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Research report

Hippocampal endocannabinoids play an important role in induction of long-term potentiation and regulation of contextual fear memory formation

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ABSTRACT

Recent studies show contradictory results regarding the contribution of endocannabinoids in fear memory formation and long-term synaptic plasticity. In this study, we investigated the effects of both cannabinoid receptor type 1 (CB1 receptor) antagonist AM281 and anandamide reuptake inhibitor AM404 on the formation of contextual fear memory in adult mice. Both i.p. and intra-hippocampal injections of AM281 promoted contextual fear memory while a high dose of AM404 inhibited it. These findings demonstrate that CB1 receptor-mediated signaling negatively contributes to contextual fear memory formation. We further investigated the induction of long-term potentiation (LTP) in CA1 pyramidal neurons of hippocampal slices and found that AM281 impaired the induction of LTP. Additionally, the blockade of LTP by AM281 was completely prevented by bath application of picrotoxin, a selective antagonist of $GABA_A$ receptor. Taken together, these results indicate that activation of CB1 receptor contributes to induction of LTP via a $GABA_A$ receptor-mediated mechanism.

1. Introduction

The endocannabinoid system is considered to be a crucial regulator in the central nervous system [15,19]. Many reports have confirmed its critical role in learning and memory of animals and synaptic transmission [18,26,30,36]. The contextual fear conditioning model is an actively studied topic in neuroscience not only because it is the most robust memory model that is associated with electrical foot shock and context, but also because it is a practical model for treating inappropriate aversive memory [26]. Recent studies show contradictory results surrounding how endocannabinoids contribute to the formation of contextual fear memory. Pamplona and Takahashi [30] showed that activating cannabinoid receptors impairs contextual fear memory. Inhibiting CB1 receptor activity, however, also causes suppression of contextual fear memory [5], although Suzuki et al. [35] have shown in a similar experiment that antagonizing CB1 receptor activity had no effect on fear memory. On the other hand, one study of CB1 knockout mice shows that the extinction but not acquisition or retrieval of fear memory is impaired [28]. The mechanism of contextual fear memory formation is closely related to the hippocampus [25,37], amygdala [18], and other brain regions including perihinal cortex and prefrontal cortex [26]. However, since CB1 receptors are widely expressed in different parts of the central nervous system including the hippocampus, amygdala, striatum and cerebellum [16,36], it is possible that CB1 receptors in different brain areas have distinct impacts on contextual fear memory [32]. Thus, injection patterns from intra-peritoneal, intra-cerebroventricular, or intra-hippocampal injection may cause different results [5,35].

Synaptic plasticity has been well accepted as a potential mechanism for various types of memory [25]. Endocannabinoids were first reported to be related to short-term plasticity [37]. Subsequent studies further show that the endocannabinoid system is also involved in long-term synaptic plasticity [4,29,41]. Either high-frequency stimulation (HFS) or theta-burst stimulation induces endocannabinoid-mediated long-term depression of γ-aminobutyric acid (GABA) transmission, and this induction profoundly affects the excitatory postsynaptic potential (EPSP)-population spike coupling and metaplasticity of excitatory synaptic transmission [29,41]. Given that CB1 receptors in different brain areas may regulate contextual fear memory and synaptic plasticity in different ways, in the current study we examined the effects of intra-hippocampal injections of CB1 receptor antagonist AM281 or anandamide reuptake inhibitor AM404 on contextual fear memory and the effects of AM281 on induction of long-term potentiation (LTP) in CA1 pyramidal neurons of hippocampal slices.

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2. Materials and methods

2.1. Animals

All experiments were conducted according to the Health Guide for the Use and Care of Laboratory Animals of Shanghai Jiao Tong University. Male mice C57BL/6s (6–8 weeks of age, 19–26 g of body weight) were housed in groups. They were maintained on a 12 h light/dark cycle and allowed *ad libitum* access to food and water. Behavior testing was performed during the dark phase of the cycle.

