Effects of the active constituents of *Crocus sativus* L. crocins and their combination with memantine on recognition memory in rats

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Crocus sativus L., is a plant cultivated in many countries of the world. Crocins are among the active constituents of _C. sativus_ and their implication in cognition has been proposed. The present study was designed to investigate in the rat the effects of crocins on distinct recognition memory components (encoding, storage and retrieval). Subsequently, the potential use of crocins as adjunctive agents for the treatment of memory disorders was examined. Thus, the effects exerted by a combination of subthreshold doses of crocins and memantine on recognition memory were evaluated. To assess the effects of compounds on memory, the novel object-recognition task (NORT) was used. In a preliminary study, the influence of the retention time (the delay between the two trials) on the performance of rats was assessed. Rats' recognition memory abilities remained intact up to 6 h, but were extinguished when a delay of 24 h was utilized. Crocins, at any dose tested (5, 15, and 30 mg/kg), did not affect rats' performance, whereas administration of higher doses (15 and 30 mg/kg) reversed delay-dependent deficits in the NORT. The combination of subthreshold doses of crocins (5 mg/kg) and memantine (3 mg/kg) did not influence the performance, but counteracted delay-dependent deficits in the NORT. These findings suggest that crocins counteract natural forgetting and may modulate different aspects of recognition memory, and that the combined use of crocins and memantine might represent a novel strategy to treat memory disorders. _Behavioural Pharmacology_ 29:400–412

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Introduction

Crocus sativus L., is a plant cultivated in many countries such as Iran, Italy, Spain and Greece. Its product is the well-known spice called saffron. Saffron is the dried red stigmas of the flower. Among the principal components of saffron, crocins are responsible for its colour, picrocrocin for its bitter taste and safranal for its characteristic aroma (Tarantilis *et al.*, 1995). The stigmas of *C. sativus* are used in folk medicine as an anticatarrhal, eupeptic, expectorant and emmenagogue. Modern preclinical pharmacological studies have shown that saffron extracts and purified chemicals possess antitumour effects, have anti-inflammatory properties and counteract atherosclerosis and hepatic damage (Rios *et al.*, 1996).

Numerous studies have indicated the involvement of saffron and its constituents in cognition. Preclinical research has shown the efficacy of *C. sativus* and crocins in attenuating memory disorders, including recognition memory deficits, in animal models related to Alzheimer's disease (AD), cerebral injuries or schizophrenia (Pitsikas, 2015). In this context, it is important to underline that recognition memory is a type of memory that is impaired in AD (Small *et al.*, 2003) and schizophrenic patients (Calev *et al.*, 1983). However, in the studies in which the beneficial effects of crocins on recognition memory were reported, no distinction was made between the effects of crocins on different mnemonic processes (i.e. acquisition or retrieval of information) (Pitsikas, 2015). Thus, it is not yet clear whether and how crocins affect the above-described different stages of memory formation.

Clinical research has assessed the efficacy of saffron, but not of crocins, in a narrow range of memory disorders (Akhondzadeh *et al.*, 2010a, 2010b; Farokhnia *et al.*, 2014; Tsolaki *et al.*, 2016). The results of these studies suggested that its effects on memory, although modest, were not dissimilar from those shown by two drugs approved for clinical use in the treatment of AD: the acetylcholinesterase inhibitor donepezil (Rogers and Friedhoff, 1998) and the *N*-methyl-D-aspartate (NMDA) receptor antagonist memantine (Reisberg *et al.*, 2003; Scarpini *et al.*, 2003). It is important to emphasize, however, that no therapy is currently available to cure memory disorders. There is therefore a clear urgent need for new molecules active against cognitive disorders, which might be combined with existing antiamnestic drugs (Cassey *et al.*, 2010). The potential role of saffron and crocins as adjunctive agents in combination

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with an acetylcholinesterase inhibitor or memantine for the treatment of cognition disorders has yet not been investigated.

Taking the above evidence into account, the aim of the present work was to study the effects of crocins and their combination with memantine on recognition memory. For these studies, the novel object-recognition task (NORT), a procedure evaluating recognition memory in rodents, was used (Ennaceur and Delacour, 1988). Previous findings had suggested that a rat's performance in this procedure was affected consistently by the inter-trial interval (ITI) used (Ennaceur and Delacour, 1988; Bartolini *et al.*, 1996). Thus, a preliminary experiment was conducted with the aim to verify which ITI (1, 6, or 24 h) was critical for the loss of the recognition memory in the rat. Subsequently, we examined the effects of crocins on recognition memory, utilizing the longest ITI at which normal rats still were able to recognize the familiar object. Further, the effects of crocins on the different mnemonic stages (acquisition, storage and retrieval of information) underlying recognition memory were studied. As crocins were expected to improve memory performance, they were tested at the ITI at which normal rats were unable to recognize the familiar object. The final goal of the present study was to determine the effects of concomitant administration of inactive doses of crocins and memantine on recognition memory. For this purpose, the effects of coadministration of subthreshold doses of crocins with memantine on recognition memory were tested using different ITIs.

Methods

Subjects

Independent groups of naive male (3-month-old) Wistar rats (Hellenic Pasteur Institute, Athens, Greece) weighing 250–300 g were used. The animals were housed in Makrolon cages (47.5 cm length × 20.5 cm height × 27 cm width; Covestro AG, Leverkuen, Germany), three per cage, in a regulated environment (21 ± 1°C; 50–55% relative humidity; 12-h/12-h light/dark cycle, lights on at 07.00 h) with free access to food and water.

The procedures that involved animals and their care were in accordance with international guidelines and national (Animal Act, P.D. 160/91) and international laws and policies [EEC Council Directive 86/609, JL 358, 1, 12 December 1987; *NIH Guide for Care and Use of Laboratory Animals*, NIH publication no. 85-23, 1985].

Novel object-recognition task

The test apparatus consisted of a dark open box made of Plexiglas (80 cm length × 50 cm height × 60 cm width; custom made) that was illuminated by a 60-W light suspended 60 cm above the box. The light intensity was equal in the different parts of the apparatus. The objects to be discriminated (in triplicate) were made of glass, plastic or metal, and had three different shapes: (i.e. metallic cubes, glass pyramids and plastic cylinders, 7 cm high) and could not be moved by the rats. In addition, these objects had no genuine significance for rats and had never been associated with a reinforcement.

NORT was performed as described previously (Ennaceur and Delacour, 1988; Boultadakis and Pitsikas, 2010). Briefly, during the week before the test, the animals were handled twice per day for 3 consecutive days. Before testing, the rats were allowed to explore the apparatus for 2 min on 3 consecutive days. During testing, a session that consisted of two 2-min trials was conducted. During the 'sample' trial (T1), two identical samples (objects) were placed in opposite corners of the apparatus in a random manner 10 cm from the side-walls. A rat was placed in the middle of the apparatus and allowed to explore the two identical objects. After T1, the rat was returned to its home cage, and an ITI followed. Subsequently, the 'choice' trial (T2) was performed. During T2, a novel object replaced one of the objects presented during T1. Accordingly, the rats were re-exposed to two objects: a copy of the familiar (F) object and the novel (N) object. All combinations and locations of the objects were counterbalanced to reduce potential bias caused by preference for particular locations or objects. To avoid the presence of olfactory cues, after each trial, the apparatus and the objects were cleaned thoroughly with 20% ethanol and then wiped with dry paper.

Exploration was defined as directing the nose towards the object at a distance of 2 cm or less and/or touching the object with the nose. Turning around or sitting on the object was not considered exploratory behaviour. The time spent exploring each object during T1 and T2 was recorded manually with a stopwatch. On the basis of this measure, a series of variables was then calculated: the total time spent exploring the two identical objects in T1 and the time spent exploring the two different objects, F and N, during T2. The discrimination between F and the N during T2 was measured by comparing the time spent exploring the familiar object with the time spent exploring the novel object. Because this time may be biased by differences in the overall level of exploration (Cavoy and Delacour, 1993), we used a discrimination index (D) to represent the preference for the novel object as opposed to the familiar object, calculated as follows; $D = N–F/N + F$ (Cavoy and Delacour, 1993). In addition, the motor activity of each animal, expressed as the total number of steps for each forelimb, during each trial was also recorded.

