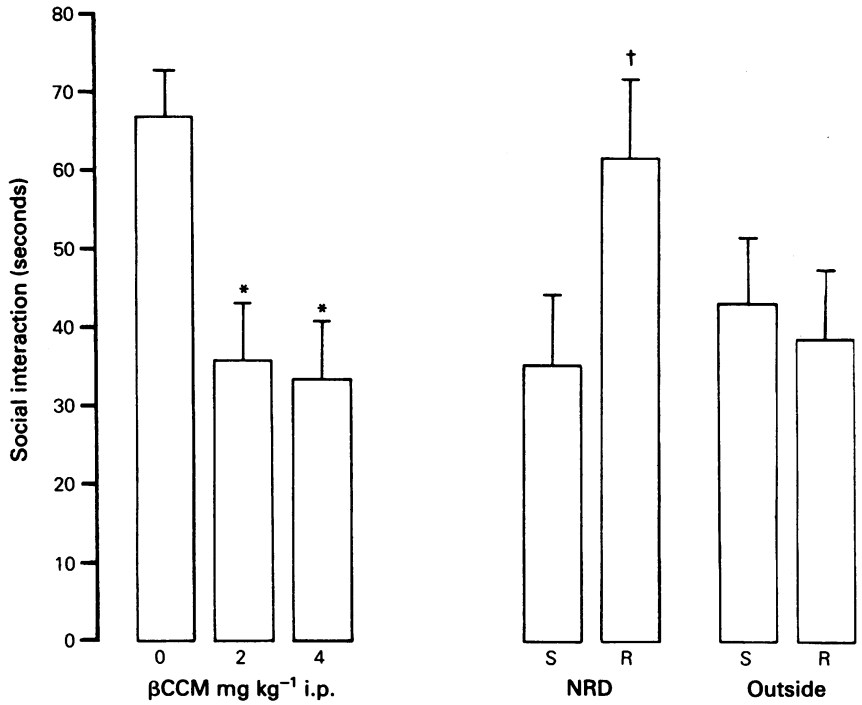

Figure 6 The effects of various treatments on social interaction scores. The first three columns represent the social interaction scores of groups of animals which received intraperitoneal injections of vehicle (0, $n = 8$), 2 mg kg^{-1} methyl β-carboline-3-carboxylate (βCCM) 5 min before testing (2, $n = 8$), or 4 mg kg^{-1} βCCM 15 min before testing (4, $n = 9$). The following 2 pairs of columns represent the social interaction scores of animals which received intraperitoneal injections of βCCM and intracerebral microinjections of saline (S) and 1 ng RO 15-1788 (R) either into the nucleus raphé dorsalis (NRD, $n = 7$) or outside the NRD (outside, $n = 10$). None of these treatments affected locomotor activity. The columns represent means and the vertical lines s.e.means. *Indicates a significant difference from intraperitoneal vehicle treatment ($P < 0.05$, analysis of variance), and † indicates a significant difference from saline microinjections ($P < 0.01$, paired t test).

boline-3-carboxylate reduces locomotor activity as well as social interaction (File *et al.*, 1982). When changes in locomotor activity occur, caution must be used in interpreting changes in social interaction as a decrease in both social interaction and locomotor activity could indicate a sedative effect (File, 1980). However, our experiments showed that microinjections of βCCM into the NRD produced similar changes in social interaction with no tendency towards a reduction of locomotor activity. Furthermore, microinjections of the antagonist RO 15-1788 into the NRD blocked the effects of i.p. βCCM on social interaction without altering locomotor activity. Therefore, it is possible that the change in social interaction is independent of the change in locomotor activity, and that the change in social interaction is not due to a sedative effect.

Twenty of the twenty-one sites where microinjections of βCCM reduced social interaction were in the NRD. There is an apparent lack of a dose-response effect from these microinjections. This may be because the low doses used were supramaximal in effect. Also the range of doses used was large, with ten fold differences in concentration between the doses.

While βCCM had no effect in 17 of the 27 sites outside the NRD there were nine sites ventrolateral to the NRD where βCCM increased social interaction, and there are corresponding sites where RO 15-1788 enhanced the anxiogenic action of βCCM (Figure 5). However, the significance of these sites is not clear as there are overlapping areas where βCCM had no effect on social interaction.

The doses of βCCM (as little as 100 pg) required to decrease social interaction were similar to the doses of chlordiazepoxide injected into the NRD by Thiébot *et al.* (1982) but were much smaller than the doses of diazepam, chlordiazepoxide or midazolam (20–60 μg) required to induce anxiolytic-like effects on microinjection into the amygdala of the rat (Shibata *et al.*, 1982). The small doses used and the lack of effect of

microinjections less than 1 mm away from the NRD make it unlikely that βCCM is producing its effects by being transported or diffusing to a distant site (Clark *et al.*, 1983).

That the NRD is an important site for the reduction of social interaction caused by the systemic administration of βCCM is shown by the attenuation of this response by microinjections of RO 15-1788 into the NRD. This was not a systemic effect of RO 15-1788 as placements outside NRD did not have any action.

Our experiments do not show whether microinjections of RO 15-1788 into the NRD produce a complete antagonism of the fall in social interaction caused by βCCM given intraperitoneally. It is likely that there are other sites in the brain where βCCM also exerts this effect. One such site could be the median raphé nucleus. This nucleus contains 5-hydroxytryptaminergic neurones which project to the forebrain (Jacobs *et al.*, 1978) and has been implicated in animal models of anxiety (Graeff, 1981). A further site of action of βCCM may be at the 5-hydroxytryptaminergic terminals of raphé nuclei cells where benzodiazepine receptor ligands may affect the release of 5-HT (Balfour, 1980). It is possible that some of the effects of βCCM are mediated by some mechanism other than the benzodiazepine receptor. This is unlikely as βCCM has a high affinity for the benzodiazepine binding site and little affinity for other binding sites (Braestrup & Nielsen, 1981).

βCCM has actions opposite to those of the benzodiazepines in the social interaction test and other animal models of anxiety (Corda *et al.*, 1983; Prado de Carvalho *et al.*, 1983). If the effects of the benzodiazepines in these tests reflect their anxiolytic actions then βCCM may have anxiogenic actions. As βCCM attenuates GABA responses elsewhere in the central nervous system (Paterson & Roberts, 1983) it is possible that βCCM is reducing GABA efficacy within the NRD and disinhibiting the projecting 5-hydroxytryptaminergic neurones. This would be consistent with the suggestion that benzodiazepine receptor ligands exert their anxiolytic or anxiogenic actions at least partially by enhancing or attenuating GABA neurotransmission within the NRD.

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