Regular Article

Chemerin-9 Peptide Enhances Memory and Ameliorates $A\beta_{1-42}$-Induced Object Memory Impairment in Mice

ZeLin Lei, YaQin Lu, Xue Bai, ZhenXiu Jiang, and Qin Yu

*Key Laboratory of Biotherapy and Regenerative Medicine, the First Hospital of Lanzhou University; Lanzhou, Gansu 730000, China: and Department of Neurology, the First Hospital of Lanzhou University; Lanzhou, Gansu 730000, China.

Received June 19, 2019; accepted October 31, 2019; advance publication released online November 19, 2019

INTRODUCTION

Alzheimer’s disease (AD) is one of the most common neurodegenerative diseases. The pathological hallmarks of AD are neurofibrillary tangles, senile plaques, synaptic dysfunction and neuronal loss in the brain. The essential component of senile plaques is aggregates of amyloid $\beta_{1-42}$ ($A\beta_{1-42})$. Accumulating evidence indicates that the infusion of $A\beta_{1-42}$ into the brain causes synaptic dysfunction and neuronal loss in the brain accompanied by neuroinflammation, which is characterized by the activation of microglia and astrocytes. Emerging data have suggested that inflammation in the pathogenesis of AD and aggregation of $A\beta$ may activate microglia cells and result in the production of various factors, including proinflammatory mediators (e.g. interleukin-1$\beta$ (IL-1$\beta$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and interleukin-6 (IL-6)). These proinflammatory cytokines may directly act on neurons to induce apoptosis. Given that AD pathogenesis includes strong interactions with immunological mechanisms in the brain, it is not restricted to the neuronal compartment. Targeting these immune mechanisms could lead to future therapeutic or preventive strategies for AD.

Some traditional anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase (COX)-2 inhibitors, are evaluating their effects in clinical trials of AD (NCT00046358, NCT00004845, NCT00013650, NCT03277573, NCT02547818, NCT00007189).

Chemerin is a secreted protein encoded by tazarotene-induced gene 2 protein (TIG2). Three receptors for chemerin, chemokine-like receptor 1 (CMKLR1), and chemerin receptor-like (CCRL2), have been identified so far. Chemerin also works as an adipokine and is associated with obesity, insulin resistance, metabolic syndrome and inflammation. The chemerin-9 (C9) peptide is a chemerin-derived nonapeptide that retains most of the agonistic activity of chemerin, but only contains the C-terminal 9 amino acids of the active form of chemerin ($^{\text{YFPGQFAFS}^{\text{Y}}}$). It has been found that the C9 peptide induces the chemotaxis of primary microglia. Recent research has shown that both full-length chemerin and the C9 peptide are able to activate CMKLR1, which is detected in the prefrontal cortex (PFC), hippocampus and cerebellum in mice. Furthermore, it has been suggested that chemerin potentially participates in the regulation of several types of inflammatory diseases, such as allergic asthma, encephalomyelitis and peritonitis. Additional experimental evidence revealed that chemerin levels in the serum are related to proinflammatory cytokines, including TNF-$\alpha$, IL-6 and C reactive protein.

Emerging data have suggested that $A\beta_{1-42}$ interacts with CMKLR1, one of the receptors of chemerin and the C9 peptide, and induces microglial chemotaxis. Given that CMKLR1 is expressed in the brain, that chemerin and $A\beta_{1-42}$ share the same receptor, and that AD, in which chemerin is involved, is associated with inflammation, we hypothesized that chemerin may have the potential to ameliorate $A\beta_{1-42}$ mediated AD disease progression.

The results showed that an intracerebroventricular (i.c.v.) injection of C9 (8 $\mu$g/kg) facilitated memory formation and improved memory retention, as evidenced by the results of both the novel object recognition test (NOR) and object location recognition (OLR) tasks. These memory-enhancing effects of C9 were also observed after C9 (2 $\mu$g/kg) was infused into the hippocampus. Moreover, we found that treatment with C9 reversed the deficits in memory and learning ability induced by oligomeric $A\beta_{1-42}$. Meanwhile, C9 also significantly inhibited $A\beta_{1-42}$-induced increases in the levels of pro-inflammatory cytokines such as interleukin-1$\beta$ (IL-1$\beta$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-6 (IL-6) in the hippocampus. The same results were obtained for Western blotting and enzyme-linked immunosorbent assay (ELISA) experiments. Finally, we observed that C9 did not affect locomotor activity, suggesting that its improvement of memory is not a false positive induced by hypolocomotion. In conclusion, C9 may facilitate memory formation, prolong memory retention, and ameliorate $A\beta_{1-42}$-induced memory impairment, suggesting that C9 may potentially represent a novel strategy for the treatment of AD.

Key words chemerin; amyloid $\beta_{1-42}$ ($A\beta_{1-42}$); hippocampus; cognitive impairment; neuroinflammation

© 2020 The Pharmaceutical Society of Japan
diated AD disease progression.

Since C9 retains the agonistic activity of chemerin, we evaluated in this study whether the intracerebral infusion of C9 affects memory performance and ameliorates \( \text{A}_{\beta_{1-42}} \) induced memory deficit. The models for detecting memory performance in this experiment are the novel object recognition test (NOR), also known as the object recognition test (ORT), and object location recognition (OLR) tests. The NOR and OLR tests are non-aversive learning paradigms that rely on the spontaneous exploratory behaviour of the animal models used to evaluate the effects of the drug on memory. Some studies have reported that hippocampal lesions produced moderate and reliable memory impairment and they reported that the hippocampus is important for recognition memory.\(^{23-25}\)

Thus, we will investigate the effects of C9 in the hippocampus. In addition, the enhancing of the locomotor activity will increase the chances of the mouse touching the object in the NOR and OLR tests, which will elicit a false positive result. At last, we will evaluate the locomotor activity of C9 in the open field.