Procedure

The experiments were conducted between 10:00 and 14:00 h in a room where only these animals were housed. Animals' behaviour was video-recorded. Data evaluation was subsequently performed by experimenters who were blinded to the pharmacological treatment of each subject. Group sizes were $n = 8$ in all experiments.

Experiment 1: effects of different intertrial intervals on object-recognition memory

The aim of this experiment was to evaluate at which delay condition (1, 6, or 24 h) recognition memory is extinguished in the rat. Animals were separated randomly into three experimental group as follows: 1, 6, and 24 h.

Experiment 2: effects of pretraining administration of different doses of crocins in the novel object-recognition task (acquisition experiment, intertrial interval 6 h)

Crocins and vehicle were administered 60 min before T1. Rats were divided randomly into four experimental groups as follows: vehicle; crocins 5 mg/kg; crocins 15 mg/kg; and crocins 30 mg/kg. For this experiment, an ITI of 6 h was used as at this delay condition, recognition memory is still intact in the untreated rat (see Results: experiment 1).

Experiment 3: effects of different doses of crocins in antagonizing delay-dependent deficits in the novel object-recognition task (acquisition experiment, intertrial interval 24 h)

To study the effects on the acquisition of information, crocins and vehicle were administered 60 min before T1. Rats were divided randomly into four experimental groups as follows: vehicle; crocins 5 mg/kg; crocins 15 mg/kg; and crocins 30 mg/kg. In this experiment, the ITI at which recognition memory disappeared in the normal rat was used. Thus, a 24 h retention was utilized (Bartolini *et al.*, 1996; Pitsikas *et al.*, 2002; see also results of experiment 1).

Experiment 4: effects of different doses of crocins in antagonizing delay-dependent deficits in the novel object-recognition task (storage experiment, intertrial interval 24 h)

It has been shown previously that crocins at 15 and 30 mg/kg counteracted delay-dependent deficits in rats in the NORT in a procedure assessing animals' storage abilities (Pitsikas and Sakellaridis, 2007). The effects exerted by a lower dose of crocins (5 mg/kg) with respect to those doses investigated previously (Pitsikas and Sakellaridis, 2007) on rats' consolidation abilities have not been studied so far. Experiment 4 was designed to answer this question. Thus, to study the effects on storage of information, compound and vehicle were administered immediately after T1 and a 24 h ITI was used. Rats were divided randomly into four experimental groups as follows: vehicle; crocins 5 mg/kg; crocins 15 mg/kg; and crocins 30 mg/kg.

Experiment 5: effects of different doses of crocins in antagonizing delay-dependent deficits in the novel object-recognition task (retrieval experiment, intertrial interval 24 h)

To study the effects on retrieval of information, crocins and vehicle were administered 60 min before T2 using a 24 h ITI. Rats were divided randomly into four experimental groups as follows: vehicle; crocins 5 mg/kg; crocins 15 mg/kg; and crocins 30 mg/kg.

Experiment 6: effects of pretraining administration of subthreshold doses of crocins and memantine in the novel object-recognition task (acquisition experiment, intertrial interval 6 h)

To examine the effects of crocins, memantine and their combination on acquisition of information, we administered the vehicle or crocins 60 min before T1. Memantine was administered 5–10 s after vehicle or crocins. The ineffective dose of crocins (5 mg/kg) was chosen on the basis of the results of experiments 3, 4 and 5. Rats were divided randomly into four experimental groups as follows: vehicle + vehicle; vehicle + crocins 5 mg/kg; vehicle + memantine 3 mg/kg; and crocins 5 mg/kg + memantine 3 mg/kg. For this experiment, an ITI of 6 h was used as at this delay condition recognition memory is still intact in the untreated rat (see results of experiment 1).

Experiment 7: effects of subthreshold doses of crocins and memantine in antagonizing delay-dependent deficits in the novel object-recognition task (acquisition experiment, intertrial interval 24 h)

To examine the effects of crocins, memantine and their combination on acquisition of information, we administered the vehicle or crocins 60 min before T1. Memantine was administered 5–10 s after vehicle or crocins. The ineffective dose of crocins (5 mg/kg) was chosen on the basis of the results of experiments 3, 4 and 5 of the present study. Rats were divided randomly into four experimental groups as follows: vehicle + vehicle; vehicle + crocins 5 mg/kg; vehicle + memantine 3 mg/kg; and crocins 5 mg/kg + memantine 3 mg/kg. In this experiment, a 24 h retention was used, being the ITI at which recognition memory disappeared in the normal rat (Bartolini *et al.*, 1996; Pitsikas *et al.*, 2002; see also results of experiment 1).

Experiment 8: effects of subthreshold doses of crocins and memantine in antagonizing delay-dependent deficits in the novel object-recognition task (storage experiment)

To examine the effects of crocins, memantine and their combination on storage of information, we administered the vehicle or crocins just after T1. Memantine was administered 5–10 s after vehicle or crocins. The ineffective dose of crocins (5 mg/kg) was chosen on the basis of the results of experiments 1, 2 and 3. Rats were divided randomly into four experimental groups as follows: vehicle + vehicle; vehicle + crocins 5 mg/kg; vehicle + memantine 3 mg/kg; and crocins 5 mg/kg + memantine 3 mg/kg. A 24 h ITI was used.

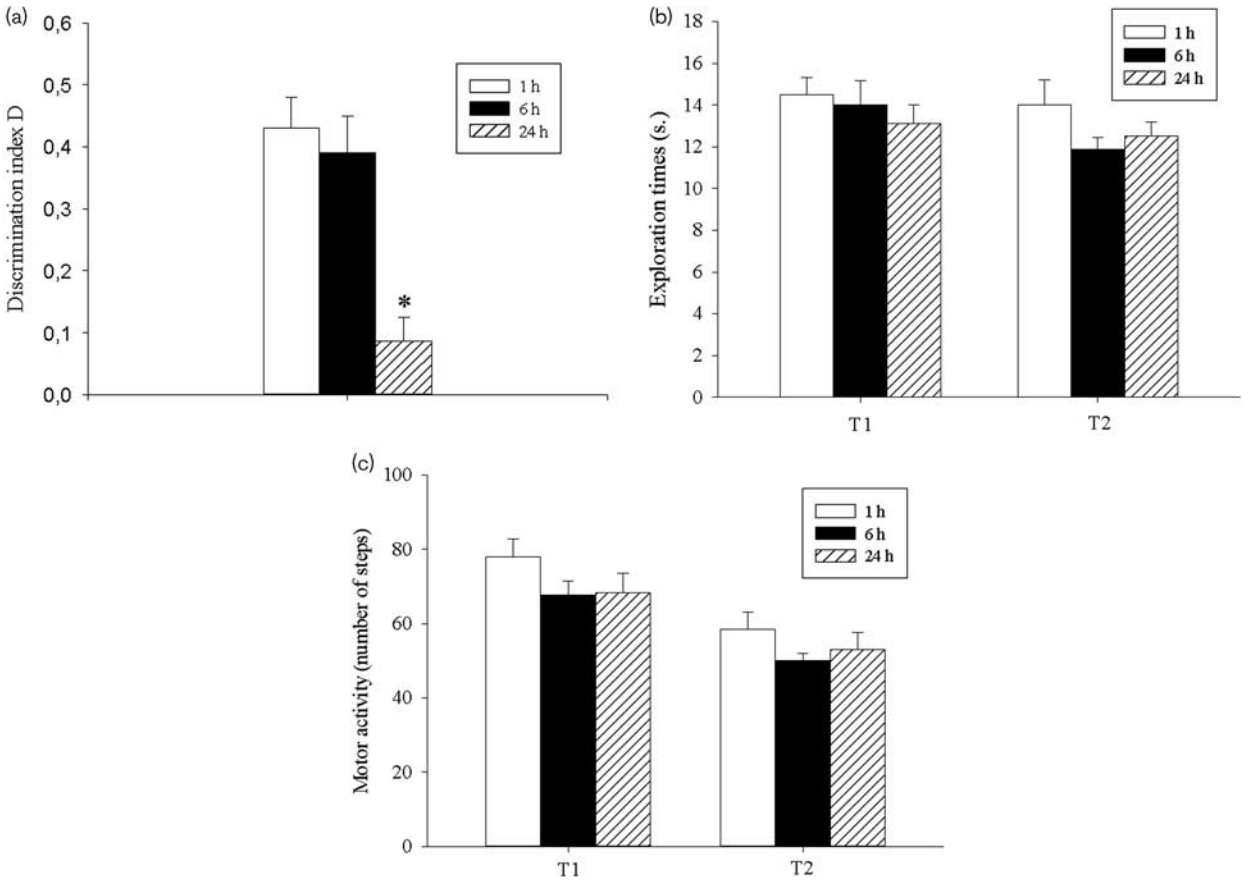
Experiment 9: effects of subthreshold doses of crocins and memantine in antagonizing delay-dependent deficits in the novel object-recognition task (retrieval experiment)

