Original Article

Midazolam-Induced Learning and Memory Impairment Is Modulated by Cannabinoid CB1 Receptor Agonist and Antagonist

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Abstract

Background: Memory impairment is a well-known effect of many benzodiazepine compounds which is mediated through their action on gamma-aminobutyric acid type A ($GABA_A$) receptors. On the other hand, cannabinoids can affect learning and memory process through presynaptic modulation of the release of both excitatory glutamate and inhibitory GABA transmitters in brain regions involved in learning and memory. The aim of the present study was to investigate the effect of cannabinoids on memory impairment and long-term potentiation (LTP) reduction properties of the short acting benzodiazepine midazolam.

Materials and Methods: One week after insertion of guide cannula by stereotaxic surgery, cannabinoid compounds or midazolam were administered by intracerebroventricular (i.c.v.) injection into lateral ventricle of male rats. Spatial memory task was evaluated using Morris water maze (MWM) test. Electrophysiological evaluation was done by field potential recording of hippocampal neurons in unconscious rats.

Results: In MWM test, while i.c.v. administration of AM251 (200 and 500 ng) per se could not change learning and memory function in rats, pretreatment of rats with AM251 (500 ng; i.c.v.) attenuated midazolam-induced memory impairment. In field potential recording, while i.c.v. administration of AM251 (500 ng) and WIN55212-2 (10 µg) did not have any effect on population spike amplitude, pretreatment of rats with both AM251 and WIN55212-2 significantly diminished midazolam-induced PS amplitude reduction in hippocampal neurons.

Conclusion: Our

Our results suggest the involvement of cannabinoid CB1 receptors in both memory impairment and LTP reduction in hippocampal neurons which was produced by midazolam. This interaction is likely through their effect on both GABAergic and glutamatergic receptors in hippocampus.

Keywords: Midazolam, AM251, WIN55212-2, learning and memory, long-term potentiation, Morris water maze

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Introduction

Gamma-aminobutyric acid type A ($GABA_A$) receptors are a family of ligand-gated ion channels that are essential for the regulation of central nervous system function. Benzodiazepines (BDZs) act via non-selective target $GABA_A$ receptors. They have become one of the most widely groups of medications for the treatment of anxiety, insomnia, and epilepsy (1). Also, BDZs are used as intravenous anesthetic agents in which sedation and amnesic effects are exploited for clinical benefits (2). The short-term use of benzodiazepines adversely affects multiple areas of cognition, the most notable one being that it interferes with the formation and consolidation of memories of new material and may induce complete anterograde amnesia (3). Prior administration of an antagonist of BDZ sites reversed BDZ-induced amnesia (4). BDZ's disruptive effect has been observed following administration either pre- or post- training in diverse learning and memory paradigms (5). Such an effect was also reported after both BDZ intra-amygdala (6), and BDZ intra-dorsal hippocampus infusions (5). Midazolam (MDZ), a short-acting drug in the benzodiazepine class, is an anxiolytic sedative used in a variety of clinical settings (7). Consistent with other findings, it has been demonstrated that MDZ could affect fear memory reconsolidation (8).

A great number of studies suggest an important role of the cannabinoid system in controlling the memory processes. High densities of CB1 receptors have been found in the hippocampus (9), a brain region that is essential for spatial/contextual learning in animals (10). Endocannabinoids (eCBs) are synthesized on demand at the post-synaptic sites of neurons after an increase in neural activity and calcium ion influx, and are then released into the synaptic cleft (11). Their main function appears to be the suppression of neurotransmitter release from presynaptic neurons (12). It has been shown that the cannabinoid agonists, WIN 55212-2, at some doses can impair memory function (13). Nonetheless, there is evidence that cannabinoid agonist can also enhance memory, depending on the route of administration, the dose used, the phase of memory and the level of emotional arousal at the time of training (14).

The discovery of Wilson and Nicoll (15), Ohno-Shosaku et al. (16), and Kreitzer and Regehr (17) revealed a retrograde modulation of both excitatory and inhibitory neurotransmission by endocannabinoids. Later, it has been shown that exogenous cannabinoids can also attenuate both inhibitory (18) and excitatory (19) neurotransmitter release though action on presynaptic cannabinoid CB1 receptors in various brain region including the hippocampus.