**MATERIALS AND METHODS**

**Experimental Animals** Male Kunming mice, a Swiss strain, eight to ten weeks old, were obtained from Lanzhou University Medical Experimental Center (Lanzhou, China). All mice were housed in 20 × 30 cm\(^2\) cages with wood shavings (5 animals/cage), and the temperature of the room was maintained at 22 ± 2°C and was accompanied by a 12-hour light–dark cycle (8 a.m.–8 p.m.). Each mouse had free access to tap water and food. All animal experiments protocols were approved by the Ethics Committee of Lanzhou University (permit number: SYXK Gan 2009-0005).

**Surgery** A 8-mm stainless-steel guide (26-gauge) cannula was implanted in the lateral ventricle or bilateral hippocampus according to a previous report.\(^{26}\) Each mouse was anaesthetized with sodium pentobarbital (intraperitoneal administration at a dose of 70 mg/kg, Sigma, U.S.A) and fixed in a stereotaxic apparatus (Leica, Germany). According to the atlas by Paxinos and Franklin,\(^{27}\) guide cannula was implanted into the right lateral ventricle (\( \text{AP} = 0.5 \text{ mm}, \text{ML} = 1.0 \text{ mm}, \text{DV} = 2.0 \text{ mm} \)), or were implanted in the bilateral hippocampus CA1 region (\( \text{AP} = 2 \text{ mm}, \text{ML} = \pm 1.5 \text{ mm}, \text{DV} = 1.2 \text{ mm} \)). Each mouse was individually housed and allowed 5–7 d to recover after surgery. All experiments were conducted between 9:00 a.m. and 6:00 p.m.

**Drugs** The C9 peptide was synthesized using a standard using Fmoc-based solid-phase method, and the structural assignment of the C9 peptide was performed by electrospray ionization time-of-flight (ESI-TOF) mass spectrometry (Fig. S1). The synthesis method of 2-(α-naphthoyl)ethyltrimethylammonium iodide (α-NETA) was shown in Fig. S4. The purity and the structure of α-NETA were checked by mass spectrometry and HPLC, and it reached the experimental requirement (Fig. S4). Purified C9 was dissolved in saline (vehicle) at a concentration of 10 mg/mL, stored at −20°C after reconstitution, and diluted to 4 mg/mL in saline immediately before being injected. Chronic injection of the \( \text{A}_{\beta_{1-42}} \) oligomer is a widely used Alzheimer’s disease model.\(^{28,29}\) In detail, \( \text{A}_{\beta_{1-42}} \) (Chinese Peptide Company Co., Ltd.) was dissolved in hexafluoroisopropanol and stored in a −80°C refrigerator overnight. On the second day, it was freeze dried to produce a powder of oligomeric \( \text{A}_{\beta_{1-42}} \). In experiment day, oligomeric \( \text{A}_{\beta_{1-42}} \) peptide was dissolved in saline containing 2% dimethyl sulfoxide (DMSO).

C9 (2 µL, 1 µL/min) or vehicle was infused into the brain over a period of 2 min by a 5 µL Hamilton syringe mounted to a syringe pumps (KD Scientific, U.S.A.). In addition, a 32-gauge stainless steel injector was lowered into guide cannula (0.5 mm beyond cannula tip). To allow the drug to diffuse, the infusion injector was left in guide cannula an additional 1 min. After drug administration, each mouse was subjected to behavioural testing immediately. C9 peptide doses were based on some previous reports.\(^{30,31}\)

**Behavioural Testing** The protocols for the learning paradigms were performed as reported by Jiang et al. previously.\(^{20,32}\) The NOR and OLR tasks consisted of two sessions: the training and test phases. In brief, the mice were tested in its sound-attenuated cages with dim lighting, the mice were handled for 3 min/d for 3 consecutive days prior to experiment. In tests, all objects were made of plastic or glass and were similar in size (4–5 cm high) but different in colour and shape. There were several copies of each object that can be used interchangeably. The objects and the location of the objects were counterbalanced and randomly permuted. At the beginning of the experiment, all objects were cleaned thoroughly with 70% ethanol to ensure the absence of olfactory cues. Exploration was defined as sniffing or touching the object with the nose or forepaws. Resting against or moving around the object was not considered exploratory behaviour. The discrimination index (DI) in the test phase was calculated as the percentage of the time spent exploring the novel object or location over the total time spent exploring the two objects or locations. A DI of 50% is equivalent to the chance level, and a higher DI indicates preferred object or location recognition memory. In addition, there were no significant differences between drug administration in the duration of the training or in the duration and total exploration time (TET) of the test phase (Table 1).

**NOR Task** During the training phase, two identical objects are placed on opposite sides of the home cage. The training trial ended when the mice explored two identical objects for 5 or 10 s in 5 min. If the TET of the training phase does not reach 5 or 10 s within 5 min, the animal is eliminated. During the testing phase, novel and familiar objects from the training trial were placed in the same position as the training phase, and the testing phase lasted 5 min. When the mouse explores two objects for a total of 25 s or after 5 min, whichever comes first, the test phase ends. Both control and drug treated mice showed good memory performance when the TET was 10 s and the mice were tested 24 h after training. When tested 3 d after training, control-treated mice failed to distinguish between novel and familiar objects, while drug-treated mice still showed good memory performance, indicating that the drug could prolong the retention of object memory. When TET was reduced to 5 s during training, control-treated mice were unable to distinguish between novel and familiar objects when tested 24 h after training. Because drug-treated mice show good memory for familiar objects, drugs may contribute to memory formation.

