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Biphasic effect of citral, a flavoring and scenting agent, on spatial learning and memory in rats

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ABSTRACT

Although some central effects of citral have been reported, cognitive effects on spatial memory have not been investigated. The evidence showed that citral can regulate the synthesis of retinoic acid (RA), which exerts a vital function in the development and maintenance of spatial memory. In this study, we applied Morris water maze to test the effect of citral on animals' spatial learning and memory. To elucidate the mechanism of this effect, we also measured the retinoic acid concentration in rats' hippocampus by high performance liquid chromatography (HPLC). Our data implied biphasic effects of citral. The low dose (0.1 mg/kg) of citral improved the spatial learning capability, and enhanced the spatial reference memory of rats, whereas the high dose (1.0 mg/kg) was like to produce the opposite effects. Meanwhile, the low dose of citral increased the hippocampal retinoic acid concentration, while the high dose decreased it. Due to the quick elimination and non-bioaccumulation in the body, effects of citral on spatial memory in this study seemed to be indirect actions. The change in hippocampal retinoic acid concentration induced by different doses of citral might be responsible for the biphasic effect of citral on spatial learning and memory.

1. Introduction

Citral, 3,7-dimethyl-2,6-octadienal, is one of the most important members of open chain monoterpenoids. Chemically, natural citral is a mixture of isomers, cis-isomer neral and trans-isomer geranial that have the same molecular formula, $C_{10}H_{16}O$, but different structures. Citral is present in the volatile oils of several plants, such as citronella (lemon grass) and verbena (Opdyke, 1979). With a strong lemon odor, citral is widely used in food, cosmetics and detergent industries as flavoring or scenting agents (Opdyke, 1979). Citral is also an important material in the manufacture of some chemicals, such as vitamin A (Ress et al., 2003). In addition to the commercial value, quite a number of other actions of citral have been investigated. The essential oils extracted from certain kinds of plants have strong antibacterial, antifungal and antiparasitic activities, and one of the most important active constituents of these essential oils responsible for these activities was proved to be citral (Fisher and Phillips, 2006; Ramachandran et al., 2008; Santoro et al., 2007). Citral is associated with some pathological processes, such as cutaneous anaphylaxis and prostatic hyperplasia (Kessler et al., 1998; Lalko and Api, 2008). There is also evidence to show that citral can interfere with the embryogenesis and carcinogenesis (Di Renzo et al., 2007; Dudai et al., 2005).

Besides multiple actions of citral mentioned above, some central effects of citral have also been reported. The sedative effect was observed in mice (do Vale et al., 2002; Vale et al., 1999), and the anxiogenic effect induced by high doses of citral was also reported (50 mg/kg i.p. in the elevated plus maze test; 200 mg/kg i.p. in the open field test) (do Vale et al., 2002). There were, however, few reports about the cognitive effects of citral, especially effects on animals' spatial learning. Spatial learning and memory are vital for animals when navigating both novel and familiar environments. Some evidence suggested that citral can influence the synthesis of retinoic acid (RA), an active metabolite of vitamin A (Di Renzo et al., 2007; Kikonyogo et al., 1999). It was proved that vitamin A and its metabolite, retinoic acid, play an important role in the development and maintenance of spatial memory (Cocco et al., 2002; Crandall et al., 2004; Ding et al., 2008). In this study, we were applying a commonly used, simple but effective paradigm, Morris water maze (MWM) task to test the effect of citral on animals' spatial learning and memory. We also measured the concentration of retinoic acid in rats' hippocampus to elucidate the potential mechanism of citral's effect on spatial learning and memory.

2. Methods

2.1. Reagents

Citral, coin oil and retinoic acid were obtained from Sigma-Aldrich. Acetonitrile, methanol and acetic acid were from Fisher Scientific, and are all high performance liquid chromatography (HPLC) grade.

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2.2. Subjects

Male SD rats, weighing 200–250 g at the beginning of the experiment, were housed two per cage in a temperature-controlled environment (24 ± 1 °C). They were maintained on a 12 h light/dark cycle with ad libitum access to food and water throughout the experiment, and all manipulations were performed during the dark phase of the cycle. All rats were habituated for one week in the vivarium prior to the commencement of the experiment. All protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and conformed to Regulations for the Administration of Affairs Concerning Experimental Animals (Hubei Province, China 2005).