Interaction between cannabinoid compounds and GABA-mediated memory impairment has been studied by Alijanpour et al., in passive avoidance learning in mice. Pre-test intra-CA1 microinjection of AM251 prevented the ethanol response on ethanol-induced amnesia while pre-test intra-CA1 microinjection of the same doses of AM251 had no effect on memory retrieval. These findings suggested the role of cannabinoid CB1 receptors of dorsal hippocampus in the effect of ethanol on passive avoidance learning (20). However, no study was performed on interaction between cannabinoids and BDZs in spatial learning and memory. The present study was performed to investigate the interaction between MDZ and the cannabinoid receptor agonist (WIN55212-2) and antagonist (AM251) on spatial memory and synaptic plasticity of hippocampal neurons.

Methods

Animals

Adult male wistar rats weighting 270-300g (Pasture Institute, Tehran, Iran) were used in this study. Animals were housed three per cage in a room under a 12:12h light-dark cycle (lights on at 7:00 a.m.) and controlled temperature (22±2°C) with free access to food and tap water except in short time during experiments. Rats were randomly divided in ten groups of 5-6 animals and each animal was used only once. The experiments were performed between 10:00 a.m. and 5:00 p.m. All procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and were approved by the local Research and Medical Ethics

Committee.

Drugs

The cannabinoid receptor agonist (WIN55212-2) and antagonist (AM251) were purchased from Sigma-Aldrich (St. Louis, USA). MDZ was a gift from Tehran Chemie Pharmaceutical Co. (Tehran, Iran). The drugs were dissolved in DMSO (Sigma-Aldrich; St Louis, USA) and injected at a constant volume of 2μL/rat. The control group received vehicle (DMSO, 2μL/rat).

Surgery

In order to evaluate the spatial memory of rats in Morris water maze test, the animals were anesthetized with intraperitoneal (IP) injection of anesthetic solution consisting of ketamine (85mg/kg) and xylazine (15mg/kg). Then, rats were placed in stereotaxic apparatus and implanted with guide cannula (8mm, 23-gauge) aimed at a site 1 mm above the right lateral ventricle according to following coordinates: 1 mm posterior and 1.6mm lateral to the bregma at a depth of 3.5 mm from the skull surface (21). Two jeweler screws were inserted into the skull and the cannula was fixed using dental cement. Then the cannula was closed with a stylet.

Morris water maze (MWM) test

The water maze was a dark circle pool (a tank made of galvanized metal, 155 cm diameter, 60cm depth) that was filled to a depth of 25cm with 22±1°C water. A clear Plexiglas platform (diameter 10 cm) was submerged 1.5cm below the surface of the water and located in the center of the arbitrary designed northeast (NE), southeast (SE), southwest (SW) and northwest (NW) orthogonal quadrants.

Behavioral training

One week after surgery, the cannula stylet was removed and injection needle (30-gauge) connected to a short piece of polyethylene tubing and a 5μl Hamilton syringe was inserted into right lateral ventricle of the conscious rat. Then 2μl of each drug or its vehicle was injected slowly over 2-3 min. In each experimental group, WIN55212-2 at the doses of 500ng and 10μg, AM251 at the doses of 200ng and 500ng or their vehicles were injected 5 min before administration of MDZ (500ng) or its vehicle. The rats were free to move in their cage during drug administration. Five min. after last drug injection, animals were subjected to training sessions. Each animal was trained during eight trials divided into two even blocks with 5 min interval between each block. For each trial, the rats were gently released into the pool, facing the wall. Four different releasing points (NE, SE, SW, and NW) were randomly selected. Rats were given a maximum of 60 s. to find the platform. After finding the platform, the rats were allowed to remain for 20 s., and were then placed in a cage for 30 s. until the start of the next trial. Animals failing to find the platform in 60 s. were gently placed on the platform and were allowed to rest for 20 s. At the end of the training sessions, the animals were returned to their home cages. Twenty-four hours later, the animals were subjected to retention test (probe test) consisted of a 60 s. swimming in tank without the presence of the platform. In order to assess the effect of drugs on rat locomotion, the velocity of swimming was measured during training sessions. In addition, the possibility of drug interference with animal vision, after probe test, rats were subjected to a 60 s. trial to find and climb the visible platform.