To examine the effects of crocins, memantine and their combination on retrieval of information, we administered the vehicle or crocins 60 min before T2 using a 24 h ITI. Memantine was administered 5–10 s after vehicle or crocins. The ineffective dose of crocins (5 mg/kg) was chosen on the basis of the results of experiments 1, 2 and 3. Rats were divided randomly into four experimental groups as follows: vehicle + vehicle; vehicle + crocins 5 mg/kg; vehicle + memantine 3 mg/kg; and crocins 5 mg/kg + memantine 3 mg/kg.

Drugs

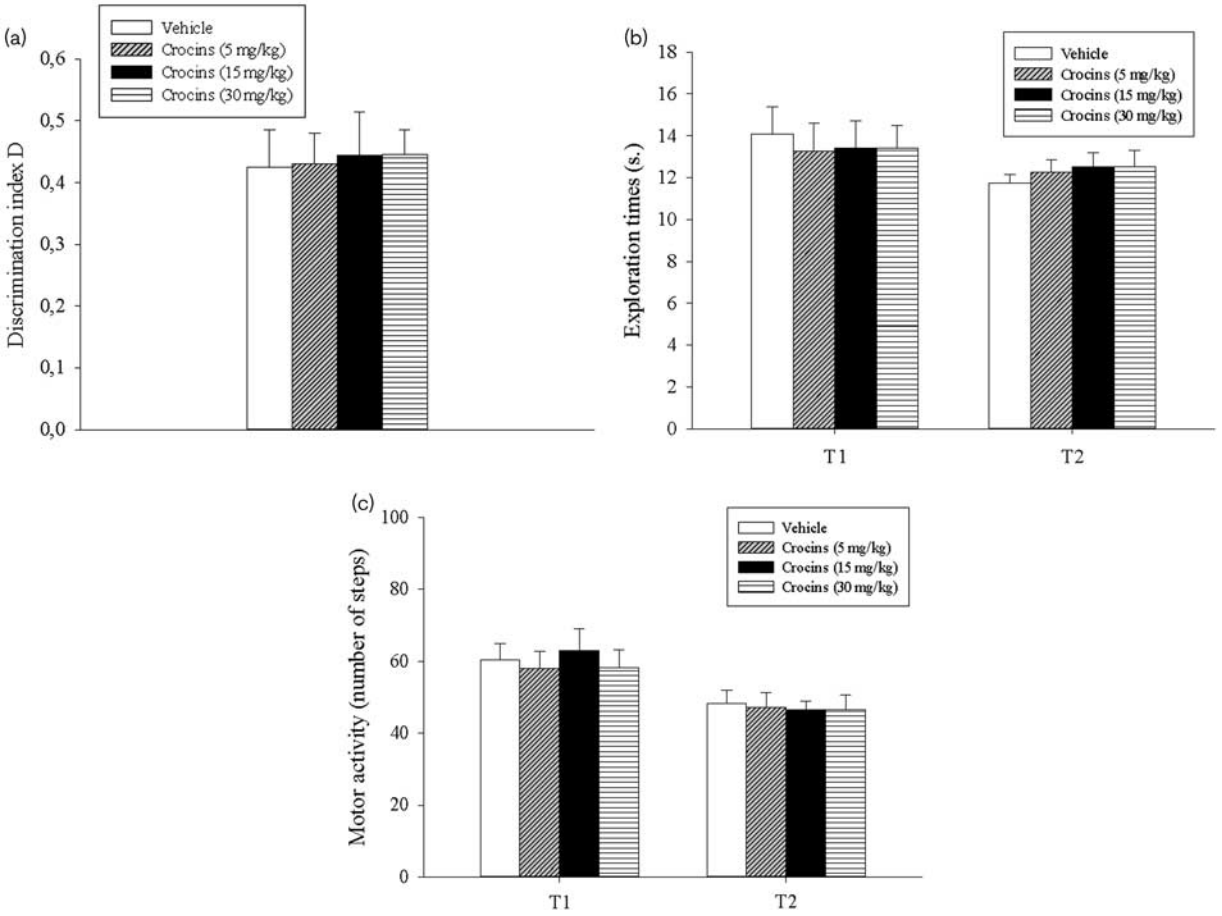
All drug solutions were freshly prepared on the day of testing. Compounds were administered intraperitoneally in a volume of 1 ml/kg. Crocins were derived from a single batch of plant material (saffron) using the same purification procedure, extraction and separation. Our plant material was kindly provided by the Cooperative of Saffron, Krokos, Kozani, Greece. Crocins were isolated from the red dried stigmas of *C. sativus* using a slightly modified method described previously (Kanakis *et al.*, 2007). They were purified from stigmas after successive and exhaustive extraction by: (a) petroleum ether 40–60° C; (b) diethyl ether (Et_2O); and (c) methanol (MeOH) 80% using ultrasound-assisted extraction. The ultrasound extraction was performed in a Sonorex, Super RK 255H type (300 mm × 150 mm × 150 mm internal dimensions) ultrasound water bath (indirect sonication, Bandelin Electronic GmbH & Co. KG, Berlin, Germany) at the fixed frequency of 35 kHz. The temperature of the sonicated water was 25°C. Procedures a and b were performed to ensure that the stigmas were free of picrocrocin and safranal, respectively. The methanol extract, after evaporation (condensed to dryness) under vacuum at room temperature, provided crocins, which are a dark red powder residue. Cocins are unusual water-soluble carotenoids (glucosyl esters of crocetin). The major component is a digentiobiosyl ester of crocetin (~60% of total crocins) (del Campo *et al.*, 2010). The purity of crocins was 81%. The remaining compounds are mainly sugars (glucose and

Fig. 1



Effects of different intertrial intervals (1, 6, and 24 h) on object-recognition memory in the 3-month-old rat. The results are expressed as mean + SEM. (a) Discrimination (*D*) index. *$P < 0.05$ versus 1 and 6 h groups. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

Fig. 2



Acquisition study. Vehicle and crocins were injected intraperitoneally 60 min before T1, with a 6-h ITI. The results are expressed as mean + SEM. (a) Discrimination (*D*) index. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

gentiobiose), crocetin (the aglycon part of crocins), dimethyl-crocetin and other carotenoids, including zeaxanthin, lycopene and α-carotenes and β-carotenes.