2.3. Drug administration

In preliminary experiments, five dose levels of citral (0.01, 0.1, 1, 10 and 100 mg/kg body weight) were examined. There was no central effect observed with 0.01 mg/kg, while 1, 10 and 100 mg/kg had similar effects on spatial memory. So 0.1 and 1.0 mg/kg were chosen as the final doses. Rats were randomly divided into three groups ($n = 20$/group, 10/group for behavioral test and 10/group for retinoic acid concentration measurement), and they were administrated with corn oil (vehicle), citral 0.1 mg/kg (low dose) and 1.0 mg/kg (high dose), respectively. The treatment was administrated i.p. at a volume of 2 ml/kg. The administration was conducted once a day over 7 consecutive days.

2.4. Apparatus

Spatial learning and reference memory were assessed in Morris water maze, and the water maze tasks started on the day following the completion of the administration. The Morris task was based on the original method described by Morris (Morris, 1984), and similar to that described elsewhere (Vorhees and Williams, 2006). The tasks used a metal circular pool with smooth white walls (120 cm in diameter and 50 cm high), filled with water maintained at 22 °C and made opaque by the addition of powdered milk. The water maze was located in the center of a dimly lit room. The pool was conceptually divided into four equal quadrants [northeast (NE), southeast (SE), southwest (SW), northwest (NW)] with four designated starting positions at the midpoint of the perimeter of each quadrant. A platform (10 cm in diameter) was located in the northeast quadrant (NE) of the pool, approximately 15 cm from the wall, and the position of the platform was fixed throughout the training tasks. The water level in the pool was adjusted so that the platform stood 2 cm below the surface of the water. Data were collected by a video camera mounted above the center of the pool and connected to a computer, and analyzed by an automated tracking system (Time, Chengdu Technology & Market Co. Ltd., China) to provide measures of swim distance, time and speed.

2.5. Behavioral procedures

Rats were trained in sets (sessions) of 4 trials per day for 5 days (20 trials total). During the training tasks, the rat was released into the pool with its head pointing towards the tank wall from one of four randomly chosen entry points around the pool's perimeter. The order of the entry points was randomly varied each day. On each trial, the rat was allowed maximum of 90 s to locate the submerged platform. Once the rat had climbed onto the platform, it was allowed to stay there for 15 s before being removed. If the rat failed to find the platform on time, it was gently guided to the platform and left on it for 15 s. Each rat was returned to its warmed holding cage for approximate 15 min (intertrial interval, ITI) until the next trial. The day after initial acquisition (Day 6), a 30 s probe trial was given for which the platform was removed. The start point for the probe trial was the farthest one (SW) from the target quadrant where the platform was placed during the acquisition training, and was the same for all rats. Here, the numbers of crossing over the original platform-site and target annulus (a circular zone surrounding and with twice diameter of the platform), time spent in the target quadrant, and swim distance in the target quadrant as well as in other quadrants were measured.

2.6. Hippocampal retinoic acid extraction

Rats were sacrificed by decapitation 24 h after the drug administration. Bilateral hippocampi were harvested, and homogenized with ice-cold normal saline (100 mg wet weight of hippocampus in 500 µl saline). The homogenate (500 µl) was mixed with 500 µl of acetonitrile containing acetic acid (1% v/v), vortexed for 30 s, and centrifuged at 4000 ×*g* at 4 °C for 10 min. The supernatant were collected, and purified by filtering through a cellulose filter (Microcon YM-3, Millipore, USA). An aliquot (20 µl) of the purified sample was directly injected into the HPLC system. All handling of samples was performed in a darkened room with red light.

2.7. High performance liquid chromatography

The analysis was performed on an HP-1100 HPLC system equipped with a vacuum degasser, quaternary pump, column heater, and a diode array detector (DAD) (Agilent Technologies, USA). Reverse-phase (RP) HPLC analysis was conducted on a C18 column (particle size: 5 µm, column size: 250 mm × 4.6 mm) (Dalian Elite Analytical instruments Co. Ltd., China). Agilent ChemStation was used for data collection and integration. The mobile phase consisted of acetonitrile, acetic acid (2% in water) and methanol (57.5:25:17.5 v/v/v). Flow rate was 1.3 ml/min. The temperature was maintained at 25 °C. Retinoic acid was detected at 354 nm. The concentration of retinoic acid was determined by the external standard method by using authentic retinoic acid dissolved in methanol.