Electrophysiological procedure

Rats were anesthetized by intraperitoneal injection of 1.5g/kg urethane and placed in a stereotaxic device. Supplementary injections of urethane (0.2–0.5 g/kg) were given when necessary to ensure full anesthesia. A heating pad was used to maintain the temperature of the animals at 36.5±0.5°C. The skin was removed from the skull and small holes were drilled in the skull at the positions of the guide cannula as well as stimulating and recording electrodes. The guide cannula was placed into lateral ventricle according to coordination previously mentioned in behavioral procedure. Then, the bipolar stainless steel recording and stimulating electrodes (0.125 mm diameter, Advent, UK) were positioned in the granular cells of dentate gyrus (AP = -3.8; ML = 2.3; DV = 2.7–3.2 mm from the skull surface) and perforant pathway (AP = −8.1; ML = 4.3; DV = 3.2 mm from the skull surface), respectively according to the atlas of Paxinos and Watson (21). In order to minimize trauma to brain tissue, the electrodes were lowered very slowly (0.2 mm/min) from cortex to the hippocampus. Correct electrode depths were determined by optimizing the evoked response. The test stimuli were delivered at 0.1 Hz (22) to the perforant pathway every 10 s. with constant current

stimuli. Stimulation intensity was adjusted to elicit a maximal field population spike (PS) and field excitatory postsynaptic potential (fEPSP). The PS amplitude was measured as the difference in voltage between the peak of the first positive wave and the peak of the first negative deflection and the fEPSP slope was measured as the maximum slope between initial point of EPSP and the first positive wave in order to measure synaptic efficacy. PS and fEPSPs were evoked in the dentate gyrus region using 0.1 Hz stimulation. Baseline recordings were taken at least 30 min. and after ensuring a steady state baseline response. Then drugs were administered by intracerebroventricular (i.c.v.) injection through the guide cannula. In each experimental group, AM251, WIN 55212-2 or their vehicle were injected 5 min before administration of MDZ or its vehicle. The doses and the volume of injection were similar to those explained in behavioral experiment. Five min. after drugs administration, the LTP was induced using a high-frequency stimuli protocol of 200 Hz (10 bursts of 15 stimuli, 0.2 ms stimulus duration, 10 s. inter-burst interval) at a stimulus intensity that evoked a PS amplitude and fEPSP slope of approximately 80% of maximum response. All potentials employed as baseline and also after high frequency stimuli were evoked at a stimulus intensity which produced 40% of this maximum. Both fEPSP and PS were recorded each 5 min. for the periods of 60 min. after the high frequency stimuli in order to determine any changes in the synaptic response of dentate gyrus neurons. For each time-point, 10 consecutive evoked responses were averaged at 10 s. stimulus interval (23).

Verification of cannula position

After termination of the behavioral tests, the rats were anesthetized and intra-cardially perfused with paraformaldehide (%4) and their brains were removed. Coronal sections with 200 μm thicknesses were provided using vibratome and injected locations were examined under a stereomicroscope. Only results obtained from animals in which tips of the injection needles were correct were considered (Figure 1). Same method was used after elctrophysiological recording for injection site verification.

Statistical analysis

The results are shown as mean±SEM. The results of MWM test as well as the results of electrophysiological recordings were evaluated using two-way ANOVA followed by Bonferroni's post-test, considering time as a factor and treatment as the other factor. Also, in order to evaluate the overall drug-induced changes during recording time, the area under the curve (AUC) of potential vs. time was calculated. Data of AUC were then analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests. Statistical analysis was performed using Graphpad Prism software (Version 5; Graphpad Inc.). The p value of less than 0.05 was considered as statistically significant.

