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CR 2249: a New Putative Memory Enhancer. Behavioural Studies on Learning and Memory in Rats and Mice

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Abstract

The effects of S-4-amino-5-[4,4-dimethylcyclohexyl)amino]-5-oxopentanoic acid (CR 2249), a new entity selected from a new series of glutamic acid derivatives, has been investigated in different paradigms for screening nootropics.

CR 2249 ameliorated the memory retention deficit produced by scopolamine in step-through-type passive avoidance in rats and by electroconvulsive shock in step-down-type passive avoidance in mice. CR 2249 was also capable of improving performance in behavioural tests of learning and memory in the absence of cholinergic hypofunction or cognitive deficit. The activity was determined using different passive and active avoidance behavioural test procedures on rats. CR 2249 was active only when given 45 min before training and did not show any effect when administered immediately after the learning training or before the retention trial. No changes in the general behaviour or motor activity of the animals were observed, indicating that CR 2249 effects cannot be attributed to sensory-motor deficit. Microdialysis experiments have shown that CR 2249 significantly increased noradrenaline release in the hippocampus of freely moving rats and reduced 3,4-dihydroxyphenylglycol efflux. These effects have led us to hypothesize that CR 2249 memory effect might be mediated by a direct or indirect action on noradrenergic transmission. These behavioural results suggest that this new agent has clinical application in memory disorders.

In recent decades the search for new drugs active against senile memory disorders has led to the development of several novel classes of active substances. These include the new class of psycho-active drugs, usually denoted nootropics or cognition activators, the prototype of which is piracetam; these drugs facilitate learning and memory in experimentally-induced brain dysfunction, but have little or no behavioural effect under normal conditions. Their mechanism of action is not yet fully understood.

Every kind of drug that can interfere with neurotransmitter functions, e.g. cholinergic (Mondadori & Etienne 1990; Mohammed 1993), dopaminergic (Dismukes & Rake 1972; Davies et al 1974; Marino et al 1990; Lazarova-Bakarova et al 1991), noradrenergic (Dismukes & Rake 1972; Lazarova-Bakarova et al 1991), serotonergic (Altman et al 1984) or GABAergic (Rayevsky & Kharlamov 1983) transmission, is likely to modify some aspects of learning and memory. Biochemical, electrophysiological and pharmacological data have recently led to the suggestion that monoaminergic and cholinergic transmission processes might play a pivotal rule in response to nootropic mechanisms (Nybäck et al 1979; Marino et al 1990; Pugliese et al 1990; Sansone et al 1990; Schmidt 1990; Bhattacharya et al 1993).

In this paper we describe the activity of CR 2249, *S*-4-amino-5-[4,4-dimethylcyclohexyl)amino]-5-oxopentanoic acid (Makovec et al 1995), a glutamic acid derivative carrying a bulky hydrophobic moiety, in some experimental paradigms used for the evaluation of drugs influencing learning and memory.

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Materials and Methods

Animals and drugs

Male Wistar rats, 175–225 g, were used in passive-avoidance, active avoidance, and open field tests; male Wistar rats, 225–250 g, were used for microdialysis experiments and for determination of CR 2249 plasma levels; male CD-1 mice, 30–40 g were used in the step-down passive-avoidance. All animals were obtained from Charles River (Calco, Italy). Animals were housed in plastic cages, with free access to standard laboratory food and water, under controlled conditions of lighting (12-h light : 12-h dark cycle) and temperature (20 ± 1°C). All the behavioural tests were performed in sound-attenuating testing rooms. Before the experiment the animals were left for 30–60 min to become acclimatized to an experimental room.

Noradrenaline, 3,4-dihydroxyphenylglycol, scopolamine hydrochloride and tacrine were from Sigma-Aldrich (Milan, Italy); [^{3}H]noradrenaline (spec. act. 70·2 Ci mmol^{-1}) was from NEN. CR 2249 was dissolved in a minimum quantity of 1 M NaOH and the final volume was made up with saline, with the pH adjusted to approximately 7·0 by addition of a few drops of 0·1 M HCl.

Determination of levels in plasma and in cerebrospinal fluid

Rats were anaesthetized by light ether inhalation and blood was drawn from the abdominal aorta, collected in heparinized tubes and centrifuged at 2000 rev min^{-1} for 10 min. Cerebrospinal fluid was drawn from the fourth ventricle through the occipital bone of the same animals.

Plasma and cerebrospinal fluid were analysed for their CR 2249 content after pre-column derivatization with *o*-phthaldialdehyde using a C_{18} reversed-phase column (Supelcosil LC-18-DB) coupled with fluorimetric detection (excitation wave-

length: 350 nm; emission wavelength: 450 nm). CR 2249 resolution was obtained using a three-solvent discontinuous gradient essentially as described by Lanza et al (1993), with a few modifications. The compositions of the solvents were: solvent A tetrahydrofuran-methanol-0·1 M sodium acetate (pH 5·8), 2 : 20 : 78; solvent B methanol-0·1 M sodium acetate (pH 5·8), 80 : 20; solvent C tetrahydrofuran-methanol-0·1 M sodium acetate (pH 6·0), 2 : 20 : 78. The gradient programme was: 0% A-25% B-75% C for 4 min from the beginning of the programme, linear step to 15% A-85% B-0% C in 2 min, isocratic step for 9 min, linear step to 100% B in 2 min, isocratic step for 17 min. The flow rate was 0·9 mL min^{-1} and the retention time of CR 2249 was 18.7 min.