2.8. Data analysis and statistics

The escape latency, path length and swim speed of 5 days acquisition trials in Morris water maze were averaged per session (four trials). For probe trial, the percentage of time spent in the target quadrant and the percentage of swim distance in the target quadrant were calculated. To exclude the confounding effect of anxiety possibly induced by citral on water maze performance, we measured the thigmotaxis during spatial acquisition and probe trail by calculating the percent time rats spent in outer zone (within 20 cm of the maze wall). Training session data were analyzed with two-way repeated measures analysis of variance (ANOVA) with dose and session as the variables. For probe trial data, one-way ANOVA with quadrant as a repeated measure was applied. Retinoic acid concentration was analyzed with one-way ANOVA. Post hoc comparisons (*Tukey*) were performed, when necessary. All the results are presented as mean ± SEM. In all comparisons, $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Spatial acquisition

Fig. 1(A) shows the mean escape latencies of three groups of animals during the training in the hidden platform water maze tasks (5 days). The learning curves indicated a significantly downward linear tendency, all three groups progressively reducing the time needed to locate the platform over the course of the training sessions [$F(4,108) = 68.35$, $p < 0.01$]. Although the downtrend of the learning curves was similar among all groups [$F(2,27) = 1.68$, $p > 0.05$], it is

worth noting that each group showed a different learning speed. The low dose group (0.1 mg/kg) showed a non-significant difference in the escape latency from the control group (vehicle). The learning curve of the low dose group was parallel to that of the control group, and the low dose group's learning curve was always below the control group's throughout the acquisition courses. Meanwhile, the learning curves of the control and high dose (1.0 mg/kg) groups started out with the similar escape latencies on the first two days, but separated on Day 3–5. The rats administrated with high dose of citral seemed to need more time than the control rats did to find the platform during the last three learning sessions (non-significant). It should be noted that the post-hoc analysis revealed statistical differences in the escape latencies between the low dose group and the high dose group on the last three training days (Day 3 $p<0.02$; Day 4 $p<0.02$; Day 5 $p<0.03$), and the escape latencies of the low dose group on Days 3–5 were significantly lower than that of the high dose group. There was not a significant dose × session interaction [$F(8,108)=0.33$, $p>0.05$].

The swim path length shown in Fig. 1(B) also revealed a similar learning evolution pattern as that of escape latencies. The path length of three groups reduced rapidly along with the procedure of the acquisition training, indicating that, across groups, rats were able to locate the platform more effectively with increased training [$F(4,108)=66.75$, $p<0.01$]. There were also non-significant differences in path length between the control group and the treatment groups (low and high dose) [$F(2,27)=3.18$, $p>0.05$]. The rats treated with low dose of citral always swam the shorter distance in the water maze to locate the submerged platform than the control group did throughout the whole training courses. The control and high dose groups had the similar path length during the first two training sessions, but the high dose group seemed to swim longer distance to locate the platform on Days 3–5 than the control rats did. The post-hoc analysis demonstrated statistical differences in path length between the low dose group and the high dose group during the last three training sessions (Day 3 $p<0.02$; Day 4 $p<0.01$; Day 5 $p<0.05$), and the low dose group swam remarkably shorter distance to find platform than the high dose group did. There was not a significant dose × session interaction [$F(8,108)=1.67$, $p>0.05$].

As shown in Fig. 1(C), there was no treatment related change in the swim speed among three groups [$F(2,27)=0.01$, $p>0.05$] over the course of spatial training [$F(4,108)=1.61$, $p>0.05$]. There was also no significant dose × session interaction [$F(8,108)=0.88$, $p>0.05$]. This finding indicated that treatment of citral with the dosage used in this study had little effect on the animals' movement and motivation in the Morris water maze task.