**OLR Task** During the training phase, two identical objects are placed in different corners of the home cage. The
locomotor activity was evaluated within 30 min. Each mouse of 80 cm. In the open field test, the novelty-induced horizontal a 50 × 50 × 40 cm³ square arena, black floor and plexiglass wall. The source of illumination is a 60W bulb with a height of 80 cm. In the open field test, the novelty-induced horizontal locomotor activity was evaluated within 30 min. Each mouse

training trial ended when the mice explored two identical objects for 5 or 10 s in 5 min. If the TET during the training phase does not reach 5 or 10 s within 5 min, the animal is eliminated. During the test phase, one of the objects was moved to a new, randomly selected corner and the test phase lasted 5 min. When the mouse detects two positions of the object for a total of 25 s, or after 5 min, whichever comes first, the test phase ends. Both vehicle and drug treated mice showed good memory performance when the TET was 10 s and the mice were tested 24 h after training. When tested 3 d after training, control-treated mice failed to distinguish between new locations and familiar locations, while drug-treated mice still showed good memory performance, indicating that the drug can prolong the retention of positional memory. When TET was reduced to 5 s during training, control-treated mice were unable to distinguish the new location from the familiar location when tested 24 h after training. Because drug-treated mice show good memory for familiar locations, drugs may contribute to the formation of locational memory.

**Locomotor Activity Tests** The open field instrument is a 50 × 50 × 40 cm³ square arena, black floor and plexiglass wall. The source of illumination is a 60W bulb with a height of 80 cm. In the open field test, the novelty-induced horizontal locomotor activity was evaluated within 30 min. Each mouse was placed in a central area and the operator sat about 1.5 m from the device. The boxes were washed with 96% ethanol between each experiment and all experiments were performed between 10 a.m. and 6 p.m. The open field test was used to

<table>
<thead>
<tr>
<th>Figure</th>
<th>Group</th>
<th>Duration of training phase</th>
<th>Duration of test phase</th>
<th>TET in test phase</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 2A</td>
<td>Vehicle</td>
<td>248.6 ± 23.8</td>
<td>300 ± 0</td>
<td>18.0 ± 1.9</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>144.9 ± 24.7</td>
<td>300 ± 0</td>
<td>13.7 ± 0.9</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Fig. 2B</td>
<td>Vehicle</td>
<td>192.7 ± 34.2</td>
<td>273.2 ± 29.7</td>
<td>16.7 ± 2.7</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>199.6 ± 28.9</td>
<td>273.3 ± 16.7</td>
<td>14.1 ± 1.3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fig. 2C</td>
<td>Vehicle</td>
<td>225.4 ± 30.4</td>
<td>291.2 ± 9.9</td>
<td>18.1 ± 1.8</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>143.1 ± 42.1</td>
<td>252.1 ± 28.7</td>
<td>17.8 ± 1.5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fig. 2D</td>
<td>Vehicle</td>
<td>285.6 ± 12</td>
<td>285.8 ± 11.8</td>
<td>16.6 ± 1.9</td>
<td>8</td>
</tr>
<tr>
<td>C9</td>
<td>128.4 ± 18.5</td>
<td>283.6 ± 14.6</td>
<td>18.1 ± 2.6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Fig. 2E</td>
<td>Vehicle</td>
<td>300 ± 0</td>
<td>300 ± 0</td>
<td>11.1 ± 0.5</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>197.6 ± 28.6</td>
<td>289.3 ± 10.7</td>
<td>15.2 ± 1.8</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Fig. 2F</td>
<td>Vehicle</td>
<td>200 ± 21.1</td>
<td>235.4 ± 28.7</td>
<td>21.0 ± 2.2</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>219.8 ± 23.4</td>
<td>292.8 ± 5.1</td>
<td>17.8 ± 2.0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fig. 3A</td>
<td>Vehicle</td>
<td>211.1 ± 33.0</td>
<td>290.1 ± 10.6</td>
<td>17.4 ± 1.7</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>275.7 ± 24.3</td>
<td>289.2 ± 10.8</td>
<td>18.6 ± 1.8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Fig. 3B</td>
<td>Vehicle</td>
<td>246.2 ± 15.5</td>
<td>300 ± 0</td>
<td>12.5 ± 0.8</td>
<td>8</td>
</tr>
<tr>
<td>C9</td>
<td>209.7 ± 18.2</td>
<td>289.7 ± 10.3</td>
<td>14.9 ± 1.8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fig. 3C</td>
<td>Vehicle</td>
<td>240 ± 24.5</td>
<td>275.1 ± 23.3</td>
<td>17.0 ± 1.8</td>
<td>8</td>
</tr>
<tr>
<td>C9</td>
<td>279.6 ± 15.8</td>
<td>300 ± 0</td>
<td>14.8 ± 1.1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Fig. 3D</td>
<td>Vehicle</td>
<td>148.8 ± 24.8</td>
<td>257 ± 35.1</td>
<td>16.1 ± 2.7</td>
<td>8</td>
</tr>
<tr>
<td>C9</td>
<td>206.5 ± 40.0</td>
<td>300 ± 0</td>
<td>13.3 ± 1.3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Fig. 3E</td>
<td>Vehicle</td>
<td>176.1 ± 16.6</td>
<td>300 ± 0</td>
<td>11.7 ± 0.2</td>
<td>8</td>
</tr>
<tr>
<td>C9</td>
<td>264.8 ± 18.5</td>
<td>300 ± 0</td>
<td>16.3 ± 1.9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Fig. 3F</td>
<td>Vehicle</td>
<td>83 ± 11.6</td>
<td>276.6 ± 20.0</td>
<td>21.6 ± 1.9</td>
<td>9</td>
</tr>
<tr>
<td>C9</td>
<td>287.7 ± 7.9</td>
<td>300 ± 0</td>
<td>16.4 ± 1.3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Fig. 4A</td>
<td>Vehicle + vehicle</td>
<td>251.8 ± 24.2</td>
<td>300 ± 0</td>
<td>13.0 ± 1.7</td>
<td>8</td>
</tr>
<tr>
<td>Aβ + vehicle</td>
<td>171.2 ± 29.7</td>
<td>265.6 ± 31.4</td>
<td>15.2 ± 2.5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Aβ + C9</td>
<td>158.3 ± 23.4</td>
<td>255.1 ± 23.7</td>
<td>18.7 ± 1.6</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Vehicle + C9</td>
<td>181.6 ± 31.8</td>
<td>300 ± 0</td>
<td>17.5 ± 1.2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Fig. 4B</td>
<td>Vehicle + vehicle</td>
<td>300 ± 0</td>
<td>182 ± 16.9</td>
<td>15.4 ± 1.5</td>
<td>8</td>
</tr>
<tr>
<td>Aβ + vehicle</td>
<td>206.5 ± 24.0</td>
<td>300 ± 0</td>
<td>13.7 ± 0.9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Aβ + C9</td>
<td>178.8 ± 36.0</td>
<td>300 ± 0</td>
<td>16.5 ± 2.0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Vehicle + C9</td>
<td>300 ± 0</td>
<td>267.2 ± 21.6</td>
<td>19.3 ± 0.9</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. There was no significant difference between groups (p > 0.05 for each comparison).
examine the locomotor activity of the mice and was conducted as previously reported. Each mouse was placed in the center of an open field device and allowed to explore for 5 min freely. The distance travelled and rearing counts were analysed using an open field test analysis system (TME, Chengdu, China).