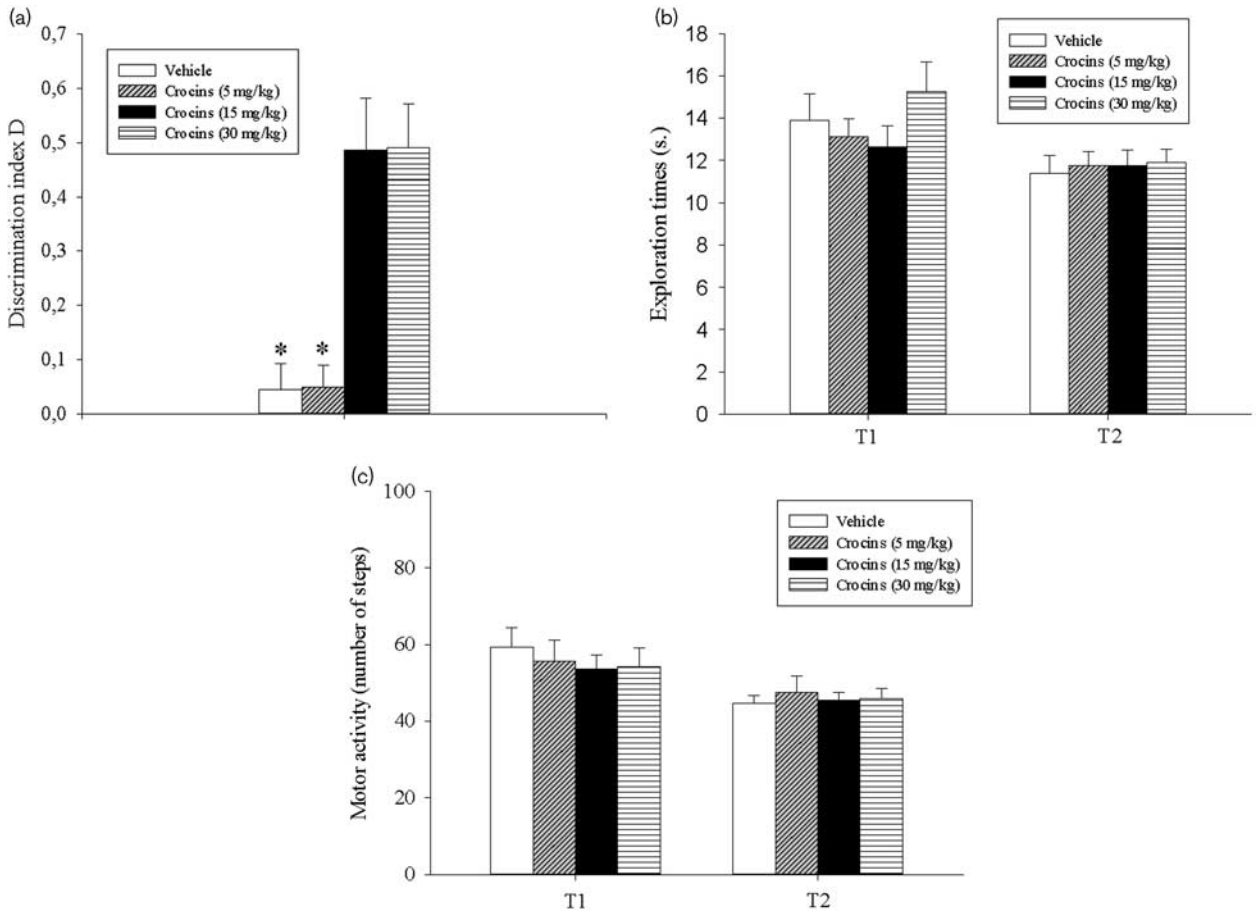
The compound was dissolved in saline (NaCl: 0.9%). The doses of crocins (15 and 30 mg/kg) were selected on the basis of a previous study (Pitsikas and Sakellaridis, 2007) and our unpublished observations (the dose of 5 mg/kg).

Memantine (1-amino-3,5-dimethyl-adamantane; Sigma, St Louis, Missouri, USA) was dissolved in saline. The dose of memantine (3 mg/kg) was chosen on the basis of a previous study in which it was found to be ineffective against time-related retention deficits in the NORT and did not produce adverse side effects (Pitsikas *et al.*, 2007). In all of the experiments, control animals received isovolumetric amounts of the vehicle (saline).

Statistical analysis

The data were expressed as mean ± SEM. Preference for objects or locations was analysed using Student's *t*-test for each experimental group. Discrimination index *D* data from experiments 1–5 were evaluated using one-way analysis of variance (ANOVA). The factor was time (experiment 1) or treatment (experiments 2–5). Discrimination index *D* data from experiments 6–9 were assessed using two-way ANOVA, with the factors crocins (two levels) and memantine (two levels). Total exploration times and motor activity data from experiments 1–3 were analysed using two-way ANOVA with the between-subjects factor delay (three levels, experiment 1) or crocins (3 levels, experiments 2 and 3) and the within-subjects factor trials (two levels). Total exploration times and motor activity data from experiments 4 and 5 were analysed using one-way ANOVA. The factor was treatment (three levels). Total exploration times and motor activity data from experiments 6 and 7 were evaluated by three-way analysis with the between-subjects factors crocins (two levels) and memantine (two levels), and the within-subjects factor trials (two levels). Total exploration times and motor activity data from experiments 8 and 9 were analysed using two-way ANOVA with the

Fig. 3



Acquisition study. Vehicle and crocins were injected intraperitoneally 60 min before T1, with a 24-h ITI. The results are expressed as mean + SEM. (a) Discrimination (*D*) index. *$P < 0.05$ versus crocins 15 and 30 mg/kg groups. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

factors crocins (two levels) and memantine (two levels). Total exploration times and motor activity data expressed by the different groups of untreated rats during the sample trial T1 of experiments 4, 5, 8 and 9 were evaluated by one-way ANOVA. Post-hoc comparisons between treatment means were made using Tukey's test, but only when a significant interaction was achieved. Values of P less than 0.05 were considered statistically significant.

Results

In all experiments, there was no significant difference within any group according to the nature of objects and their locations.

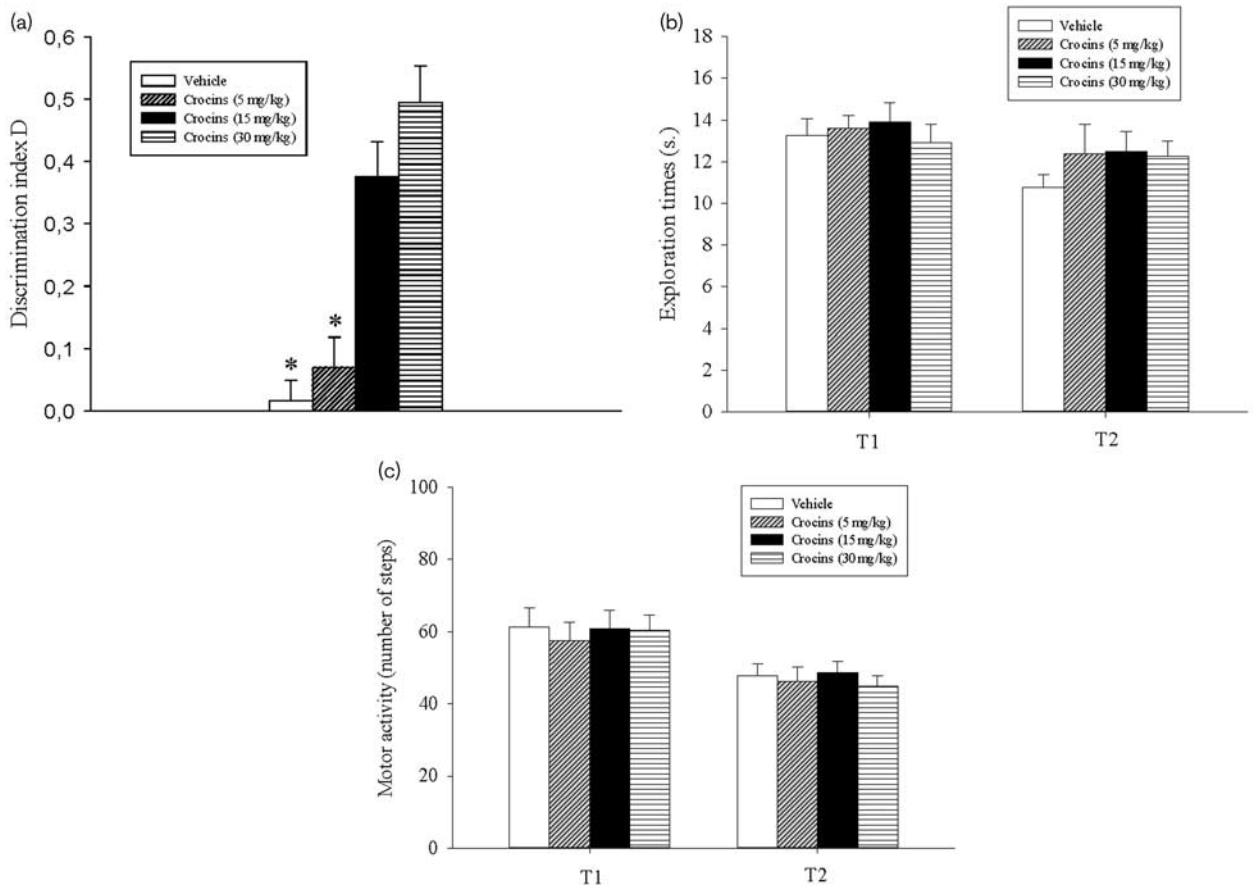
Experiment 1: effects of different intertrial intervals on object-recognition memory

Data for index *D* (Fig. 1a) showed a significant effect of delay [$F_{(2, 21)} = 14.3$, $P < 0.001$]. Post-hoc comparisons indicated that the 24 h rats showed a lower index *D* compared with their 1 and 6 h counterparts ($P < 0.05$). The overall analysis of total object exploration times showed no significant main effect of trials or delay or the trials × delay interaction (Fig. 1b). The overall analysis of motility data indicated a significant main effect of trials [$F_{(1, 21)} = 51.4$, $P < 0.001$], but no significant main effect of delay or trial × delay interaction (Fig. 1c).