Receptor binding studies

Binding studies were performed according to the experimental conditions listed in Table 1.

Release studies from slices

The animals were killed by decapitation and the hippocampi were quickly removed. Coronal slices, 0·40 mm thick, were prepared from the hippocampus using a McIlwain tissue chopper. Slices were labelled with 0·01 μM [^{3}H]noradrenaline, 20 min at 37°C, in a medium with the composition (mM): 125 NaCl, 3 KCl, 1·2 $CaCl_2$, 1·2 $MgSO_4$, 1 NaH_2PO_4, 22 $NaHCO_3$ and 10 glucose (aerated with 95% CO_2 and 5% O_2 at 37°C), pH 7·2–7·4. The incubation medium contained the serotonin uptake inhibitor, 6-nitroquipazine (0·1 μM), to prevent possible false labelling of serotonergic terminals. After washing with tracer-free medium, slices were transferred to parallel superfusion chambers (Danuso, Milan, Italy; 1 slice per chamber) and superfused at 1 mL min^{-1}, at 37°C, with a medium from which Mg^{2+} ions were omitted. After 60-min superfusion to equilibrate the system, seventeen 10-min fractions were collected. Samples and superfused slices (dissolved with Soluene) were then counted for radioactivity in a liquid scintillation counter.

CR 2249 was added throughout the experiments starting at 20 min after the end of the equilibration period of perfusion.

Step-through type passive-avoidance in the rat

Acquisition and retention trials were performed in an automated passive-avoidance apparatus (Ugo Basile, Comerio, Italy). The apparatus consisted of a two-compartment box, each compartment measuring 24 × 21 × 21 cm. One was made of transparent perspex and was illuminated by a 12-W light bulb, the other was made of black perspex. The floor of the compartment was made from 19 stainless-steel rods spaced 12 mm apart. The two compartments were separated by an 8 × 8 cm guillotine door, controlled by an electronic device that closed the door as soon as the animal passed entirely beyond it.

Acquisition training. Each rat was placed in the centre of the illuminated compartment facing the wall opposite the closed door. After a 4-s delay, the door was opened and the latency time, i.e. the time taken by the rat to enter with all four paws into the dark compartment, was recorded. Rats with latencies to enter the dark compartment greater than 60 s were discarded.

As soon as the rat entered the dark compartment, the door was closed and an inescapable, scramble foot-shock was delivered through the grid floor for 4 s. Immediately after completion of this training trial, the rat was removed from the dark compartment and put back into its home cage.

Retention test. In the retention test each rat was placed in the illuminated compartment and the same acquisition training procedure was followed except that no shock was applied through the grid floor. The time for each rat to enter the dark compartment was measured as step-through latency. When a rat did not enter the dark compartment for at least 120 s, a score of 120 s as maximum latency time was assigned.

In a first protocol the effect of different foot-shocks (0–0·12 mA) was measured by testing retention at different times (24–72 h) after the acquisition trials.

In the second protocol, in which CR 2249 was tested, we used a foot-shock of 0·09 mA delivered for 4 s in the acquisition training and the retention was measured 72 h after the acquisition trial. To assess the influence of CR 2249 on acquisition, the substance was administered intraperitoneally 45 min before training. To evaluate the effect of CR 2249 on the consolidation process, the compound was administered intraperitoneally immediately after training, whereas its influence on retrieval processes was determined by intraperitoneal injection 45 min before the retention test. Control animals received saline given at the same time as CR 2249. In order to check the basic latency to enter the dark compartment we used two control groups both treated with saline but with just one of the groups receiving a foot-shock.

Table 1. Experimental conditions used for receptor-binding studies.