Histology At the end of the behavioural studies, the positioning and the permeability of the cannula were confirmed. Following the behavioural studies, each mouse was sacrificed by cervical dislocation after methylene blue was injected via the implanted cannula. Data on the diffusion of the methylene blue in the ventricles were analysed by statistical evaluation. Each mouse was used only once. However, the position of the cannula in the hippocampus was checked by histology. Histological examination was performed as described by Jiang et al. Whole brains were dissected and fixed in 4% paraformaldehyde overnight at 4°C. Coronal sections (60 µm) were cut by a vibratome and stained with hematoxylin–eosin (H&E). The slides were observed under microscope to examine the cannula placement. A representative photomicrograph of the needle track terminating in the hippocampus is shown (Fig. 1). Data on the correct hippocampal injection positioning in the mice were analysed by statistical evaluation.

Quantitative Real-Time PCR (RT-PCR) Fifteen minutes after infusion of C9 or vehicle into the hippocampus, the hippocampus of each mouse was dissected. Total RNA was extracted using TRIzol (TaKaRa, Japan). RT-PCR was performed according to the method published by Lai et al. and the manufacturer’s instructions (TaKaRa). PCR was run under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s and 72°C for 30 s. The expression levels of the TNF-α, IL-6, IL-1β and CMKLR1 genes were examined. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer pairs used for each gene (BGI, Beijing, China) are shown in Table 2.

Enzyme-Linked Immunoassay (ELISA) Total proteins from the hippocampus of mice from each group were extracted with lysis buffer (RIPA) containing protease inhibitor (Gibco, U.S.A.). Total protein was measured by a BCA TM protein assay kit (Sangon Biotech, China). IL-1β, IL-6 and TNF-α levels in the hippocampus tissue were measured by an ELISA kit (Elabscience, China) according to the manufacturer’s instructions.

Western Blot The samples were minced and homogenized in standard RIPA buffer by passing the tissue through a 20-gauge needle 15 times. The homogenized lysate was incubated on ice for 10 min and centrifuged for 15 min at 13000 rpm in a refrigerated centrifuge. The cleared lysate
was transferred to a new tube and used for Western blotting to detect the indicated proteins. A 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was used to separate the proteins. Western blotting was carried out according to the manufacturer’s instructions. Briefly, total protein was extracted with RIPA buffer containing protease inhibitor (Gibco, U.S.A.). The protein samples were blocked with 5% fat-free milk in PBS for 2 h and incubated overnight at 4°C with the following specific antibodies at the indicated concentrations: anti-TNF-α (cat number: sc-52012, 1:500, Santa Cruz, U.S.A.), anti-IL-6 (cat number: sc-57315, 1:500, Santa Cruz), GAPDH (cat number: 5174S, 1:2000, Cell Signaling) were used as a loading control. For each group of samples, n = 5.

**Experimental Design** Firstly, we investigated the enzymatic stability experiment of the C9 peptide in mouse brain homogenates and calculate its half-life. Under the experimental conditions of the protein concentration of cerebrospinal fluid of 1 mg/kg and the concentration of C9 peptide of 200 µM, the half-life of the C9 peptide was determined to be approximately 32.6 min (Fig. S2). Figure 1 shows the experimental design of the entire article. Intracerebroventricular (i.c.v.) injection of C9 or Aβ1-42 does not use any anesthesia. Each mouse was handled for 3 min per day for three consecutive days prior to i.c.v. injection. The NOR and OLR tasks consisted of two sessions: the training phase and the test phase. Injecting drugs immediately after training, the test phase will be carried out after 24h. Previous reports demonstrated that the infusion of Aβ1-42 (800 pmol; 4 µL; i.c.v.) 14 d before training impaired memory in mice in the NOR and OLR tasks if the delay between training and test phases was 1 d and the TET in training phase was 10 s. Here, we verified whether C9 could inhibit the memory-impairing effects of Aβ1-42 and its underlying mechanisms.