Experiment 2: effects of pretraining administration of different doses of crocins in the novel object-recognition task (acquisition experiment, intertrial interval 6 h)

Evaluation of index *D* did not indicate any significant effect of treatment (Fig. 2a) with control or crocins-treated rats showing similar levels of discrimination performance. Statistical analyses of total exploration levels did not show significant main effects of treatment or trials or a significant treatment × trials interaction (Fig. 2b). The analysis of total motor activity data showed a significant main effect of trials [$F_{(1, 28)} = 22.9$, $P < 0.001$, Fig. 2c], but not of crocins, and no sigificant crocins × trials interaction.

Fig. 4



Storage study. Vehicle and crocins were injected intraperitoneally immediately after T1, with a 24-h ITI. The results are expressed as mean + SEM. (a) Discrimination (*D*) index. **P* < 0.05 versus crocins 15 and 30 mg/kg groups. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

Experiment 3: effects of different doses of crocins in antagonizing delay-dependent deficits in the novel object-recognition task (acquisition experiment, intertrial interval 24 h)

The index *D* results showed a significant effect of treatment [$F_{(3, 28)} = 13.4$, $P < 0.001$]. Post-hoc comparisons indicated that the vehicle-treated and crocins 5 mg/kg-treated rats had lower discrimination levels compared with their counterparts that received crocins 15 and 30 mg/kg ($P < 0.05$, Fig. 3a). Overall analysis of total exploration times and of motor activity data showed no significant main effect of crocins or crocins × trials interaction. A significant main effect of trials, however, was found for both exploration times [$F_{(1, 28)} = 10.1$, $P < 0.002$, Fig. 3b] and motility [$F_{(1, 28)} = 24.8$, $P < 0.001$, Fig. 3c].

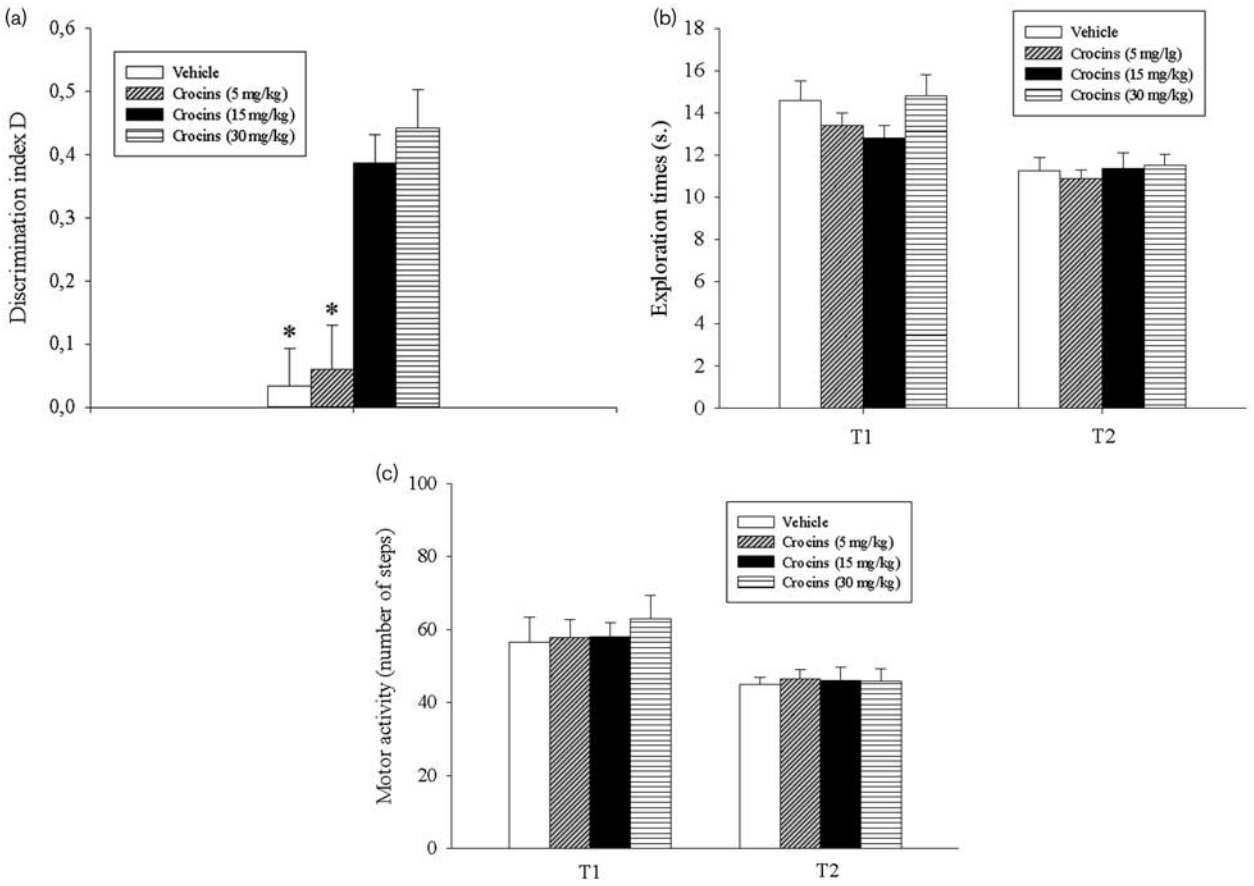
Experiment 4: effects of different doses of crocins in antagonizing delay-dependent deficits in the novel object-recognition task (storage experiment, intertrial interval 24 h)

Data for index *D* (Fig. 4a) showed a significant effect of treatment [$F_{(3, 28)} = 21.5$, $P < 0.001$]. Post-hoc comparisons indicated that the crocins 15 and 30 mg/kg-treated rats explored the novel object more than the familiar one during T2 with respect to their counterparts that received the vehicle or crocins 5 mg/kg ($P < 0.05$). The statistical analyses of locomotor activity and total object exploration time data from trial T2 showed no significant effects of crocins on these measures of performance. Similarly, levels of exploratory and motor activity were not significantly different among the various groups of untreated animals during the sample trial T1 (Fig. 4b and c, respectively).

Experiment 5: effects of different doses of crocins in antagonizing delay-dependent deficits in the novel object-recognition task (retrieval experiment, intertrial interval 24 h)

The index *D* results (Fig. 5a) showed a significant effect of treatment [$F_{(3, 28)} = 13.2$, $P < 0.001$]. Post-hoc comparisons showed that rats treated with crocins at 15 and 30 mg/kg had higher index *D* values compared with their counterparts that received the vehicle or crocins 5 mg/kg ($P < 0.05$). Total exploration levels and motility data

Fig. 5



Retrieval study. Vehicle and crocins were injected intraperitoneally 60 min before T2, with a 24-h ITI. The results are expressed as mean + SEM. (a) Discrimination (*D*) index. **P* < 0.05 versus crocins 15 and 30 mg/kg groups. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

during T2 did not show any significant effect of treatment. In addition, exploratory activity and motility did not differ significantly among the various groups of animals during T1 (Fig. 5b and c, respectively).