Receptor	[^{3}H]-Ligand concentration	Tissue and species	Displacing agent for non-specific binding	Reference
NMDA	[^{3}H]CGS 19755 (10 nM)	Forebrain/rat	L-Glutamic acid (1 mM)	Murphy et al (1988)
Kainate	[^{3}H]Kainic Acid (10 nM)	Forebrain/rat	Kainic acid (0·1 mM)	London & Coyle (1979)
Quisqualate	[^{3}H]AMPA (20 nM)	Forebrain/rat	L-Glutamic acid (1 mM)	Murphy et al (1987)
NMDA/glycine	[^{3}H]5,7-dichlorokinurenic acid (10 nM)	Whole brain/rat	D-Serine (0·1 mM)	Baron et al (1991)
NMDA (non-competitive)	[^{3}H]MK-801 (2 nM)	Cortex/rat	MK-801 (0·1 μM)	Foster & Wong (1987)
α_1-Adrenergic	[^{3}H]Prazosin (0·5 nM)	Whole Brain/rat	Prazosin (1 μM)	Miach et al (1980)
α_2-Adrenergic	[^{3}H]Yohimbine (1·5 nM)	Whole Brain/rat	Yohimbine (10 μM)	Brown et al (1990)
Cholecystokinin/CCK_B	[^{3}H]pBC 264 (0·2 nM)	Cortex/guinea pig	CCK-8 (1 μM)	Durieux et al (1992)
5-HT_{1A}	[^{3}H]8-OH-DPAT (0·5 nM)	Hippocampus/rat	5-HT (10 μM)	Hall et al (1985)
5-HT_2	[^{3}H]Ketanserin (0·6 nM)	Prefrontal Cortex/rat	Ketanserin (3 μM)	Leysen et al (1982)
Cholinergic M_1	[^{3}H]Pirenzepine (1 nM)	Forebrain/rat	Atropine (1 μM)	Watson et al (1983)

Step-through type passive-avoidance: scopolamine-induced amnesia in the rat

In the acquisition training of the experiments in which we used scopolamine for memory disruption, a foot-shock of 0·2 mA for 4 s was given and a retention test was performed 24 h after training. Scopolamine (1·5 mg kg^{-1}, i.p.) was administered 30 min before training; CR 2249 and tacrine were administered intraperitoneally 15 min before scopolamine.

Step-down passive-type avoidance in the mouse

The passive-avoidance apparatus consisted of a rectangular 21 × 21 × 40 cm perspex box with a floor made from 29 stainless-steel rods spaced 1·2 cm apart. A Coulbourn Instrument Grid Floor Shocker (Palo Alto, California) was connected to the grid to provide a scrambled foot-shock. A wooden platform (4 × 4 × 4 cm) was placed in the centre of the grid floor. The electroconvulsive shock was delivered by an electroconvulsive unit (ECR unit 7801; Ugo Basile, Comerio, Italy).

Training. Each mouse was placed gently on the wooden platform set in the centre of the grid floor. When the mouse stepped down the platform and placed all its paws on the grid floor, the scrambled constant current shock (0·2 mA) was delivered continuously until the animal returned to the platform to escape the shock. The step-down latency and escape latency were measured. Animals showing step-down latency and escape latency in the criterion range (3–30 s and 3–60 s, respectively) were used for the retention test.

Immediately after training mice received an electroconvulsive shock, a series of 20 mA, 50 Hz impulses each lasting 1 ms at 20-ms intervals, for a total duration of 0·4 s, by means of two electrodes placed on the two corneas.

Retention test. The retention test was performed 24 h after training, in a similar way, except that the electric shock was not applied to the grid floor. Each mouse was placed again on the wooden platform and the step-down latency was recorded with an upper cut-off time of 120 s.

CR 2249 and tacrine were administered orally by gavage 60 min before training.

Step-through active-avoidance conditioning

Apparatus. Active-avoidance learning was performed in a shuttle box apparatus (Ugo Basile, Comerio, Italy) consisting of two identical compartments measuring 24 × 21 × 21 cm separated by an 8 × 8 cm gate. The floor of each compartment consisted of 19 stainless-steel bars spaced 1·2 cm apart. Scrambled constant current shock (0·4 mA) was delivered through the grid floor and acted as an unconditioned stimulus. The conditioned stimulus consisted of a small lamp over the compartment occupied by the rat and of a tone generator mounted over the centre of the apparatus.

Procedure. Each of the 40 rats used (10 animals/group) in the experiment was subjected to five sessions of conditioning avoidance with an interval of 24 h between them. They were given an intraperitoneal injection (5 mL kg^{-1}) of physiological saline or CR 2249 solution once a day, 45 min before each session. Each rat was placed in the shock compartment, submitted to the conditioned stimulus for 3·5 s and immediately afterwards to the unconditioned stimulus for 3·5 s. The conditioned and unconditioned stimuli remained on either until the rat escaped or for a maximum of 7 s. The conditioned and unconditioned stimuli terminated simultaneously with the escape response. After a 23-s inter-trial interval, a new avoidance learning cycle started again, each session consisting of 40 trials. Conditioned avoidance responses, defined as a crossing within 3·5 s of conditioned stimulus, uncoordinated escape responses, defined as a crossing within 3·5 s of unconditioned stimulus and spontaneous inter-trial responses, defined as crossing in the absence of the conditioned stimulus and unconditioned stimulus, were automatically recorded for each session.

Motor activity

Each animal was placed individually in a 60 × 60 × 30 cm box. A television camera was located approximately 1 m above the cage to frame the whole box. Motor activity was measured by means of a Videotrack 512 (View Point, Lyon, France) automated system. The Videotrack system tracks, in real time, the movements of laboratory animals by means of a television camera connected to an image processor and a personal computer.