All three groups were progressively reducing the time swimming in the outer zone during training sessions [% time in outer zone of three groups: (control: Day 1 60.53 ± 5.19, Day 2 43.27 ± 5.76, Day 3 36.23 ± 2.84, Day 4 36.03 ± 2.23, Day 5 33.05 ± 3.95); (low dose: Day 1 60.99 ± 3.13, Day 2 39.68 ± 3.25, Day 3 33.48 ± 2.12, Day 4 39.11 ± 3.20, Day 5 31.96 ± 3.92); (high dose: Day 1 57.54 ± 4.50, Day 2 41.30 ± 3.00, Day 3 36.10 ± 4.14, Day 4 40.17 ± 1.30, Day 5 36.55 ± 3.87)] [$F(4,108)=20.44$, $p<0.01$]. There was no significant difference in thigmotaxis among three groups [$F(2,27)=0.17$, $p>0.05$]. There was no session × dose interaction [$F(8,108)=0.45$, $p>0.05$].

3.2. Probe trials

Rats' spatial reference memory was assessed by probe trials conducted 24 h following the last acquisition trial. Rats from all three groups swam more time in the target quadrant where the platform was located during the training sessions than they did in the other quadrants [$F(3,87)=66.59$, $p<0.01$]. However, as shown in Fig. 2(A), it is notable that there was a significant difference in percent time rats spent in target quadrant among three groups [$F(2,27)=15.35$, $p<0.01$]. The post-hoc analysis revealed that the low dose group spent significantly more time in the target quadrant than the control rats did ($p<0.04$), whereas rats treated with high dose of citral spent remarkably less time in the target quadrant than the control group did ($p<0.02$). Similarly, all three groups swam longer distance in target quadrant than they did in other quadrants [$F(3,87)=32.06$, $p<0.01$]. There was also a significant difference among three groups in the percentage of distance in the target quadrant [$F(2,27)=16.61$, $p<0.01$]. The post-hoc analysis showed that the low dose group swam the markedly longer distance in the target quadrant than the control rats did ($p<0.02$). Meanwhile, the high dose group swam notably shorter distance in the target quadrant than the control group did ($p<0.02$). There were also significant differences between the low dose group and the high dose group in the percent time and distance in the target quadrant (% time in target quadrant: $p<0.01$, % distance in target quadrant: $p<0.01$), and the low dose group showed evidently stronger preference for the target quadrant than the high dose group did. Fig. 3 shows the representative swimming tracks of three groups for the probe trials. In comparison to the control rats, the rats administrated with low dose of citral were more prone to swim in the target

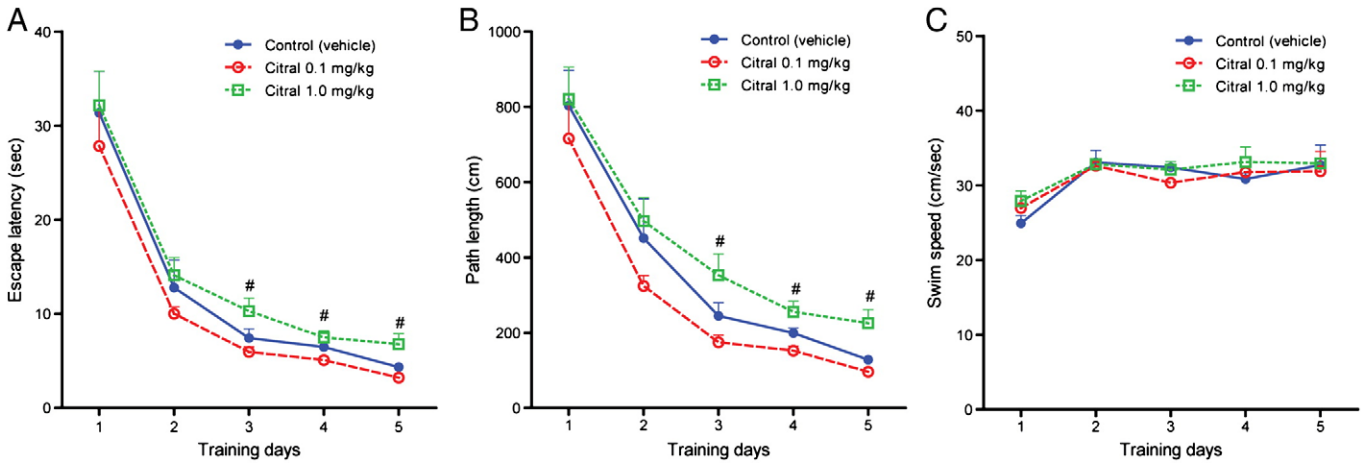

Fig. 1. Effects of citral on the spatial learning ability on the acquisition training phase of Morris water maze tasks with invisible platform. Escape latency (A), path length (B) and swim speed (C) were averaged at each time point (Days 1–5) for each group ($n=10$/group), and are shown as mean ± SEM. $^{\#}p<0.05$ between the low dose group (0.1 mg/kg) and the high dose group (1.0 mg/kg).