Results

Training sessions in MWM test – changes in distance to platform

The results were shown in figure 2. One-way ANOVA revealed a significant difference between groups [$F(7,63)=6.341$, $p<0.0001$; Figure 2A]. Further analysis by Dunnett's test showed a significant increase in group treated with MDZ (500ng; $p<0.001$) compared to control group. This effect of MDZ was attenuated by co-administration with AM251 (500ng), but not AM251 (200ng). Furthermore, treatment of rats with WIN55212-2 (10ug) significantly increased distance to platform compared to control group ($p<0.01$). However, co-administration of WIN55212-2 (10ug) and MDZ (500ng) did not alter the effect of each drug per se.

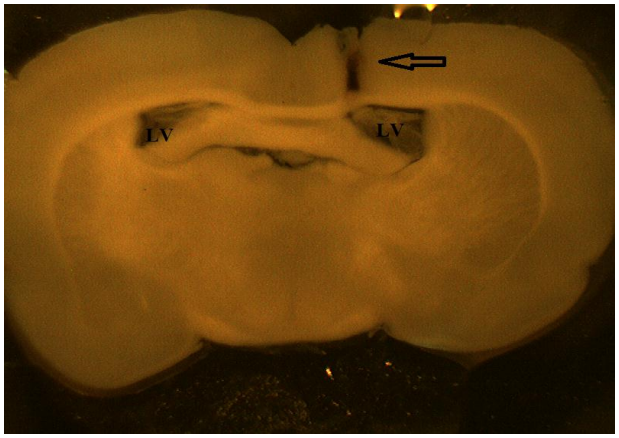

Fig. 1. A typical photo micrograph of a coronal section through the injection site (arrow) in the lateral ventricle. The tip of the guide cannula can be seen on the right side.

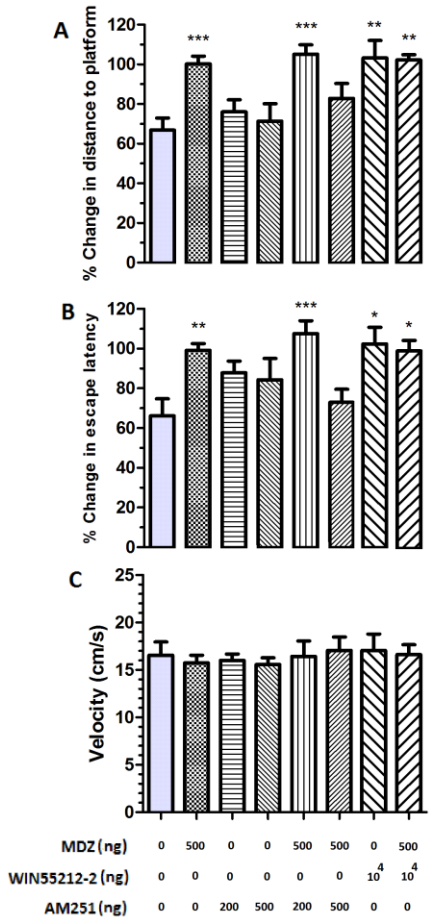

Fig. 2. The effect of i.c.v. administration of WIN55212-2, AM251 or their vehicle 5 min before i.c.v. administration of MDZ (MDZ) or its vehicle on learning of rats in MWM test. Rats were subjected to 8 training sessions 5 min after last drug injection. The mean distance of swimming from the platform (A), the mean time of swimming to find the platform (i.e. escape latency; B) and the mean velocity of swimming (c) during eight training sessions were depicted. Values are mean± SEM of 5-6 rats.
*p<0.05, **p<0.01, ***p<0.001 compared to control (vehicle) group.

Training sessions in MWM test – changes in escape latency

One-way ANOVA revealed significant change between groups [F(7,65)]=4.131, p=0.0008; Figure 2B]. Further analysis showed a significant increase in escape latency in group treated with MDZ (500ng; p<0.01).The effect of MDZ was attenuated by co-administration AM251 (500ng), but not AM251 (200ng). Moreover, treatment of rats with WIN55212-2 (10ug) significantly increased escape latency compared to control group (p<0.05). Co-administration of WIN55212-2 (10ug) and MDZ (500ng) did not alter the effect of each drug per se.