**Statistical Analysis** Statistical analysis was conducted using SPSS 18.0 (IBM Corp.). Data are presented as the mean ± standard error of the mean (S.E.M.). A t-test was used to examine whether the DI of each group was significantly different from 50%. Two groups, differences were assessed by one-way ANOVA followed by Bonferroni’s post-hoc test. p < 0.05 were considered statistically significant.

## RESULTS

### C9 Facilitates Object Recognition Memory Formation and Prolongs Object Recognition Memory Retention

We first tested the effect of the C9 peptide on memory formation and retention. When the TET was 5 s in the training phase, mice injected with vehicle (2 µL, i.c.v.) could not distinguish the novel object from the familiar object when tested 24 h after training. The C9 (8 µg/kg, i.c.v., 2 µL)-treated mice could distinguish the novel object from the familiar object and showed good memory performance (t-test: ***p < 0.001 for the C9 group) (Tukey’s test: F3,33 = 7.250, p = 0.0015 < 0.01 for between the vehicle group and the C9 group, Fig. 2A), indicating that C9 facilitated the formation of object memory when animals explored the object for a relatively short time. However, when the TET reached 10 s during the training phase, both the vehicle and C9 (8 µg/kg, i.c.v.) treated mice demonstrated good object recognition memory performance when tested 24 h after training (t-test: **p < 0.01 for the vehicle group, ***p < 0.001 for the C9 group) (vehicle, 62.3%; C9, 66.3%, Fig. 2B). To check the effects of the C9 peptide on object recognition memory retention, the NOR test with a TET of 10 s was used, and the animals were tested 3 d after training. As shown in Fig. 2C, the vehicle-treated mice could not make a distinction between the familiar object and the novel one, while the C9 (8 µg/kg, i.c.v., 2 µL)-treated group still showed good object recognition memory (***p < 0.001 the C9 group) (vehicle, 62.3%; C9, 66.3%, Fig. 2C), suggesting that C9 prolonged the retention of object recognition memory.

Using the similar experimental procedures, when the TET was 10 s, mice bilaterally injected with either vehicle (0.5 µL/ side) or C9 (2 µg/kg, iCA1) into the hippocampus displayed good object recognition memory performance when they were tested 24 h after training (t-test: **p < 0.01 for the vehicle group, ***p < 0.001 for the C9 group, Fig. 2E) (vehicle, 61.8%; C9, 66.3%, Fig. 2E). In contrast, when the TET in the training phase was 5 s, the mice that received an infusion of C9 (2 µg/kg, iCA1) into the hippocampus displayed better object recognition memory than the mice infused with vehicle when tested 24 h after training (t-test: ***p < 0.001 for the C9 group) (Tukey’s test: F3,33 = 3.428, p = 0.029 < 0.05 for between the vehicle group and the C9 group, Fig. 2C), suggesting that C9 prolonged the retention of object recognition memory.

### C9 Facilitates Location Recognition Memory Formation and Prolongs Location Recognition Memory Retention

The same conclusions were also drawn from the object location recognition test. The OLR test was performed on mice injected with vehicle or the C9 peptide (8 µg/kg, i.c.v., 2 µL) as well. After 24 h, in the test sessions, the vehicle-treated mice failed to distinguish the new location from the familiar location when the TET was 5 s in the training period, whereas the C9 (8 µg/kg, i.c.v., 2 µL)-treated mice could still showed
good location memory when tested 24 h after training (t-test: \( p < 0.01 \) for the C9 group) (Tukey’s test: \( F_{3, 23} = 4.159, \# p = 0.0192 < 0.05 \) for between the vehicle group and the C9 group, Fig. 3A). When the TET in the training phase was 10 s and the mice were tested 24 h after training, both the vehicle and C9 (8 \( \mu \)g/kg, i.c.v.) treated mice could distinguish the relocated object from the stationary object (t-test: \( * p < 0.01 \) for the vehicle group, \( ** p < 0.001 \) for the C9 group) (vehicle, 68.4%, C9, 65.7%, Fig. 3B). When the TET was 10 s and the mice were tested 3 d after training, the vehicle-treated mice failed to distinguish the novel location from the familiar location, while the C9 (8 \( \mu \)g/kg, i.c.v.)-treated mice exhibited significantly better location recognition memory (t-test: \( *** p < 0.001 \) for the C9 group) (Tukey’s test: \( F_{3, 27} = 9.882, ### p = 0.0002 < 0.001 \) for between the vehicle group and the C9 group, Fig. 3C) than that of the vehicle treated mice, indicating that C9 improved location memory retention.

Similar to the results of i.c.v. administration of C9, as shown in Fig. 3E, no significant difference was found between the vehicle- and C9 peptide (2 \( \mu \)g/kg, iCA1, 0.5 \( \mu \)L/side)-treated groups when the TET was 10 s and the mice were tested 24 h after training (t-test: \( *** p < 0.001 \) for both groups) (vehicle, 66.3%, C9, 71.9%, Fig. 3E). However, when the TET was reduced to 5 s, the C9 peptide (2 \( \mu \)g/kg, iCA1, 0.5 \( \mu \)L/side)-treated mice showed better location recognition memory performance compared to that of their vehicle-treated counterparts (t-test: \( *** p < 0.001 \) for the C9 group) (Tukey’s test: \( F_{3, 27} = 22.04, ### p < 0.001 \) for between the vehicle group and the C9 group, Fig. 3D). Memory retention was better in the C9 treated mice than in the vehicle-treated mice when tested 3 d after training (t-test: \( ** p < 0.01 \) for the C9 group) (Tukey’s test: \( F_{3, 27} = 20.53, ### p < 0.001 \) for between the vehicle group and the C9 group, Fig. 3F). These results were consistent with the NOR tests.