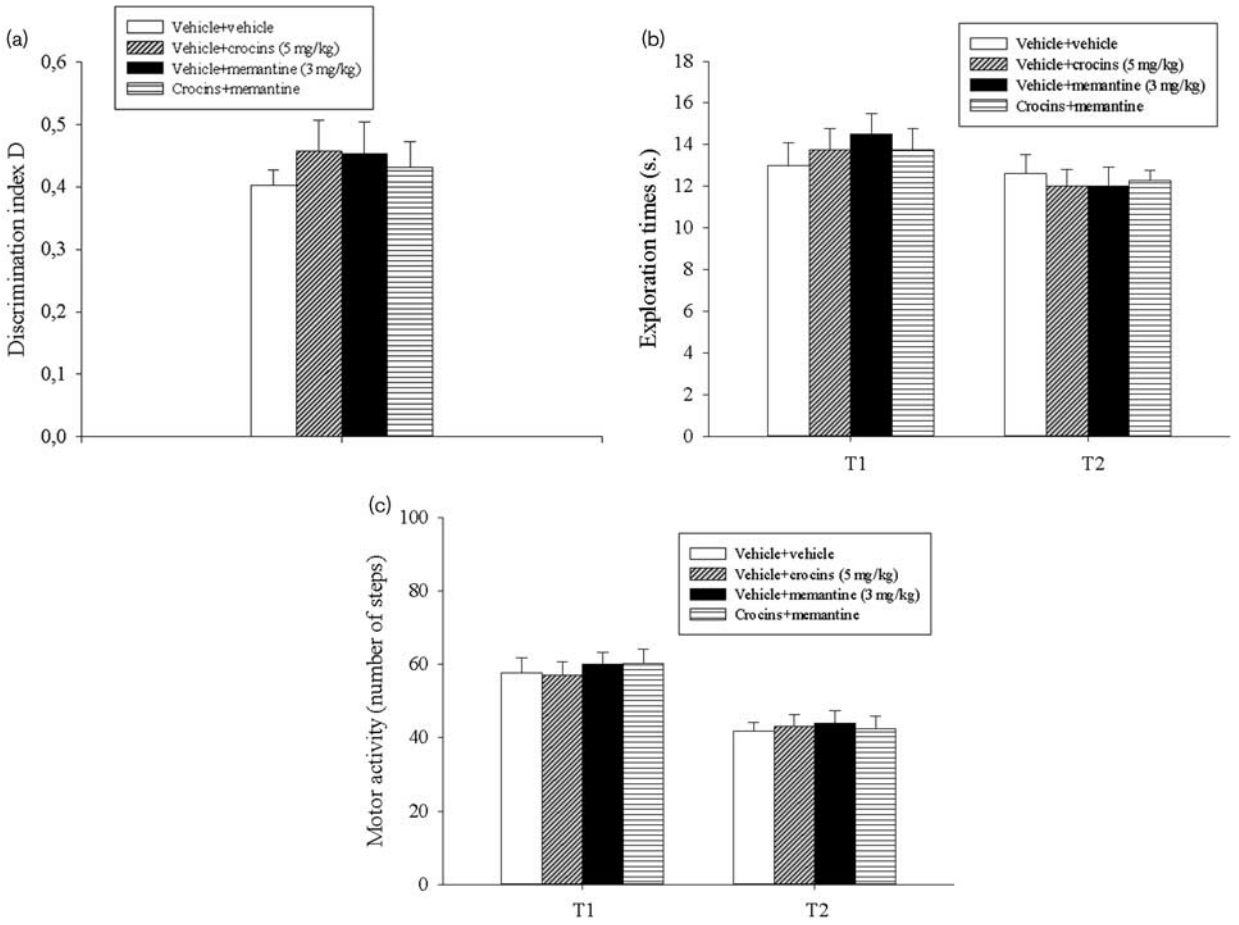
Experiment 6: effects of pretraining administration of subthreshold doses of crocins and memantine in the novel object-recognition task (acquisition experiment, intertrial interval 6 h)

Data for index *D* did not show any significant difference among the various experimental groups independent of their treatment (Fig. 6a). Overall analysis of total exploration times and motor activity data showed a nonsignificant three-way crocins × memantine × trials and two-way crocins × memantine, crocins × trials and memantine × trials interactions. There were significant main effects of trials, but not of crocins or memantine, for exploration times [$F_{(1, 28)} = 5.5$, $P < 0.05$, Fig. 6b] and locomotor activity [$F_{(1, 28)} = 43.1$, $P < 0.001$, Fig. 6c], respectively.

Experiment 7: effects of subthreshold doses of crocins and memantine in antagonizing delay-dependent deficits in the novel object-recognition task (acquisition experiment, intertrial interval 24 h)

The analysis of the *D* index data showed significant main effects of crocins [$F_{(1, 28)} = 7.1$, $P < 0.02$] and memantine [$F_{(1, 28)} = 6.4$, $P < 0.02$] and a significant crocins × memantine interaction [$F_{(1, 28)} = 6.3$, $P < 0.02$]. Post-hoc comparisons showed that the vehicle + vehicle, the vehicle + crocins (5 mg/kg) and the vehicle + memantine (3 mg/kg) groups had a lower index *D* compared with the crocins + memantine group ($P < 0.05$, Fig. 7a). Overall analysis of total exploration times data showed nonsignificant three-way and two-way interactions, and a nonsignificant main effect of crocins, memantine and trials was evidenced (Fig. 7b). Analysis of motor activity data showed nonsignificant three-way and two-way interactions. There was a significant main effect of trials [$F_{(1, 28)} = 13.1$, $P < 0.001$, Fig. 7c], but not of crocins or memantine.

Fig. 6



Acquisition study. Vehicle, crocins and memantine were injected intraperitoneally 60 min before T1, with a 6-h ITI. The results are expressed as mean + SEM. (a) Discrimination (*D*) index. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

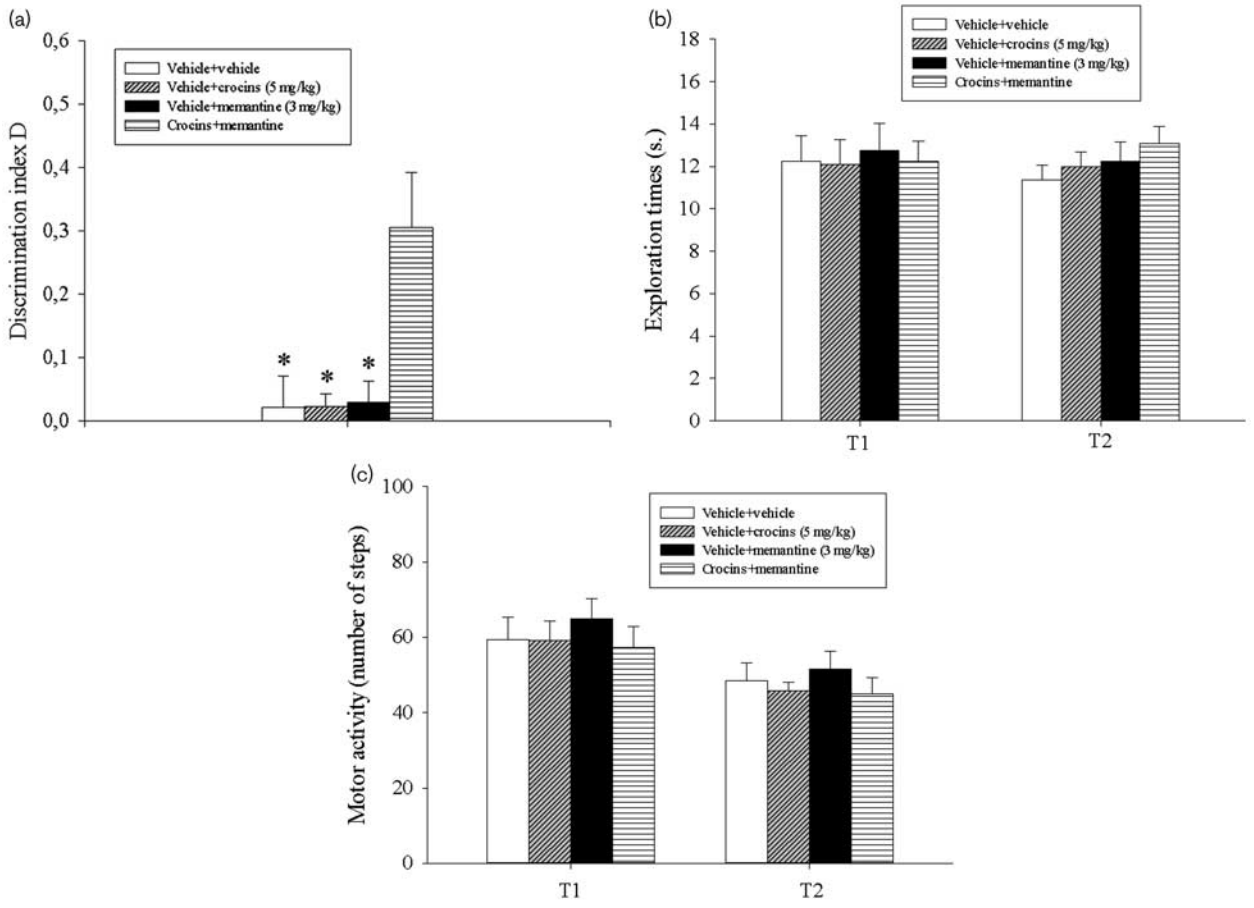
Experiment 8: effects of subthreshold doses of crocins and memantine in antagonizing delay-dependent deficits in the novel object-recognition task (storage experiment, intertrial interval 24 h)