2.2. Drugs

AM281 (Tocris, UK) (1-[2,4-Dichlorophenyl]-5-[4-iodophenyl]-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) as a stock solution (2.5 mg/ml) and stored at −30 °C. For the behavior study, the anandamide reuptake inhibitor, AM404 (*N*-(4-Hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraena-mide) (Tocris, UK), was dissolved in Tocrisolve™ 100 and kept at −4 °C. The stock solutions were freshly diluted in saline (0.9%). The final doses of drugs were based on previous reports: 2.5 mg/kg AM281 for i.p. injection [33], 0.05 μg AM281, 1 μg AM404, and AM281 (0.05 ng) plus AM404 (1 μg), for intrahippocampal injections, respectively [5].

For electrophysiological analysis, the stock solution of AM281 was diluted in ice-cold artificial cerebral-spinal fluid (ACSF), yielding a final concentration of 0.1% DMSO and 500 nM AM281 [29]. AM404 was dissolved in DMSO as a stock solution (20 mM) and the final concentration in the ACSF was 20 μM [21].

2.3. Cannula implanting and intra-hippocampal injection

Mice were anesthetized with 1% pentobarbital sodium (7.5 ml/kg, i.p.) and fixed in a stereotaxic instrument. A craniotomy was made, and the tip of the guide cannula (22 GA) was implanted into the CA1 region of the dorsal hippocampus (AP: −2.0 mm, ML: −2.0 mm, V: −1.5 mm from the skull surface) [31]. The cannula was fixed with acrylic cement and filled with a metal obturator before the behavior study. All animals were allowed 1 week to recover from surgery and to clear the anesthetic. The animals were randomly assigned to different groups. Each mouse was lightly restrained during the drug administration. For injections, an internal 30 GA, stainless steel, cannula was inserted extending 1 mm below the guide cannula to a final depth of 2.5 mm from the skull. Then, 0.2 μl drug containing 0.05 μg AM281, 1 μg AM404, or AM281 (0.05 ng) plus AM404 (1 μg), respectively, was injected over 100 s using a 5 μl microsyringe (Shanghai GaoKe, China) connected to an infusion pump (Shanghai Alcott, China) through a short polyethylene catheter. The needle was left in the cannula for an additional 2 min at the end of each injection.

2.4. Contextual fear memory test

The contextual fear conditioning test was performed with a computerized fear conditioning system (Coulbourn Instruments, USA) as described previously [40]. Mice were placed in the experimental room and acclimatized for at least 2 h before the experiments. Each mouse was placed in a conditioning chamber (17.8 cm × 17.8 cm × 30.5 cm) inside a sound-attenuated room and allowed to explore freely for 3 min. Three electrical foot shocks (1 s, 2 mA) were delivered with 30 s inter-stimulus intervals. After 24 h, the mice were placed in the conditioning chamber again for 2 min without any foot shocks, while the freezing time was assessed during the 30–90 s period. Freezing was defined as the absence of any movement except respiration. All the animals were behaviorally tested only once in order to avoid the possible influence of previous fear conditioning tests on the next test.

In order to confirm whether the drug had been injected into dorsal hippocampus, the mice treated with intra-hippocampal injections were anesthetized after the behavioral tests, and their brains were removed immediately after that. Only those mice with a proper trace of the needle in the right hippocampus were included in the data analysis.

2.5. Preparation of hippocampal slices

Hippocampal slices were prepared as described previously [22]. Briefly, mice were deeply anesthetized with halothane, and both hippocampi were quickly removed from the brain after decapitation. Transverse hippocampal slices (400 μm thick) were prepared with a Vibratome tissue slicer and placed in a humidified interface-type holding chamber for at least 2 h in the presence of gas (95% O_2, 5% CO_2)-saturated ACSF. The composition of ACSF was as follows (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$ and 11 glucose.