Animals were randomly assigned to treatment groups; CR 2249 or saline were administered intraperitoneally 45 min before the test. The animals were placed in the box and left there for 10 min. Total distances covered by animals were recorded by computer.

Microdialysis experiments

Animals were anaesthetized with equitesine (pentobarbital 40 mM, chloral hydrate 250 mM, magnesium sulphate 85 mM, propylene glycol 40% v/v, ethanol 10% v/v, distilled water 60% v/v; 3·6 mL kg^{-1}, i.p.) and placed in a stereotaxic frame (David Kopf Instruments). The skull was exposed and a small hole was drilled to enable implantation of a vertical probe into the hippocampus (AP: −5·80 mm; ML: −4·80 mm; DV: 6·80 mm relative to bregma according to the atlas of Paxinos & Watson (1982). The probe was fixed to the skull with two screws and dental cement. Concentric dialysis 'I' probes were made as previously described (Hutson et al 1985) with some modifications (Fedele & Foster 1993). The length of the membrane (Hospal AN 69) exposed to the brain hippocampal tissue was 4.5 mm.

After a 16-h recovery period, modified Ringer's solution (145 mM NaCl, 3·0 mM KCl, 1·26 mM $CaCl_2$, 1·0 mM $MgCl_2$, 1·4 mM Na_2HPO_4, 0·6 mM Na_2HPO_4; pH = 7·4) was continuously perfused through the probe at a rate of 5·0 μL min^{-1} via a fluid swivel (CMA/Microdialysis AB) that enabled relatively unrestricted movement of the animal. Dialysis samples were collected at 20-min intervals in plastic vials (final volume 100 μL). Before starting any experimental manipulation, noradrenaline and 3,4-dihydroxyphenylglycol efflux were monitored for a maximum of 80 min in order to ensure stable baseline values. At the end of the baseline-monitoring period all animals were treated with drugs or saline. The samples were then analysed for their noradrenaline or 3,4-dihydroxyphenylglycol content, by use of high performance liquid chromatography with electrochemical detection. The mobile phase (0·1 M acetate buffer pH 3·75, 0·1 mM EDTA, 0·34 mM octyl sulphonic acid) was filtered and

degassed before pumping at a flow rate of 0·8 mL min^{-1} through a Supelcosil LC-18-DB (150 mm × 4·6 mm i.d.) column. Electrochemical detection was performed using a Coulochem Esa II detector with a model 5011 electrode cell in oxidation/reduction mode with the oxidizing and reducing electrodes set at +375 mV and −325 mV, respectively. In-vitro experiments were performed to test the recovery of noradrenaline and 3,4-dihydroxyphenylglycol through the dialysis probes. In these experiments, dialysis membranes were perfused with Ringer's modified medium, placed in a bath containing the same Ringer's solution and noradrenaline or 3,4-dihydroxyphenylglycol, and perfused at 5 μL min^{-1}. Fractions were collected every 20 min and analysed for noradrenaline and 3,4-dihydroxyphenylglycol. The mean relative recoveries (± s.e.m.) of noradrenaline and 3,4-dihydroxyphenylglycol were 10·78 ± 0·92% (n = 7) and 14·31 ± 1·09% (n = 7), respectively.

Statistics

Passive avoidance latencies were expressed as median with 25–75 percentile and analysed using a survival analysis technique (Kaplan and Meier function) with log-rank test to compare the different treatments. These data included a cut-off time, considered to be a censored observation; the log-rank test is a statistical method for analysing 'time to event' data that might include censored observations. In the active avoidance experiment data were plotted as mean percentage (± s.e.m.) of responses referring to the maximum possible values (i.e. 100%) of 40 responses/day for the conditioned and uncoordinated escape responses. Inter-trial response data were represented merely as mean ± s.e.m. Data were analysed by the analysis of variance split-plot procedure using values transformed as difference from baseline (day 1) levels. Levels of hippocampal noradrenaline and 3,4-dihydroxyphenylglycol were expressed as mean percentage (± s.e.m.) referring to baseline values and compared with Student's *t*-test after obtaining homogeneous variances by means of reciprocal transformation.

Plasma and cerebrospinal fluid levels of CR 2249 were analysed using Siphar/Win (Simed), a software package for pharmacokinetic data analysis; area under the plasma concentration-time curve (AUC), time to maximum concentration (T_{max}), lag-time and half-life ($t_{\frac{1}{2}}$) were calculated.

Results

Plasma and cerebrospinal fluid levels of CR 2249 after oral and intraperitoneal administration

The average (± s.e.m.) plasma and cerebrospinal fluid levels of CR 2249, after a single intraperitoneal or oral administration of 30 mg kg^{-1}, plotted against time (h), are given in Fig. 1. Pharmacokinetic parameters are listed in Table 2.

CR 2249 appeared in plasma shortly after both oral and intraperitoneal administration, with a lag-time of 0·12 h. The plasma T_{max} was recorded 60 min after both oral and intraperitoneal administration, although plasma levels of CR 2249 given orally were already close to plateau 15 min after administration.