The analysis of the *D* index data showed significant main effects of crocins [$F_{(1, 28)} = 12.4$, $P < 0.001$] and memantine [$F_{(1, 28)} = 16.3$, $P < 0.001$] and a significant crocins × memantine interaction [$F_{(1, 28)} = 14.1$, $P < 0.001$]. Post-hoc comparisons showed that the vehicle + vehicle, the vehicle + crocins (5 mg/kg) and the vehicle + memantine (3 mg/kg) groups had a lower index *D* compared with the crocins + memantine group ($P < 0.05$, Fig. 8a). Overall analysis of total exploration time and motor activity data during T2 did not show any signifcant effect of crocins or memantine or a significant crocins × memantine interaction. There were no significant differences in the total exploration levels and motility data of untreated rats during T1 (Fig. 8b and c, respectively).

Experiment 9: effects of subthreshold doses of crocins and memantine in antagonizing delay-dependent deficits in the novel object-recognition task (retrieval experiment, intertrial interval 24 h)

The analysis of the *D* index data showed significant main effect of crocins [$F_{(1, 28)} = 5.3$, $P < 0.05$] and memantine [$F_{(1, 28)} = 7.1$, $P < 0.02$] and a significant crocins × memantine interaction [$F_{(1, 28)} = 6.7$, $P < 0.02$]. Post-hoc comparisons showed that the vehicle + vehicle, the vehicle + crocins and the vehicle + memantine groups had a lower index *D* compared with the crocins + memantine group ($P < 0.05$, Fig. 9a). Analysis of total exploration time and motor activity data during T2 did not show significant effects of crocins or memantine or a significant crocins × memantine interaction on these measures of performance. There were no significant differences in total exploration levels and motility data of untreated rats during T1 (Fig. 9b and c, respectively).

Fig. 7



Acquisition study. Vehicle, crocins and memantine were injected intraperitoneally 60 min before T1, with a 24-h ITI. The results are expressed as mean + SEM. (a) Discrimination (*D*) index.*$P < 0.05$ versus crocins + memantine group. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

Discussion

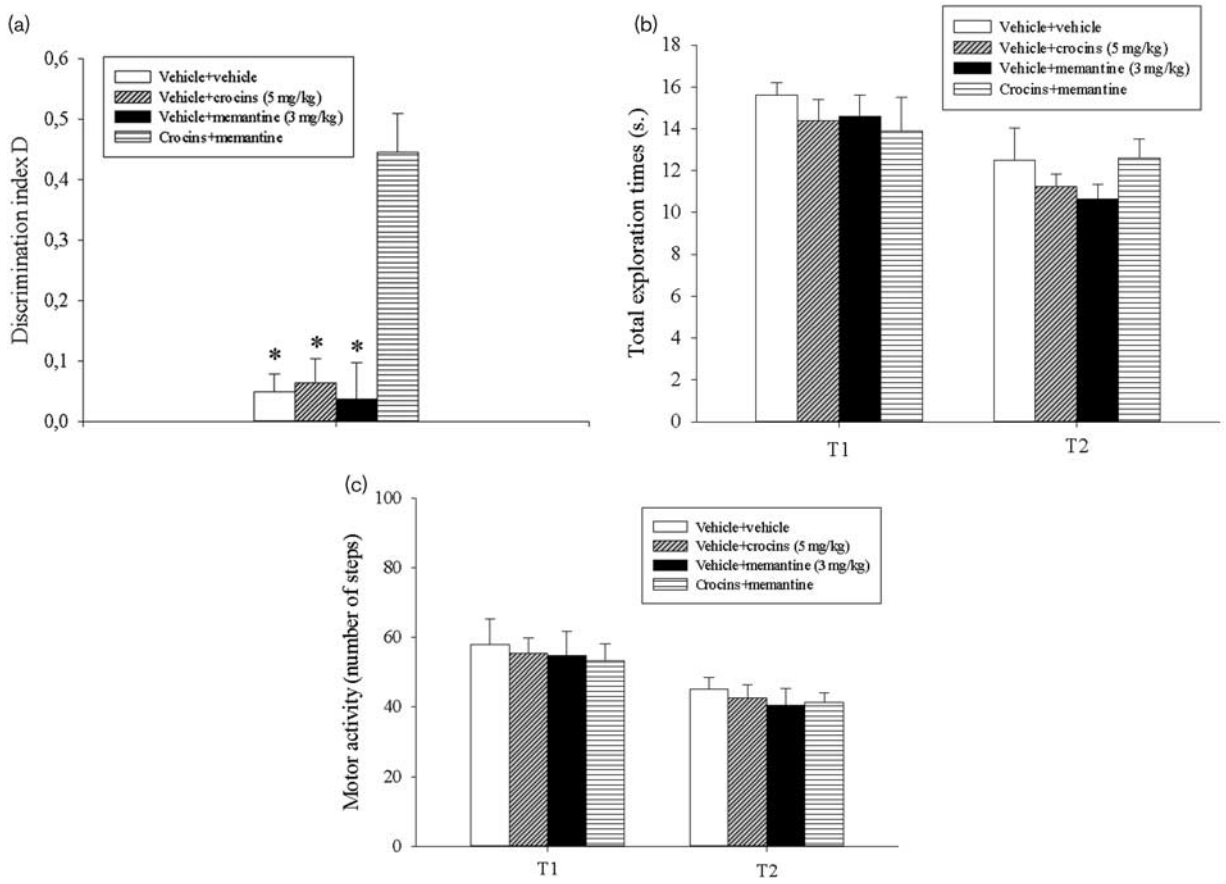
NORT is a recognition memory paradigm that it is based on spontaneous exploratory behaviour in rodents (Ennaceur and Delacour, 1988). This behavioural procedure does not involve explicit reward or punishment, but relies on the natural curiosity of rodents and their preference for novelty (Robbins, 1977), which does not appear to be influenced by reinforcement/response contingencies (Dere *et al.*, 2007). This paradigm shares similarities with procedures utilized in clinical research and has a significant level of predictive validity (Ennaceur and Delacour, 1988).

Consistent with previous reports (Bartolini *et al.*, 1996; Pitsikas *et al.*, 2002), recognition memory remained intact up to a 6-h delay, but dissipated with a 24 h interval between initial exposure to the objects and the testing phase. Rats treated with crocins (5, 15, and 30 mg/kg) expressed similar levels of discrimination abilities to their vehicle-treated counterparts at the 6-h delay. Animals that received 15 and 30, but not 5 mg/kg crocins or vehicle, could discriminate between familiar and new objects after an ITI as long as 24 h.

Rats treated with the inactive doses of crocins (5 mg/kg) memantine (3 mg/kg) and their combination showed similar levels of discrimination levels to their vehicle-treated counterparts when a delay of 6 h was used. Pretraining and post-training coadministration of subthreshold doses of crocins (5 mg/kg) and memantine (3 mg/kg) counteracted time-dependent recognition memory deficits with a 24 h ITI. Neither memantine (3 mg/kg) nor crocins (5 mg/kg) alone reduced delay-dependent deficits.