2.6. Electrophysiology

Field EPSP (fEPSP) recording was performed as described previously [40]. Individual slices were transferred into a submerge-type recording chamber, fixed with a nylon net, and submerged beneath the continuously perfusing gas (95% O_2, 5% CO_2)-saturated ACSF at the rate of 2.0 mL/min. To record the fEPSP, a glass pipette filled with 3 M NaCl (2–8 MΩ resistance) was placed in the stratum radiatum of the CA1 region, and a bipolar tungsten stimulating electrode was placed along the Schaffer collateral-commissural fibers, 120 μm away from the recording glass pipette. The intensity of the stimulations was adjusted to produce a fEPSP with an initial slope between 0.15 and 0.20 mV/ms and with an amplitude between 0.45 and 0.6 mV. The test stimulation was delivered to the stimulating electrode every 15 s by an Iso-Flex stimulus isolation unit (A.M.P.I., Jerusalem, Israel) with 0.2-ms constant-current pulse triggered by a Master-8 pulse generator (A.M.P.I.). At least 30 min of stable baseline recordings were obtained prior to the delivery of drugs or high frequency stimulation (HFS). HFS consisted of a 1-s train of 100 Hz at the same stimulation intensity used to evoke the baseline response. LTP values were set as the ratio of the average of stable responses after induction of LTP (typically 50–60 min after HFS) to that before the induction of LTP (0–30 min before HFS). Post-tetanic potentiation (PTP) was calculated as the ratio of the average of initial fEPSP slopes at 1 min after applying HFS to that 0–30 min before applying HFS.

Field potential index of HFS-evoked fEPSP was calculated as follows:

$$\text{Charge transfer index} = \frac{\text{Mean depolarization from 7 to 9 ms after each stimulus}}{\text{Peak of HFS-evoked fEPSP}}$$

During the HFS, the mean amplitudes from 7 to 9 ms after each stimulus were calculated and were normalized to the peak amplitude of HFS-evoked fEPSP. HFS-evoked Charge transfer index was calculated as the ratio of the area of HFS-evoked fEPSP to the area of averaged test stimulus-evoked fEPSP (0–10 min before HFS).

In some experiments, the $GABA_A$ receptor antagonist picrotoxin (PTX, 100 μM) was applied to isolate the excitatory synaptic transmission. In picrotoxin-treated slices, a cut was made to separate the CA3 region from the CA1 region in order to avoid the epileptiform activity. All experiments were performed at 25 ± 0.5 °C. Recordings were performed with a MultiClamp 700B (Molecular Devices, USA) and analyzed (filtered at 3 kHz, sampled at 10 kHz) on a personal computer using pCLAMP 10 (Molecular Devices, USA).

2.7. Statistical analysis

All the values were expressed as mean ± SEM. Field potential indexes between AM281- and DMSO-treated slices were compared using two-way ANOVA. The LSD *post hoc* test was employed to make multiple, pair-wise group comparisons. Unless stated, other data were analyzed with Student's *t*-test. For all analyses, $p < 0.05$ was considered as statistically significant.

3. Results

3.1. CB1 receptor-mediated signaling inhibits contextual fear memory formation

In order to investigate how the CB1 receptor regulates the fear memory, we examined the effect of AM281, an antagonist of CB1 receptor [33], on the formation of contextual fear memory. AM281 (2.5 mg/kg) was i.p. injected 15 min prior to the conditioning session. There was no significant difference in freezing response between AM281- and vehicle injected mice during the conditioning session (Fig. 1a). After 24 h, however, AM281-treated mice showed longer freezing times than those in the vehicle-treated group in the contextual test session (AM281, 67.3 ± 2.8%, $n = 9$; vehicle, 53.1 ± 5.2%, $n = 10$; $p = 0.033$) (Fig. 1b). Since CB1 receptors are widely distributed, we cannot rule out the possibility that CB1 receptors in other brain areas, rather than the hippocampus, contributed to the promotion of contextual fear memory. In order to examine the function of the CB1 receptor in the hippocampus, we injected AM281 (0.05 μg) through a pre-implanted cannula into dorsal hippocampus 15 min prior to the conditioning session. In consistent with the result of i.p. injection, AM281-treated mice showed longer freezing behavior than those in the vehicle-treated group (AM281, 46.9 ± 5.8%, $n = 10$; vehicle, 27.9 ± 4.3%, $n = 9$; $p = 0.019$) (Fig. 1c and d). Taken together, these results indicate that hippocampal CB1 receptor-mediated signaling negatively regulates the formation of contextual fear memory.

There are two main endocannabinoids in central nervous system, 2-arachidonoylglycerol (2-AG) and anandamide. Thus, we next investigated how the transporter inhibitor of anandamide, AM404, regulates contextual fear memory. AM404 (1 μg) was injected through a pre-implanted cannula into dorsal hippocampus 15 min prior to the conditioning session. AM404 significantly