CR 2249 crossed the blood-cerebrospinal fluid barrier very rapidly, showing lag times of 0·1 and 0·23 h after oral and intraperitoneal administration, respectively. The maximum concentration of CR 2249 in the cerebrospinal fluid was reached 45–60 min after oral and intraperitoneal administration. The cerebrospinal fluid/plasma AUC ratios were 47% after intraperitoneal administration and 60% after oral administration. These data showed that CR 2249 has high absorption across the blood-cerebrospinal fluid barrier, and suggested that behavioural tests should be performed 45–60 min after administration.

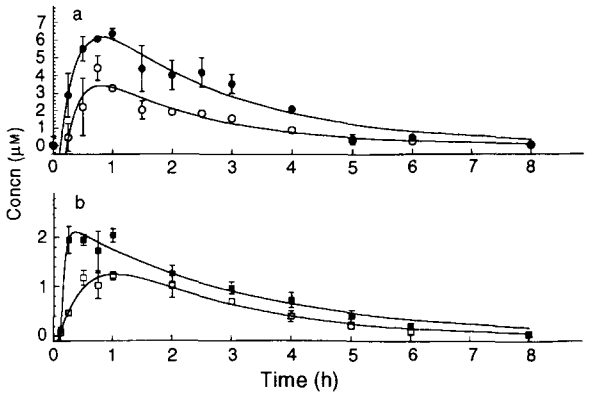

FIG. 1. Plasma (closed symbols) and cerebrospinal fluid (open symbols) levels (μM) after single intraperitoneal (a) and oral (b) doses of CR 2249 (30 mg kg^{-1}). Data are means ± s.e.m. (n = 3).

In-vitro receptor binding studies

As CR 2249 is a glutamic acid derivative, we first of all tested its affinity to the subtypes of acidic amino acid receptors. CR 2249 bound very weakly to the recognition site of NMDA receptor (IC50 0·7 mM) and had no significant binding affinity to the two other subtypes, i.e. AMPA (DL-[^{3}H]α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and kainic receptors. On the other hand, CR 2249 showed moderate affinity for the strychnine-insensitive glycine receptor (K_i = 10 μM) and increased the binding of [^{3}H]MK-801 in well washed membranes of rat cerebral cortex (EC50 11.6 μM). These results suggest that CR 2249 is endowed with agonist properties for the strychnine-insensitive glycine site or for the polyamine site of NMDA receptor complex, or for both.

The other binding assays demonstrated that the compound had no significant binding affinity to the adrenergic (α_1, α_2), 5-HT-ergic (5-HT_{1a}, 5-HT_2), cholecystokinin (CCK_B) or muscarinic (M_1) receptors. Taken together, these studies seem to indicate that CR 2249 interacts selectively with the NMDA receptor complex.

Release studies from slices

NMDA receptor activation in rat hippocampus is associated with the enhanced release of neurotransmitters such as noradrenaline (Vezzani et al 1987). In order to confirm the agonist properties shown by CR 2249 on NMDA receptor binding assays, we studied its effect on the release of [^{3}H]noradrenaline from slices of rat hippocampi. In this assay system CR 2249 was used in the range 10–1000 μM. It proved to be efficacious, producing a dose-dependent increase of [^{3}H]noradrenaline release in comparison with controls, with a maximum effect of 120% increase at 1000 μM concentration. The calculated EC50 was 104·5 ± 17·8 μM (± s.e.m.; n = 12).

Table 2. Plasma and cerebrospinal fluid pharmacokinetic parameters of CR 2249 in the rat.

Parameter	CR 2249 (p.o.) Plasma	Cerebrospinal fluid	CR 2249 (i.p.) Plasma	Cerebrospinal fluid
Lag time (h)	0·12 ± 0·02	0·10 ± 0·01	0·12 ± 0·01	0·23 ± 0·03
AUC (μM h)	16·3 ± 1·12	9·9 ± 0·45	41·4 ± 2·08	19·8 ± 0·85
Absorption half-life (h)	0·04 ± 0·01	0·34 ± 0·07	0·19 ± 0·03	0·17 ± 0·04
Elimination half life (h)	2·10 ± 0·14	1·42 ± 0·06	1·66 ± 0·07	1·33 ± 0·01
Time of maximum plasma concentration (h)	1·00 ± 0·33	1·00 ± 0·09	1·00 ± 0·12	0·75 ± 0·05
Maximum plasma concentration (μM)	2·13 ± 0·21	1·22 ± 0·11	6·42 ± 0·31	4·41 ± 0·25

The dose of CR 2249 was 30 mg kg^{-1}. Values are from mean curves (± s.e.m. n = 3).

Step-through type passive-avoidance performance in the rat

Latency to enter the dark chamber at re-test increased when higher foot-shock intensities were applied to animals during the training, and decreased when longer delays between training and re-test were employed. Because the best conditions to study a drug that could increase memory would be training animals with a foot-shock not producing too marked an increase in the main avoidance acquisition (Venault et al 1986), we checked memory recall after different periods of time from training to observe the effect of different intensities of foot-shock. On the basis of these results we selected 0·09 mA foot-shock for all passive-avoidance experiments and performed the retention test 72 h after training.