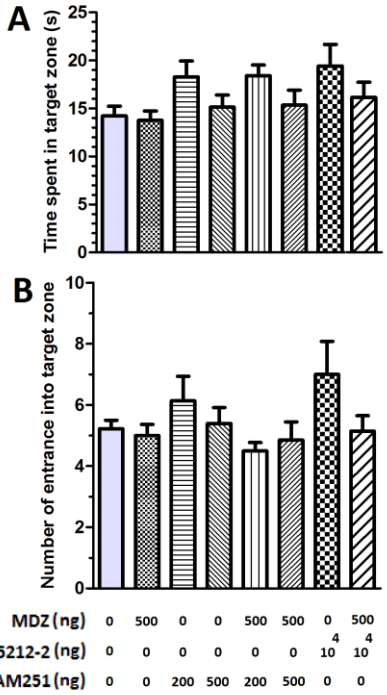

Fig. 3. The effect of i.c.v. administration of AM251, WIN55212-2 or their vehicles 5 min before i.c.v. administration of MDZ (MDZ) or its vehicle on memory of rats in MWM test. Rats were subjected to probe test 24h after last training session. The time spent in target quadrant (A), and the number of entrance into target quadrant (B) during probe test were depicted. Values are mean± SEM of 5-6 rats.

Training sessions in MWM test – changes in swimming velocity

In order to evaluate the effect of drugs on animal locomotion, the velocity of swimming was compared between groups. One-way ANOVA revealed no significant change in swimming velocity between groups [F(7,63)=0.2102, p=0.9818; Figure 2C].