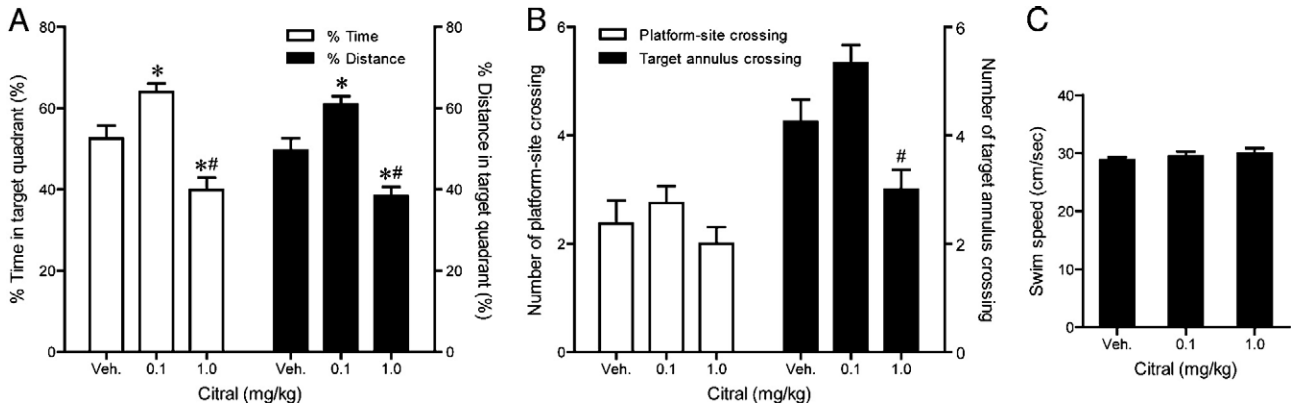

Fig. 2. Effects of citral on the spatial reference memory tested by probe trials in Morris water maze with platform removed. % time in target quadrant (open bars) and % distance in target quadrant (filled bars) (A), number of platform-site crossing (open bars) and number of target annulus crossing (filled bars) (B), and swim speed (C) are presented as mean ± SEM. $^{*}p<0.05$ vs. control group (veh.); $^{\#}p<0.05$ between the low dose group (0.1 mg/kg) and the high dose group (1.0 mg/kg).

quadrant during the probe trial, while the high dose group showed weaker preference for the target quadrant.

The numbers of platform-site and target annulus crossing were also recorded during the probe trials [number of platform-site crossing: $F(2,27)=1.09$, $p>0.05$; number of target annulus crossing: $F(2,27)=8.26$, $p<0.01$]. As shown in Fig. 2(B), there was a similar trend of changes in them as in the percent time and distance in the target quadrant induced by different doses of citral. Compared to the control, the low dose of citral slightly raised the numbers of the platform-site crossing and the target annulus crossing, whereas the high dose caused the modest reduction of these numbers. According to the post-hoc analysis, the numbers of the target annulus crossing of the low dose group was significantly higher than that of the high dose group ($p<0.01$). The analysis of the swim speed during the probe trials showed no significant treatment effects [$F(2, 27)=0.45$, $p>0.05$] (Fig. 2(C)).

There was no significant difference in thigmotaxis observed among groups (% time in outer zone: control 51.76 ± 3.82; low dose 50.40 ± 3.56; high dose 51.59 ± 3.93) [$F(2,27)=0.57$, $p>0.05$], while all groups showed evident preference for the outer zone [$F(2,58)=74.64$, $p<0.01$] (The platform was located in the outer zone during training sessions).