Pre-training administration of CR 2249 significantly increased the retention latencies in comparison with saline-treated rats, at intraperitoneal doses of 10 and 30 mg kg^{-1} ($P < 0·01$). On the other hand, CR 2249 (3, 10 or 30 mg kg^{-1}, i.p.) administered immediately after training or 45 min before re-test failed to increase retention latency in all the groups of treated animals (Table 3).

Scopolamine-induced memory impairment in passive-avoidance performance in the rat

CR 2249 was tested to determine whether it could revert scopolamine-induced memory decrement in the passive-avoidance paradigm with rats. The scopolamine-treated animals had a significantly shorter latency than the saline-shocked controls ($P < 0·01$) during the retention test. CR 2249 (1 and 10 mg kg^{-1}, i.p.) dose-dependently increased latency; the effect was significant ($P < 0·05$) at a dose of 10 mg kg^{-1}. The cholinesterase inhibitor tacrine, used as a memory enhancer standard reference (Mohs et al 1985; Summers et al 1986), also reversed the scopolamine effect at 10 mg kg^{-1}, the effect being statistically significant ($P < 0·05$) (Table 4).

Step-down type passive-avoidance in the mouse

In this model the saline-treated animals easily learnt the test whereas control mice receiving electroconvulsive shock had a significantly lower latency ($P < 0·01$), showing that the amnesic treatment had been efficient and that mice in this group could not pass the test. CR 2249 at oral doses of 3, 10 and 30 mg kg^{-1} dose-dependently increased step-down latency; for doses of 10 and 30 mg kg^{-1} the effect was statistically significant ($P < 0·05$ and $P < 0·01$, respectively). Tacrine at oral doses of 1, 3 and 10 mg kg^{-1} did not have any effect (Table 5).

Effects of CR 2249 on step-through active avoidance performance

The acquisition of active avoidance behaviour was evaluated during a daily session of 40 trials for 5 consecutive days. Mean

Table 3. Effect of CR 2249 on step-through type passive avoidance in the rat.

	Pre-training treatment (n = 28)	Post-training treatment (n = 10)	Pre-retention treatment (n = 10)
Saline	11·7 (9·2–91·9)	10·1 (8·2–26·8)	6·8 (4·6–24·6)
Saline + foot-shock	22·0 (12·1–36·5)	22·2 (7·7–30·2)	27·7 (4·4–107·9)
Saline + foot-shock + CR 2249 3 mg kg^{-1}	33·5 (12·4–117·8)	23·4 (8·7–36·9)	16·6 (12·1–77·4)
Saline + foot-shock + CR 2249 10 mg kg^{-1}	105·1‡ (24·5–120·0)	17·8 (8·9–33·5)	26·4 (16·4–41·7)
Saline + foot-shock + CR 2249 30 mg kg^{-1}	64·4‡ (28·9–120·0)	18·9 (10·6–44·8)	23·2 (11·7–102·4)

Values represent the median latency (s) to enter the shock compartment and interquartile ranges. ‡$P < 0·01$ vs saline + (FS) (log rank test).

Table 4. Effect of tacrine and CR 2249 on scopolamine-induced memory impairment in step-through type passive avoidance in rat.

	Tacrine (n = 20)	CR 2249 (n = 30)
Saline	18·2 (5·1–46·0)	15·0 (2·7–31·6)
Saline + foot-shock	120·0 (38·2–120·0)	120·0 (69·5–120·0)
Scopolamine + foot-shock	48·4† (24·2–120·0)	44·4‡ (6·3–120·0)
Scopolamine + foot-shock + drug 1 mg kg^{-1}	54·0 (11·0–120·0)	66·8 (25·2–120·0)
Scopolamine + foot-shock + drug 10 mg kg^{-1}	101·0† (33·7–120·0)	105·5† (23·2–120·0)

Values represent the median latency (s) to enter the shock compartment and interquartile ranges. †$P < 0·05$ vs scopolamine + (FS), ‡$P < 0·01$ vs saline + (FS) (log rank test).

Table 5. Effect of tacrine and CR 2249 on step-down type passive avoidance in mouse.