Probe test

The results were shown in Figure 3. One-way

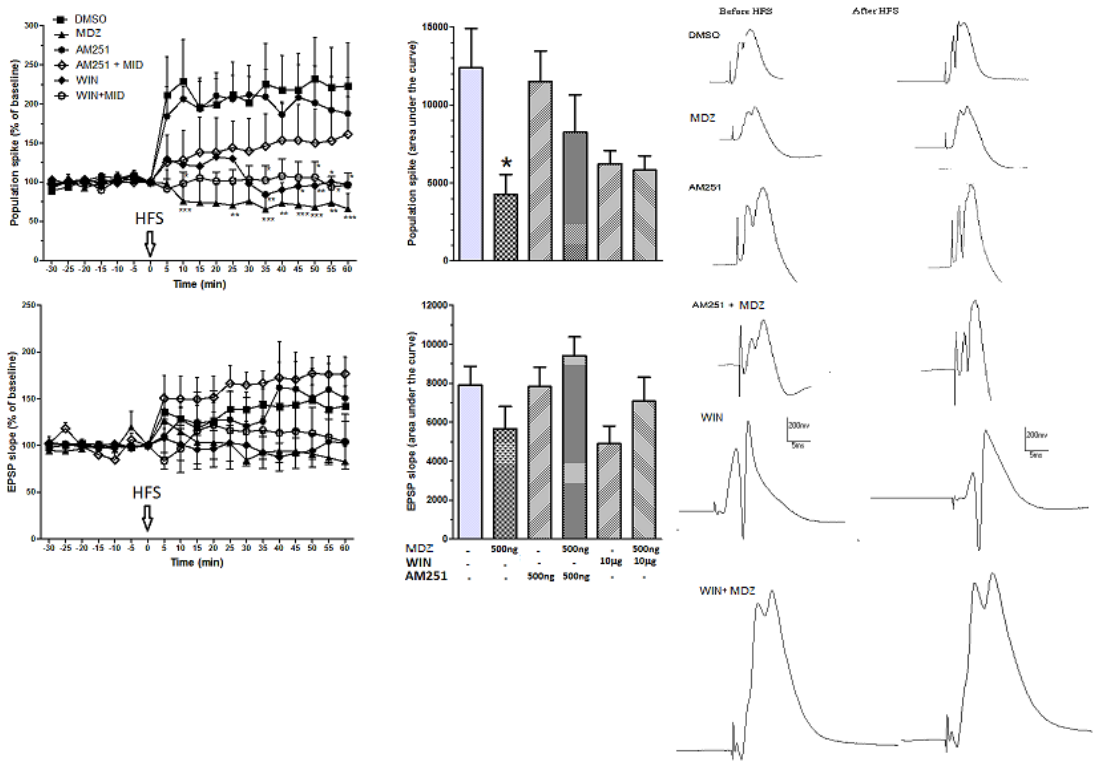

Fig. 4. Left: The effects of i.c.v. administration of AM251, WIN55212-2 or their vehicles, 5 min before i.c.v. administration of MDZ (MDZ) or its vehicle on high frequency stimulation (HFS)-induced LTP in the dentate gyrus of hippocampus in rats. The population spike (top) and EPSP slope (bottom) were measured every 5 min from 30 min before until 60 min after HFS induction. Data are plotted as the average percentage change from baseline responses. Values are %mean±S.E.M. Middle) Area under the curve of plots depicted on left panel. Right: Sample traces representing the effect of drug administration on fEPSP and PS before and after LTP induction (average of 5 responses)
*p<0.05, **p<0.01, ***p<0.001 compared to control (vehicle) group (N= 5 for each group).

ANOVA revealed no significant difference in time spent in target zone [F(7,63)=2.053, p=0.062; Figure 3A]. Moreover, there was no significant difference in frequency of entrance into target zone between control and treated groups [F(7,63)=1.028, p=0.042; Figure 3B].

Visual test

All of the animals were able to find the visible platform during 60s. time of visual test (data not shown).

Field potential recording

The results were shown in figure 4. Two-way ANOVA revealed a significant effect of treatment [F(5,304)=33.21, p<0.0001; Figure 4A] and time [F(18,304)=3.192, p<0.0001; Figure 4A] on LTP population spikes. Further analysis by Bonferroni's post-test revealed a significant decrease in population spikes at various time courses after HFS application in MDZ-treated group, WIN55212-2-treated group, and the group received co-administration of WIN55212-2 and MDZ compared to control group. Moreover, comparison of AUC of the LTP population spikes using one-way ANOVA revealed significant difference between treated groups [F(5,16)=3.113, p=0.038; Figure 4B]. Post hoc analysis using Dunnett's test showed a significant decrease in MDZ-treated group (p<0.05) compared to control group.

For EPSP slope of LTP, two-way ANOVA revealed significant effect of treatment [F(5,285)=17.70, p<0.0001; Figure 4C] and time [F(18,285)=2.816, p=0.0002; Figure 4C]. However, further analysis using Bonferroni's post-test revealed no significant change in treated groups compared to control group. Moreover, comparison of the AUC of

EPSP slope of LTP using one-way ANOVA revealed no significant change in treated groups [F(5,15)=2.48, p=0.08; Figure 4D].

Discussion

The results of this study showed that CB1 receptor is important in amnesic effect of MDZ. Administration of MDZ into lateral ventricle, impaired learning of rats compared to the control group. Administration of AM251 (either 200ng or 500 ng) alone did not affect learning and memory in rats. Previous studies showed that microinjection of both WIN55212-2 (5μg/side) and AM251 (6ng/side) impaired not only spatial learning in water maze test but also the LTP in the Schaffer collateral-CA1 projection (24).

Co-administration of AM251 (200ng) did not change MDZ effects on learning, but co-administration of AM251 (500ng) and MDZ diminished MDZ-induced learning impairment. Administration of WIN55212-2 (10μg) per se impaired spatial learning and memory of rats, which is consistent with the results of previous studies showing memory impairment following intra-hippocampal administration of WIN55212-2 at the doses of 500ng in a step-down type inhibitory avoidance task (25, 26). Also, microinjection of WIN55212-2 into basolateral amygdala impaired both fear acquisition and consolidation, but not retrieval in the aversive contextual fear task (27). In our study, co-administration of WIN55212-2 (10μg) and MDZ, however, did not change MDZ effect on learning of the rats. These effects seems to be mnemonic since the drugs showed no impairment on motor performance which was evaluated by measurement of swim velocity.