3.3. The concentration of retinoic acid in hippocampus

As shown in Fig. 4(A), the concentration of retinoic acid in hippocampus was significantly changed in rats administrated with both doses of citral [$F(2,27)=16.92$ $p<0.01$]. The post-hoc analysis revealed that hippocampal retinoic acid concentration in rats administrated with low dose of citral was evidently higher than that in control rats ($p<0.03$). Compared to the control, the high dose of citral remarkably decreased retinoic acid concentration in hippocampus ($p<0.05$). There was also a statistical difference in retinoic acid concentration between the low dose group and the high dose group ($p<0.01$). The representative HPLC chromatogram of three groups was shown in Fig. 4(B).

4. Discussion

Thus, our data implied that different dose of citral seemed to have different effect on the spatial learning and reference memory of rats. The low dose (0.1 mg/kg) of citral in this experiment might have a stimulant action. Namely, small dose of citral could possibly improve the spatial learning capability of rats, and enhance the animals' spatial reference memory in Morris water maze. However, the high dose (1.0 mg/kg) of citral was like to produce an opposite effect, that is high dose of citral could probably repress the spatial learning ability and spatial reference memory of rats, and the repressive action on animals' spatial learning capability might be more obvious during the late stage of learning courses (Days 3–5). Actually, we were not the first one to find the biphasic actions of citral. Price and Berry proved that citral with low concentration (10^{-4} M) had excitative effects on spontaneous firing in dorsal unpaired median neurons of cockroach,

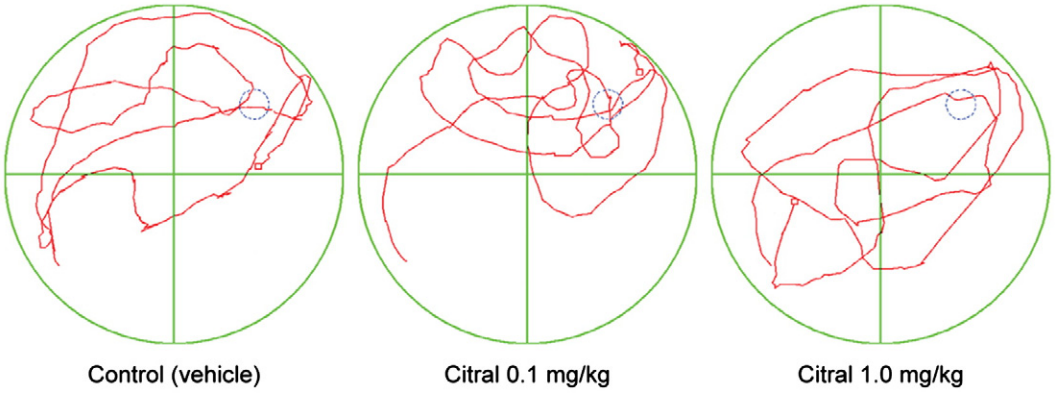

Fig. 3. Representative swim tracks of three groups for the probe trials: control (vehicle), low dose (0.1 mg/kg), and high dose (1.0 mg/kg) groups. The original location of the removed platform was marked with the dashed circle.