	Tacrine (n = 20)	CR 2249 (n = 36)
Saline	120·0 (54·2–120·0)	91·2 (71·8–120·0)
Saline + electroconvulsive shock	38·2** (29·2–52·4)	27·2** (23·5–60·1)
Drug 1 mg kg^{-1} + electroconvulsive shock	36·0 (22·4–57·4)	–
Drug 3 mg kg^{-1} + electroconvulsive shock	40·2 (27·0–94·2)	54·0 (21·0–82·9)
Drug 10 mg kg^{-1} + electroconvulsive shock	41·4 (28·4–67·2)	61·2† (44·9–120·0)
Drug 30 mg kg^{-1} + electroconvulsive shock	–	72·0‡ (49·7–120·0)

Values represent the median latency (s) to enter the shock compartment and interquartile ranges. **$P < 0.01$ vs saline no ECS, †$P < 0.05$ and ‡$P < 0.01$ vs saline + ECS (log rank test).

percent conditioned avoidance responses are reported in Fig. 2. CR 2249 given intraperitoneally in the dose-range 1–10 mg kg^{-1} improved the percent of conditioned avoidance at 3 and 10 mg kg^{-1}. Split-plot analysis of variance showed a statistically significant dose-effect interaction ($F(1,76) = 5.32$; $P = 0.024$). The treatment × days interaction was also significant ($F(9,228) = 1.95$; $p = 0.046$). This interaction was further investigated and found to result from the control vs treated × days interaction ($F(3,228) = 2.8$; $P = 0.041$). The regression × days interaction was, on the other hand, not significant and, consequently, the observed regression was independent of time, demonstrating that the dose-effect relationship was constant over the 5-day experiment. CR 2249 was, moreover, ineffective in uncoordinated escape responses and spontaneous inter-trial responses, clearly indicating that this compound did not alter spontaneous motor activity in rats at the doses used.

Motor activity

As shown in Table 6, intraperitoneal CR 2249 at 10 and 30 mg kg^{-1} did not affect the spontaneous motility of rats.

Microdialysis experiments

Baseline release of noradrenaline and 3,4-dihydroxyphenylglycol from the hippocampus of freely moving rats was, respectively, 39·77 ± 7·04 and 1927·66 ± 70·35 fmol/100 µL (n = 10); it remained stable in saline-treated animals throughout the whole experiment. As shown in Table 7, CR 2249 locally applied to hippocampus through the dialysis probe (100 µM), significantly increased noradrenaline release to approximately 225% of the baseline value after a 90-min perfusion (226·21 ± 28·41%, n = 6, compared with 97·03 ± 3·36, n = 4). On the other hand, 3,4-dihydroxyphenylglycol values gradually reduced the baseline to 69·00 ± 13·79% (n = 6) compared with 92·33 ± 8·56% (n = 4).

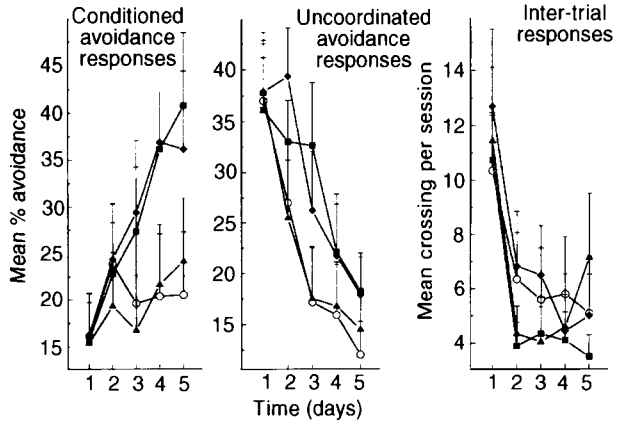

FIG. 2. Effect of CR 2249 on step-through type active avoidance in the rat. Data plotted represent the average (± s.e.m.; n = 10) percentage of conditioned avoidance responses and uncoordinated avoidance responses referred to the maximum value of 40 responses for each session. Also plotted are the mean values (± s.e.m.; n = 10) of spontaneous inter-trial responses for each daily session. ○ Saline; ▲ CR 2249 1 mg kg^{-1}; ■ CR 2249 3 mg kg^{-1}; ◆ CR 2249 10 mg kg^{-1}.

Discussion

The behavioural experiments results described in this paper seem to provide pharmacological evidence in support of the hypothesis that CR 2249 enhances memory. CR 2249 ameliorated the retention deficit produced by scopolamine in step-through type passive avoidance in rat and by electroconvulsive shock in step-down type passive avoidance in mice. CR 2249 was also capable of improving performance in the behavioural

Table 6. Effect of CR 2249 on motility in rats in the open field test.

Treatment	Dose (mg kg^{-1})	Total distance (cm)	Effect compared with saline (%)
Saline	–	3329·6 ± 173	–
CR 2249	10	2829·4 ± 317	−15·0
CR 2249	30	3227·6 ± 250	−3·1

CR 2249 was administered intraperitoneally 45 min before the test. Data represent the total distance covered by animals in 10 min, and are expressed as means ± s.e.m. (n = 10).

Table 7. Effect of 100 µM CR 2249 on hippocampal noradrenaline and 3,4-dihydroxyphenyl glycol release (%) in in-vivo microdialysis experiments.

	Noradrenaline	3,4-Dihydroxyphenylglycol
Saline	97·03 ± 3·36	92·33 ± 8·56
100 µM CR 2249	226·21 ± 28·4‡	69·80 ± 13·79†

CR 2249 was dissolved in modified Ringer's solution and perfused throughout the experiment. The effect of CR 2249 was analysed in the sample collected 70–90 min after starting drug perfusion. Values represent means ± s.e.m. (n = 4 for saline; n = 6 for CR 2249-treated animals). ‡$P < 0.001$, †$P < 0.05$ compared with saline, Student's t-test.

tests of learning and memory in rat in the absence of cholinergic hypofunction or cognitive deficit.