In electrophysiological study, i.c.v. administration of MDZ 5 min. before high frequency stimulation significantly reduced PS amplitude which could be considered as prevention of LTP induction. On the other hand, while administration of AM251 (500ng) produced no effect on fEPSP nor on PS amplitude per se, however, it could diminish MDZ-induced LTP impairment when administered before i.c.v. administration of MDZ. It is well established that agonists at the benzodiazepine site show anxiolytic and amnesic properties whereas inverse agonists, such as β-carbolines, exert anxiogenic and learning-enhancing actions (28). MDZ is an anxiolytic and sedative agent which is used in a variety of clinical settings. MDZ-induced amnesia is through facilitating the action of GABA on postsynaptic neurons. Furthermore, BDZs are known to reduce LTP in the hippocampus. It has been demonstrated that diazepam can reduce hippocampal LTP in Schaffer collateral-CA1, mossy fiber-CA3 and perforant path-dentate gyrus synapses (29). Also, it has been shown that MDZ had little influence on baseline synaptic responses but was very effective in blocking LTP through modulation of $GABA_A$ receptors (30). Same results were also obtained in our study in which MDZ did not alter the baseline synaptic function while LTP induction was significantly reduced.

Many studies have demonstrated that cannabinoids impairs learning and memory processes (31, 32). However there are some controversies about the involvement of CB1 antagonists in behavioral memory tests and LTP induction. Administration of the cannabinoid CB1 receptor antagonist did not produce significant effect upon memory of mice in inhibitory avoidance task (33) nor in high frequency stimulation-induced LTP induction (34, 35). In contrast, Carlson et al. (36) and de Oliveira Alvares et al. (37) have found that a CB1 receptor antagonist inhibits LTP induction in hippocampal CA1 neurons. In our study, no effect was observed after i.c.v. administration of AM251 per se on LTP-induction in hippocampal neurons. Moreover, our results also showed that AM251 per se also had no effects on spatial learning of rats in MWM task. On the other hand, administration of WIN55212-2 (10μg) either alone or in combination with MDZ significantly reduced PS-amplitude at some time points after HFS induction, though the overall change in PS-amplitude, which was shown by the area under the curve, did not changed significantly between WIN55212-2-treated and control group.

A high density of $GABA_A$ receptors exists in brain areas which are important in learning and memory process, such as the hippocampus (38). There is few evidence regarding interaction between the cannabinoid CB1 receptor and the GABAergic

system in learning and memory process (39). In this study, we suggested the possible involvement of the cannabinoid CB1 receptor in the amnesic action of MDZ in MWM task as well as its effect on plasticity of hippocampal neurons. The BDZs produce their clinical effects by acting on $GABA_A$ receptors. The activation of the $GABA_A$ receptors results in neuronal hyperpolarization via the opening of chloride-permeable ion channels. High levels of CB1 receptors are expressed in both GABAergic and glutamatergic neurons in the hippocampus. The activation of the cannabinoid CB1 receptor in this region decreases both inhibitory GABAergic and excitatory glutamatergic neurotransmission via presynaptic inhibition of neurotransmitter release (40).

The finding that the blockade of cannabinoid CB1 receptor by AM251 counteracts the action of MDZ both in the behavioral and electrophysiological tests supports the key role of these receptors in the action of BDZs. Consistent with our findings, García-Gutiérrez et al. showed that CB1 receptors are involved in alprazolam-induced amnesia (39).

Although the activation of cannabinoid receptor could suppress the release of both glutamate and GABA in hippocampal neurons, however, in this study, it seems that the effects of CB1 agonist and antagonist were primarily through CB1 receptors located at glutamatergic synapses. In this regard, the CB1 antagonist AM251 could primarily inhibit CB1 receptors at glutamatergic synapses, results in increase of glutamate release and physiologically attenuation of GABA-mediated effects of MDZ. Likewise, WIN55212-2 primarily activates CB1 receptors at glutamatergic synapses and inhibition of glutamate release could physiologically enhance MDZ effect.

Conclusion

Our results showed an inhibitory effect of AM251 on MDZ-induced both learning and memory impairment and reduction in LTP formation. On the other hand, no enhancement of MDZ effect was observed when co-administered with WIN55212-2 neither on learning and memory nor on LTP formation. Our results suggest that the effects of cannabinoid compounds, at least at the doses used in this study, were through their effects on glutamatergic system, but not through their action on GABAergic system which is basically is modulated by BDZs such as MDZ.

Acknowledgment

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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