**I.c.v. Administration of C9 Improves the Object Recognition Memory Impairment and Location Recognition Memory Deficit Induced by \( A\beta_{1-42} \)** In Alzheimer’s disease, one of the most notable and severe symptoms is memory loss and impairment. \( A\beta_{1-42} \) aggregation is found in the brains of AD patients and may lead to AD. Thus, we established a mouse model of \( A\beta_{1-42} \)-induced AD. Mice were injected with vehicle or \( A\beta_{1-42} \) at a concentration of 800 pmol 14 d prior to training, followed by the administration of vehicle or the C9 peptide (8 \( \mu \)g/kg, i.c.v., 2 \( \mu \)L) immediately after training. As shown in Fig. 4A, after 24 h, in the test sessions, the vehicle + vehicle-treated group could clearly discriminate the novel object from the familiar object when the TET was 10 s in the training period, with a DI (64.3%) notably higher than the 50% (t-test: \( p < 0.05 \) and \( *** p < 0.001 \) compared to the vehicle group with one-way ANOVA. The dashed line indicates the 50% chance level.

Fig. 3. C9 Facilitates the Formation and Retention of Object Location Recognition Memory

A: The injection of C9 (8 \( \mu \)g/kg; i.c.v.) immediately after training enhanced location memory when TET was 5 s and it was tested 1 d after training. B: The injection of vehicle and C9 (8 \( \mu \)g/kg; i.c.v.) immediately after training elicited good location memory performance when TET was 10 s in the training and it was tested 1 d after training. C: C9 (8 \( \mu \)g/kg, i.c.v.) treatment prolonged the retention of location memory. In the training, TET was 10 s, the OLR test results from 3 d. D: The infusion of C9 (2 \( \mu \)g/kg, iCA1) immediately after training enhanced object location memory when it was tested 1 d with a TET of 5 s in the training phase. E: The OLR results for mice tested 3 d with a TET of 10 s in training phase. F: The OLR results for mice tested 3 d with a TET of 10 s in training phase. **p < 0.01 and ***p < 0.001 compared to 50% chance level with t-test, #p < 0.05 and ###p < 0.001 compared to the vehicle group with one-way ANOVA. The dashed line indicates the 50% chance level.
**p < 0.01 for the vehicle + vehicle group). However, Aβ1-42 treatment significantly impaired object recognition memory, and the DI (49.7%) of the Aβ1-42-treated group was remarkably lower than that of the vehicle + vehicle group (p < 0.05 between the vehicle + vehicle group and the Aβ1-42 + vehicle group). An i.c.v. injection of C9 (8 μg/kg) in Aβ1-42-treated mice improved the object recognition memory impairment induced by Aβ1-42 (t-test: ***p < 0.001 for the Aβ1-42 + C9 group, Tukey’s test: Aβ1-42 + vehicle group × Aβ1-42 + C9 group interaction, F1, 28 = 10.52, ***p < 0.001, Fig. 4A). Consistent with the results of the control group, C9 alone injection did not impair object recognition memory, with a DI (66.8%) notably higher than the 50% (t-test: ***p < 0.001 for the vehicle + C9 group). Meanwhile, there is not a significant difference between C9 + Aβ1-42 and Aβ1-42 + vehicle (p = 0.9771 > 0.05, Fig. 4A), indicating that C9 alleviated the object recognition memory-deficit effects of Aβ1-42.

Similar results were found in the OLR tests. As shown in Fig. 4B, Aβ1-42 (800 pmol, i.c.v.) administration impaired object location memory recognition (DI of 68.3% for the control group, t-test: ***p < 0.001 for the vehicle + vehicle group; 48.6% for the Aβ1-42 + vehicle group) (p < 0.05 between the vehicle + vehicle group and the Aβ1-42 + vehicle group). However, C9 (8 μg/kg, i.c.v., 2 μL) administration rescued object recognition memory impairment (DI of 61.2% for the Aβ1-42 + C9 group, t-test: ***p < 0.001 for the Aβ1-42 + C9 group; Tukey’s test: Aβ1-42 + vehicle group × Aβ1-42 + C9 group interaction, F1, 31 = 4.812, #p < 0.05, Fig. 4B). Meanwhile, C9 (8 μg/kg, i.c.v., 2 μL) alone injection did not impair object recognition memory, with a DI (70.6%) notably higher than the 50% (t-test: ***p < 0.001 for the vehicle + C9 group), and there is not a significant difference between C9 + Aβ1-42 and Aβ1-42 + vehicle (p = 0.3748 > 0.05, Fig. 4B), indicating that C9 alleviated the object location recognition memory-imparing effects of Aβ1-42.