The results presented indicate that CR 2249 facilitates passive-avoidance behaviour in the rat when administered 45 min before the learning trial, but not when administered 45 min before the retention test or immediately after the learning trial. One explanation of the observed inability of CR 2249 to influence memory consolidation processes in the learning trial might be found in the pharmacokinetic characteristics of the compound. It has, in fact, been reported that the process of memory consolidation occurs immediately after training (Mondadori et al 1986; Serra et al 1989; Vanderwolf & Cain 1994). As the concentration of CR 2249 was below detectable levels up to 7 min after administration, and the maximum absorption of CR 2249 in plasma and cerebrospinal fluid after intraperitoneal administration occurred 60 min after treatment, it is reasonable to speculate that during the memory consolidation period CR 2249 had not reached plasma and cerebrospinal fluid concentrations high enough to influence learning processes. In addition, the finding that CR 2249 failed to facilitate passive-avoidance behaviour when administered before the retention test, suggests that this compound affects only one component of long-term memory (acquisition), but does not facilitate memory recall (Venault et al 1986).

Pre-trial CR 2249 treatment also led to an improvement in the acquisition of a two-way active avoidance conditioning task as compared with that shown by control rats. This improvement became statistically significant for daily intraperitoneal doses of 3 and 10 mg kg^{-1} of CR 2249. This increased avoidance was not correlated with a possible increase of locomotor activity induced by the compound, because the inter-trial responses were not affected by CR 2249 treatment. In the same way, in the open field model in the rat, we did not see any sign of motor alteration at the same doses used for passive and active avoidance experiments. The effects we observed in behavioural tests could not, moreover, be explained by a possible analgesic effect, because hot-plate experiments in mice demonstrated that CR 2249 had no effect on pain threshold (data not shown).

The ultimate mechanism by which learning processes could be increased after treatment with CR 2249 cannot be concluded from the results obtained so far. We believe, however, that the data presented support the hypothesis that the effects of CR 2249 on acquisition might depend, at least in part, on increasing the amount of catecholamines at CNS synapses, as demonstrated by the augmented release of [^{3}H]noradrenaline from hippocampal slices and by microdialysis studies showing a 2.5-fold increase in noradrenaline after perfusion of 100 μM CR 2249. These results correlate with the hypothesis that endogenous noradrenaline release facilitates learning (Sahakian et al 1985; Desai et al 1995).

Preliminary biochemical studies on striatal slices (data not shown) seem to exclude the possibility that CR 2249 operates by increasing acetylcholine release. Because it has been reported that scopolamine-induced cognitive disruption decreases hippocampal and cortical noradrenaline levels in the rat brain (Matsumoto et al 1991; Oishi et al 1991), the effect of CR 2249 on the amelioration of the retention deficit produced by scopolamine in passive avoidance in the rat, might also be explained in terms of the increased release of catecholamines induced by the compound.

It is not yet clear if the enhanced release of noradrenaline induced by CR 2249 is totally NMDA-mediated, as can be speculated from the results of binding studies in which it was shown that CR 2249 binds with moderate affinity to strychnine-insensitive glycine receptors and that it stimulates [^{3}H]MK-801 binding in well washed rat cortical membranes.

It might be worth noting that a specific molecular mechanism linking noradrenaline release with nootropic drugs is not yet known, even if one recent report (Pittaluga et al 1995) proposed the reversal of kynurenic acid antagonism at hippocampal NMDA receptors, sited on noradrenergic neurons, as a possible model for investigation of putative cognition enhancers. The interaction between glutamatergic receptors and noradrenergic or cholinergic transmission might be an interesting approach for evaluation of the activity of nootropic drugs.

We have analysed both noradrenaline and 3,4-dihydroxyphenylglycol because simultaneous measurement of these two compounds provides a comprehensive assessment of sympathetic nervous function (Eisenhofer et al 1988). The moderate but significant decrease of 3,4-dihydroxyphenylglycol induced by perfusion of 100 μM CR 2249 might be a consequence of a noradrenaline releasing action or a noradrenaline uptake inhibitory effect, or both (Itoh et al 1990). Biochemical experiments to elucidate the possible molecular mechanism by which CR 2249 increases noradrenaline levels and possible indirect interactions with cholinergic transmission are currently in progress.

Finally it is worth nothing that CR 2249 seems to be particularly safe toxicologically (LD50 mouse = 381 mg kg^{-1} i.v. and 1676 mg kg^{-1} p.o.) and there was no evidence of neurologic disfunction or behavioural alterations at the doses used.

In conclusion, although much work is still necessary for further characterization of CR 2249, the behavioural data reported here suggest that CR 2249 might have potential clinical application in cases where cognitive functions are compromised.

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