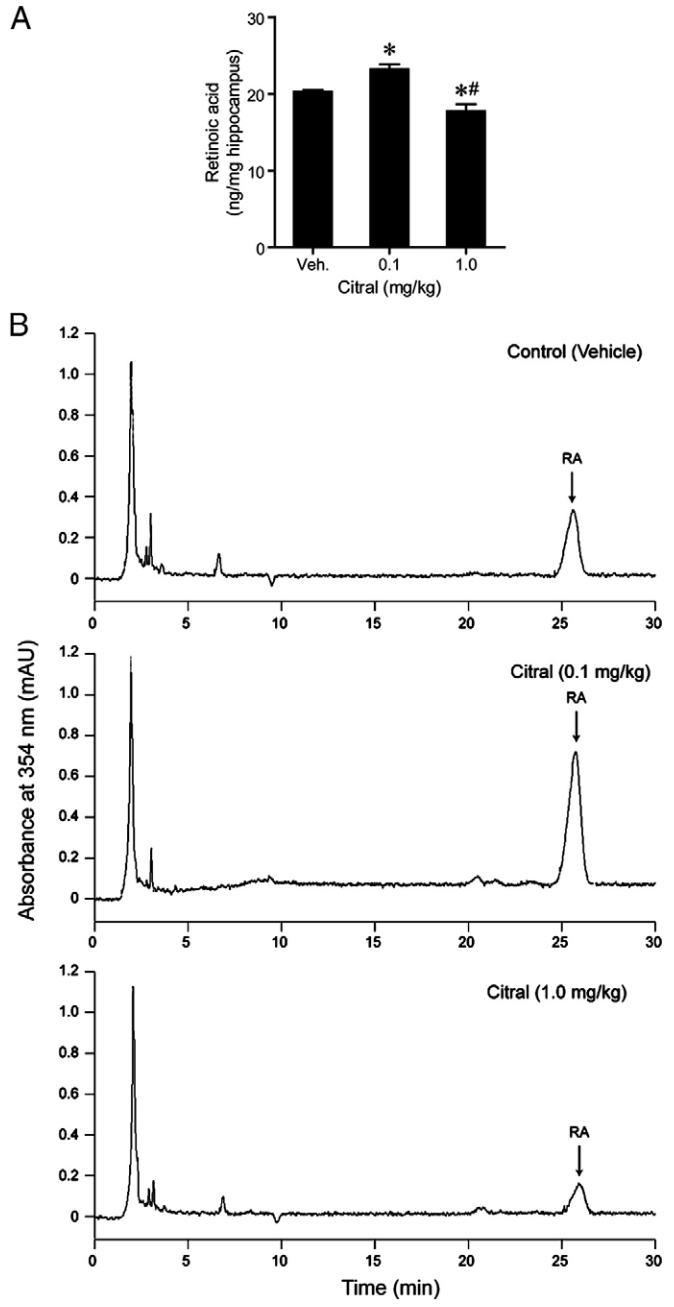

Fig. 4. Effects of citral on retinoic acid (RA) concentration in hippocampus and the representative HPLC chromatogram of three groups. (A) The concentration of retinoic acid in rats' hippocampus was presented as mean ± SEM. $^{*}p<0.05$ vs. control group (veh.); $^{\#}p<0.05$ between the low dose group (0.1 mg/kg) and the high dose group (1.0 mg/kg). (B) Representative HPLC chromatogram of three groups: control (vehicle), low dose (0.1 mg/kg), and high dose (1.0 mg/kg) groups.

while the high concentration of citral (2×10^{-3} M) produced depressive effects (Price and Berry, 2006).

The metabolism and elimination of citral administrated via various routes were both rapid and extensive. Approximately 50% of the dose after a single p.o. exposure was eliminated by urine within 24 h, and there was no bioaccumulation observed with repeated exposure to citral (Diliberto et al., 1990, 1988). In this study, the acquisition training started 24 h after the completion of the administration, and the probe trials were even later. Thus, the blood concentration of citral seemed unable to be maintained at a level to produce an effective action during the whole procedure of Morris water maze tasks. So the effects of cital on the spatial learning and reference memory we observed might not be the direct action, but more like an intermediation triggering other pathway to influence the spatial memory. Just as stated above, citral can influence the synthesis of retinoic acid by regulating the activity of retinoic acid synthetases, retinaldehyde dehydrogenases (RALDHs) (Di Renzo et al., 2007; Kikonyogo et al., 1999). Our previous study also showed that certain isoforms of RALDHs were abundantly expressed in adult human brain (Xi and Yang, 2008). It is well known that retinoic acid, a potent signaling molecule, not only controls the procedure of embryonic development (Bowles and Koopman, 2007; Soprano et al., 2007), but also plays an important role in adult, including the regeneration and maintenance of the adult nervous system (Maden, 2007). There was evident to show that retinoic acid exerts a vital function in the development and maintenance of spatial memory (Cocco et al., 2002; Crandall et al., 2004; Ding et al., 2008). In this study, our data showed that citral had a biphasic effect on the concentration of retinoic acid in rats' hippocampus. The low dose of citral induced remarkable increment in hippocampal retinoic acid concentration, while the high dose caused a significant reduction in it. Given all that, the change in hippocampal retinoic acid concentration induced by different doses of citral might be responsible for the biphasic effect of citral on spatial learning and memory.

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