C9 Mitigates the Aβ1-42-Induced Production of Pro-inflammatory Cytokines C9 was reported to be involved in the regulation of several inflammation processes and the aggregation of Aβ1-42 may also induce inflammation. To study the role of C9 in the inflammatory process induced by Aβ1-42, RT-PCR was used. The results showed that the levels of TNF-α, IL-6 and IL-1β, as shown in Figs. 5A–C, were dramatically increased after the injection of Aβ1-42 into the hippocampus (Tukey’s test: TNF-α: *p < 0.05 for Aβ1-42 vs. vehicle, IL-1β: **p < 0.01 for Aβ1-42 vs. vehicle, IL-6: ***p < 0.001 for Aβ1-42 vs. vehicle). C9 treatment significantly decreased the expression levels of TNF-α (Tukey’s test: #p < 0.01 between Aβ1-42 and Aβ1-42 + C9, F3, 29 = 7.316, Fig. 5A), IL-1β (Tukey’s test: #p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 29 = 6.718, Fig. 5B) and IL-6 (Tukey’s test: **p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 29 = 4.03, Fig. 5C) in the hippocampus. In addition, the ELISA experiment showed that the protein levels of TNF-α, IL-6 and IL-1β were higher in the hippocampus of the Aβ1-42-induced AD mice (Tukey’s test: TNF-α: **p < 0.01 for Aβ1-42 vs. vehicle, IL-1β: **p < 0.01 for Aβ1-42 vs. vehicle, IL-6: ***p < 0.001 for Aβ1-42 vs. vehicle), and C9 treatment markedly downregulated the expression of TNF-α, IL-6 and IL-1β (Tukey’s test: #p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 31 = 3.815; IL-6: #p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 31 = 6.965; and IL-1β: ***p < 0.001 for between Aβ1-42 and Aβ1-42 + C9, F3, 31 = 7.28, Fig. 5D–F). The results of WB experiments were consistent with the results of ELISA, Aβ1-42 treatment significantly increases the expression levels of TNF-α (Tukey’s test: ***p < 0.001 for Aβ1-42 vs. vehicle, IL-6 (Tukey’s test: ***p < 0.001 for Aβ1-42 vs. vehicle) and IL-1β (Tukey’s test: #p < 0.01 for Aβ1-42 vs. vehicle). C9 treatment markedly downregulated the expression of TNF-α (Tukey’s test: **p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 31 = 7.305), IL-6 (Tukey’s test: **p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 31 = 6.09) and IL-1β: **p < 0.01 for between Aβ1-42 and Aβ1-42 + C9, F3, 31 = 4.28). (Fig. 6). These data indicate that C9 reduces the production of pro-inflammatory cytokines.

The Anti-inflammatory Effects of C9 May Be Related to the CMKLR1 Receptor in Mice Firstly, in Aβ1-42-induced AD model mice, stimulation of Aβ1-42 significantly up-regulated the expression of CMKLR1, and the applica-
The expression of C9 did not decrease its expression (Fig. S3, \( p < 0.05 \)), which is consistent with the Peng et al. report.\(^{13}\) Figures 5 and 6 showed the anti-inflammatory effects of C9 in mice. According to Zabel and colleagues reports,\(^{36,37}\) we synthesized and purified a novel CMKLR1 small molecule antagonist (\( \alpha \)-NETA). The synthesis method, mass spectrometry and HPLC analysis of \( \alpha \)-NETA are shown in Fig. S4.

Meanwhile, in vivo, pretreatment with suitable doses of \( \alpha \)-NETA (10 mg/kg, s.c.), the anti-inflammatory effects of C9 were significantly blocked in \( \alpha \beta_{1-42} \) induced AD model mice (Tukey’s test: TNF-\( \alpha \): \( ^{\#} p < 0.05 \) for between \( \alpha \beta_{1-42} + C9 \) and \( \alpha \beta_{1-42} + C9 + \alpha \)-NETA; IL-6: \( ^{\#\#} p < 0.01 \) for between \( \alpha \beta_{1-42} + C9 \) and \( \alpha \beta_{1-42} + C9 + \alpha \)-NETA; and IL-1\( \beta \): \( ^{\#\#} p < 0.01 \) for between \( \alpha \beta_{1-42} + C9 \) and \( \alpha \beta_{1-42} + C9 + \alpha \)-NETA; inducible nitric oxide synthase (iNOS): \( ^{\#\#} p < 0.05 \) for between \( \alpha \beta_{1-42} + C9 \) and \( \alpha \beta_{1-42} + C9 + \alpha \)-NETA, Figs. 7A–D). Similar to the results of in vivo experiments, after pretreatment with \( \alpha \)-NETA (5 \( \mu \)M) on BV2 cells, C9 (5 \( \mu \)M) did not inhibit \( \alpha \beta_{1-42} \) (10 \( \mu \)M) induced inflammatory response (Fig. S5). These data indicate that C9 reduces the production of pro-inflammatory cytokines via CMKLR1 pathway.

**Effects of the i.c.v. Administration of C9 on Locomotor Activity in Mice** If C9 enhances the locomotor activity in the mice, the chances of the mouse touching the object in the NOR experiment increases, increasing the possible of eliciting a false positive result. Therefore, we used the open field test to rule out this possibility. As evidenced by the open field test, C9 (8 \( \mu \)g/kg, i.c.v.) failed to significantly increase the locomotor activity of the mice, including the distance travelled compared to that of the vehicle-treated animals (Fig. 8).
DISCUSSION

In our study, the results showed that the i.c.v. administration of C9 facilitated memory formation and retention in the NOR and OLR tasks, as well as C9 infusion of C9 directly into the hippocampus, these effects were equally robust. In
addition, our results suggested that C9 prevented Aβ1-42
induced memory impairment in both the NOR and OLR tasks. To our knowledge, this is the first report of such results.