These results suggest that the concomitant administration of inactive doses of crocins and memantine might be relevant for improving recognition memory. The high doses of crocins (15 and 30 mg/kg) and the combined administration of inactive doses of crocins and memantine influenced animals' performance during retention, reflecting a modulation of different memory processes (acquisition, consolidation and retrieval of information).

Fig. 8



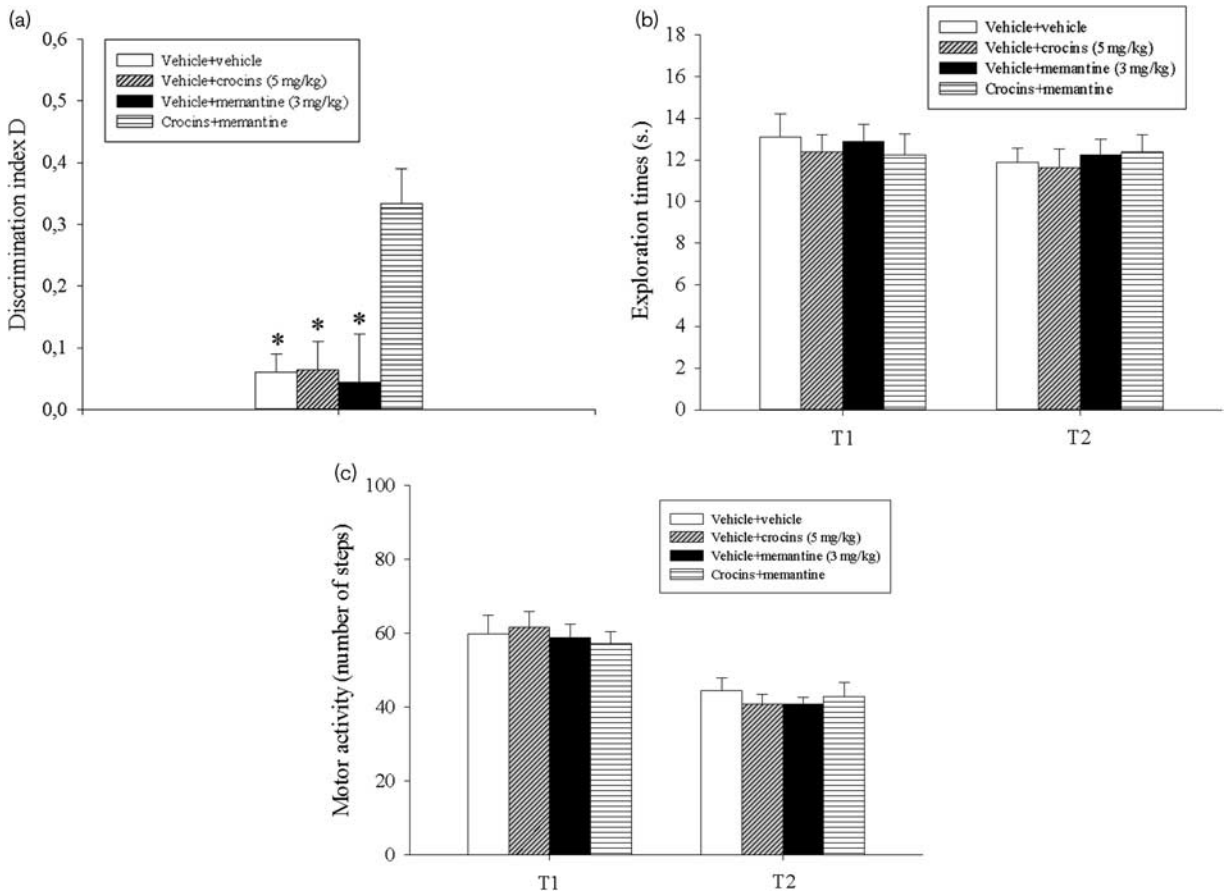
Storage study. Vehicle, crocins and memantine were injected intraperitoneally immediately after T1, with a 24-h ITI . The results are expressed as mean + SEM. (a) Discrimination (*D*) index.*$P < 0.05$ versus crocins + memantine group. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

No data are available on pharmacokinetic interactions between crocins and memantine treatments. Both drugs were administered only once, and their half-life is around 2 (Asai *et al.*, 2005) and 3 h (Spanagel *et al.*, 1994), respectively. We cannot, therefore, exclude that compounds administered before T1 might have influenced not only acquisition but also early consolidation.

Crocins and memantine were delivered peripherally, and nonspecific factors such as arousal or sensorimotor deficits may have influenced rats' cognitive performance in the present studies. In this context, it has been suggested that a rewarding effect of novelty might underlie the effects of compounds on memory (Bevins and Besheer, 2005). In addition, as NORT is based on spontaneous exploration and as exploratory activity may reflect anxiolytic or anxiogenic processes (Ennaceur and Delacour, 1988), the compounds that we used could have acted on performance through anxiolytic or anxiogenic mechanisms. However, no differences in terms of exploration levels and locomotor activity were observed at either T1 or T2 among the various experimental groups (untreated or treated animals). Under some circumstances, (experiments 2, 3, 6 and 7), exploratory and motor activities were decreased in all animals, independent of their treatment condition, across trials, which could reflect habituation. Although these animals showed lower levels of exploration or of motility across trials, importantly, their exploration and motor activity levels were similar within the choice trial T2. Altogether, the pattern of the present results suggests that the involvement of nonspecific factors in the effects of the compounds on cognitive performance seems unlikely.

The mechanism(s) by which crocins exert their effects on cognition is still a matter of investigation. In this context, it has been reported that crocins promoted hippocampal long-term potentiation (LTP) (Sugiura *et al.*, 1994), a form of activity-dependent synaptic plasticity that may underlie learning and memory (Lynch and Staubli, 1991), and it has recently been shown that aqueous extracts of saffron increased the levels of brain-derived neurotrophic factor (BDNF) in rat hippocampus (Ghasemi *et al.*, 2015). Memantine is a noncompetitive NMDA receptor antagonist of moderate affinity that could decrease pathological

Fig. 9



Retrieval study. Vehicle, crocins and memantine were injected intraperitoneally 60 min before T2, with a 24-h ITI. The results are expressed as mean ± SEM. (a) Discrimination (*D*) index.*$P < 0.05$ versus crocins + memantine group. (b) Total exploration times. (c) Total motor activity. ITI, intertrial interval.

activation of NMDA receptors without affecting physiological NMDA receptor activity (Scarpini *et al.*, 2003). It has been reported that memantine also increased BDNF levels (Marvanova *et al.*, 2001) and prolonged the duration of LTP in the aged rat (Barnes *et al.*, 1996). This augmentation of BDNF concentrations induced by both saffron and memantine might be of importance as this neurotrophic factor has been shown to facilitate synaptic plasticity and enhances the induction of hippocampal LTP (Figurov *et al.*, 1996; Patterson *et al.*, 1996).

The combination of drug therapy to improve cognitive impairments has received considerable attention recently. An important advantage of combined drug therapy is that it offers the prospect of additive or synergistic benefits and the potential to decrease doses of agents that may cause undesired side effects (Wise *et al.*, 2007). There is no information, however, on other behavioural and/or biochemical effects of the combined administration of crocins with memantine and, hence, the underlying mechanism(s) of action. The stimulatory effect of both crocins and memantine on synaptic plasticity (Sugiura *et al.*, 1994; Barnes *et al.*, 1996) might be a plausible explanation for the beneficial action of their combination on recognition memory. Intensive research, however, is required to properly address this important issue.

Conclusion

In summary, the present results show that crocins and the combination of their subthreshold doses with inactive doses of memantine counteracted delay-dependent recognition memory deficits in rats. Further, the current findings suggest that crocins are involved in different stages of recognition memory. The combined use of crocins and memantine could represent a novel strategy to treat memory disorders. It is important to underline, however, that the promnesic effects of the combination of crocins with memantine evidenced in the present study are limited to recognition memory in young rats. Further preclinical investigations utilizing different memory paradigms (e.g. spatial memory tasks) and the use of genuine models of memory deficits (e.g. old

rodents, transgenic animals) are mandatory to evaluate whether crocins will be suitable adjunctive agents for the treatment of cognitive problems. In this context, it is important to take into account the good safety profile of saffron that was reported in different clinical studies (Pitsikas, 2015).

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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