In detail, the enhancement of memory formation by C9 was illustrated by the NOR and OLR tasks with a short TET time of 5 s. When the TET was 10 s during the training phase, both the C9- and vehicle-treated mice showed good memory performance when tested 1 d after training in the NOR and OLR tests. In this case, it is difficult to identify whether C9 has a memory enhancement effect in the object recognition memory retention. This difference may reflect the existence of a ceiling effect of C9. Thus, according to the articles,29,32 we have used an extended interval of 3 d. The results showed that memory was preserved in the C9-treated mice at an interval of 3 d, but this was not the case in the vehicle-treated mice. These data reveal that C9 administration may enhance memory formation and prolong memory. Previous reports have indicated that chemerin regulates the extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) signalling pathways, which are involved in triggering long-term potentiation (LTP) in the CA1 region of the hippocampus.12,38–40 Additionally, multiple reports have indicated that CMKLR1, the receptor of C9, is expressed in the hippocampus.13,15 Thus, we investigated the CMKLR1 expression after C9 injection. The result showed that the expression level of CMKLR1 mRNA in normal mice was up-regulated after C9 application, which was consistent with the memory-enhancing effects of C9. The hippocampus plays a very important role in cognition, and its dysfunction might damage the process of learning and memory.41,42 Thus, we evaluated the effects of C9 in the hippocampus of mice. The present data indicate that C9 infused directly into the hippocampus is able to improve object and location memory retention, suggesting that C9 improves memory possibly by activating CMKLR1 in the hippocampus. The good outcome of C9 administration indicates its pharmaceutical potential in treating dementia.

AD is the most common form of dementia and is characterized by the deposition of Aβ in the brain as well as progressive cognitive impairment and memory loss.43 The deposition of Aβ appears to be a key factor in the pathogenesis of AD. i.c.v. administration of Aβ1-42 has been widely used in experimental AD mouse models for testing learning and memory.4,44,45 In this study, an i.c.v. injection of Aβ1-42 impaired memory function in mice. This is consistent with the effect of Aβ1-42 on memory described in previous reports.35,46 Interestingly, we found that an i.c.v. injection of C9 significantly ameliorated the memory deficits induced by Aβ1-42 in object and location memory tests, suggesting that C9 may be a potential treatment for enhancing memory in AD patients. Currently, the role of C9 in inflammation remains controversial. Some reports have indicated that chemerin plays a pro-inflammatory role in various inflammation processes.47–49 Other studies have suggested that chemerin may play an anti-inflammatory role.15,50,51 The underlying mechanisms of the function of C9 in cognition and AD are still unclear. We hypothesize that it is closely related to the inflammation status in the brain. Meanwhile, the mechanism of neuroinflammation induced by Aβ1-42 has not been fully elucidated. Previous studies have shown that increased levels of proinflammatory cytokines are frequently observed around Aβ plaques, which lead to the release of inflammatory factors such as TNF-α and IL-6 that may be toxic to neurons and may lead to the onset of AD, in the brains of AD patients.63 To test this hypothesis, we investigated the release of proinflammatory factors (TNF-α, IL-1β and IL-6) induced by Aβ1-42. The mRNA and protein levels of TNF-α, IL-1β and IL-6 increased in the hippocampus after Aβ1-42 was infused into the bilateral hippocampus. Then, TNF-α, IL-1β and IL-6 were significantly reduced after an infusion of C9. Western blotting experiments were used to detect the expression of these proinflammatory cytokines, and the results showed that they were dramatically increased in the hippocampus of Aβ1-42-treated mice. Furthermore, these pro-inflammatory factors were significantly downregulated after C9 application. In addition, our data showed that stimulation of Aβ1-42 significantly up-regulated the expression of CMKLR1, which were similar to the reports in many publications.15,52 Some studies suggested that Chemerin treatment inhibited the production of inflammatory cytokines and that this inhibitory effect mainly depended on CMKLR1.52–54 Thus, pretreatment with suitable doses of α-NETA, a novel small molecule antagonist of CMKLR1,36,37 the anti-inflammatory effects of C9 were significantly blocked in Aβ1-42 induced AD model mice. In BV2 cells, similar to the results of in vivo experiments, after pretreatment with α-NETA, C9 did not inhibit Aβ1-42 induced inflammatory response. These data indicate that the anti-inflammatory effects of C9 may be related to the CMKLR1 receptor. In addition, we found that in Aβ1-42 induced AD model mice, stimulation of Aβ1-42 significantly up-regulated the expression of CMKLR1, which is consistent with the Peng et al. report.15 They found that the transcript of the chemerin receptor CMKLR1 was upregulated in the brain of AD patients and in mouse brain tissue following systemic lipopolysaccharide (LPS) administration.13 However, the application of C9 did not decrease CMKLR1 expression, and other studies involving Chemerin/CMKLR1 showed similar results.53 In hypoxic–ischemic encephalopathy (HIE) disease model, compared to sham pups, the expression of chemR23/CMKLR1 was significantly upregulated in a time-dependent manner and peaked 72 h after HIE. Pretreatment with chemerin, expression of chemR23/CMKLR1 was increased in HIE model12,53 and the specific mechanism is still unclear.

Given the movement-enhancing effects of the drug, the chances of the mouse touching the object in the NOR experiment increased. Therefore, we examined the effects of C9 on locomotor activity by an open field test to rule out the possibility of this false positive. The results indicated that C9 did not increase locomotor activity. In other words, the memory-improving effects of C9 are unlikely to be changes in locomotor activity.

In conclusion, the present investigation demonstrated that the central administration of C9 enhances memory performance. Likewise, C9 provides neuroprotection against Aβ-induced neuroinflammation and the memory impairment induced by Aβ1-42. Collectively, the results suggest that chemerin may be a new potential target for enhancing memory and treating cognitive decline in AD patients.

Acknowledgments We are grateful for the Grants from the Gansu province Natural Science Foundation of China (1506RJJA278).

Conflict of Interest The authors declare no conflict of
interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

37) Kumar V, LaJevic M, Pandrala M, Jacobo SA, Malhotra SV, Zabel


