CB1 Cannabinoid Receptor Activation Rescues Amyloid β -Induced Alterations in Behaviour and Intrinsic Electrophysiological Properties of Rat Hippocampal CA1 Pyramidal Neurones

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Key Words

Alzheimer's disease • Neuronal excitability • Ca^{2+} Currents • After hyperpolarization • Neurotoxicity

Abstract

Background: Amyloid beta ($\text{A}\beta$) is believed to be responsible for the synaptic failure that occurs in Alzheimer's disease (AD), but there is little known about the functional impact of $\text{A}\beta$ on intrinsic neuronal properties. Here, the cellular effect of $\text{A}\beta$ -induced neurotoxicity on the electrophysiological properties of CA1 pyramidal neurons and the mechanism(s) of neuroprotection by CB1 cannabinoid receptor activation was explored. **Methods:** A combination of behavioural, molecular and electrophysiological approaches was used. **Results:** Bilateral injections of the $\text{A}\beta$ peptide fragment (1-42) into the prefrontal cortex caused a significant impairment in the retention and recall capability in the passive avoidance tasks and significantly increased the level of active caspase-3 in the hippocampus. Whole-cell patch clamp recordings revealed a significant reduction in the intrinsic action potential (AP) frequency and an increase in the discharge irregularity in the absence of synaptic inputs in $\text{A}\beta$ treated group. $\text{A}\beta$ treatment

induced also significant changes in both the spontaneous and evoked neuronal responses. However, co-treatment with ACEA, a CB1 receptor agonist, preserved almost the normal intrinsic electrophysiological properties of pyramidal cells. **Conclusions:** *In vivo* $\text{A}\beta$ treatment altered significantly the intrinsic electrophysiological properties of CA1 pyramidal neurons and the activation of CB1 cannabinoid receptors exerted a strong neuroprotective action against $\text{A}\beta$ toxicity.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which is characterised by cognitive deterioration, irreversible memory loss and changes in behaviour [1]. Neuropathological hallmarks of AD are senile plaques, amyloid β ($\text{A}\beta$) accumulation [2, 3] and the dysregulation of calcium homeostasis [4, 5]. Whereas the exact underlying cellular mechanism responsible for amyloid β toxicity is still not entirely known, it is commonly accepted that $\text{A}\beta$ disrupts the neuronal Ca^{2+} homeostasis by increasing the intracellular Ca^{2+}

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concentration [6, 7] and thereby inducing Ca^{2+} neurotoxicity and cell death. Although there are several pharmacotherapy options available for AD, including acetylcholinesterase inhibitors, none of these therapies effectively alter the pathophysiology of dementia [8], which results from progressive synaptic loss and neuronal degeneration [9]. Knowing the mechanisms responsible for the neuronal cell death and neurodegeneration in several brain disorders, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, a more promising therapeutic approach could be used to reduce or prevent neuronal dysfunction and death. In this regard, the use of potential neuroprotective agents, including cannabinoids, should be considered [10-12]. Although the electrophysiological mechanisms underlying this neuroprotection have not yet been fully elucidated, it appears to be linked to either the activation of CB1, CB2 and unknown receptor(s) or receptor-independent pathways. The present study first aims to investigate how the neurotoxic A β peptide (1-42) alters the intrinsic electrophysiological properties of pyramidal neurons from the CA1 region of the hippocampus in a rat model of Alzheimer's disease. Second, the neuroprotective effect of cannabinoid receptor agonists against A β -induced alterations in the intrinsic properties of CA1 pyramidal neurons were assessed using whole-cell patch clamp recording in combination with molecular and behavioural approaches.

Based on previous reports demonstrating an important role for intrinsic neuronal excitability in neuroplasticity [13], we hypothesised that A β (1-42) strongly alters the intrinsic electrophysiological characteristics of hippocampal pyramidal neurons.

The effect neurotoxic A β has on the neuronal excitability is controversial. Yun and colleagues [14] reported that A β (1-42) reduced the neuronal excitability in mouse dentate gyrus, whereas Minkeviciene et al. [15] found that A β caused hyperexcitability, which was associated with progressive epilepsy. A β -induced changes in neuronal excitability could be due to the destabilisation of calcium homeostasis [4, 7] or the inhibition of K^{+} channels [16]. Other studies have emphasised the disturbances in neuronal synchronisation [17-20] due to changes in chemical synaptic networks. However, there are no detailed reports on the action of A β peptides on the intrinsic electrophysiological properties of neuronal cells.

In contrast, cannabinoids have been shown to inhibit voltage-activated Ca^{2+} channels [21-23] and cause a hypoglutamatergic condition by inhibiting the release of glutamate [24]. The direct activation of voltage-gated K^{+} channels, including fast transient (A-type) and large con-

ductance Ca^{2+} -activated K^{+} channels by cannabinoids, has also been reported [25, 26].

Based on these studies, the present study assessed whether A β causes plastic changes in the intrinsic excitability of CA1 pyramidal cells and whether cannabinoid receptor activation can prevent the A β -induced alterations in the neuronal intrinsic properties.

Materials and Methods

In this study male, adult Wistar rats (150–200 g) were used. They were housed in pairs under a 12 h light/dark cycle with food and water ad libitum. The experiments were conducted in accordance with the animal care and use guidelines approved by the Institutional Ethic Committee (IEC) at Shahid Beheshti University of Medical Sciences. All efforts were made to minimise the number of animals used and their pain and suffering.

Surgery

Rats were anaesthetised for stereotaxic surgery with an i.p. injection of ketamine (80 mg/kg) and xylazine (20 mg/kg). The rats were then bilaterally administered a stereotaxic injection of the A β peptide fragment (1-42) (Sigma, UK) (3 μl for each side) into the frontal cortex (3.2 mm AP, 2 mm DV relative to the bregma; depth 3 mm) according to previous published studies [12, 27, 28]. A β (1-42) was dissolved in sterile normal saline [29, 30] to the concentration of 10 ng/ μl , and 3 μl was injected (n=7) on each side using a Hamilton syringe. The peptide was still perfectly soluble even in the sham group, an equivalent volume of normal saline was injected without A β (n=6). One additional group served as the control (intact) group (n=7).

In vivo treatment with cannabinoid receptor ligands

Six hours post-surgery, two groups of A β -treated rats were given i.p. with either WIN-55212-2 (3 mg/kg, n=7), a non-selective cannabinoid receptor agonist, or ACEA (1 mg/kg, n=7), a selective CB1 receptor agonist. Next, the daily injection of agonists was repeated for 12 days. In addition, to define the role of the cannabinoid receptor that is involved in the neuroprotection against A β -induced toxicity, four groups of A β -treated rats (n=7 in each group) received either WIN+AM251 (CB₁ receptor antagonist, 1 mg/kg), WIN+AM251+AM630 (CB₂ receptor antagonist, 1 mg/kg), ACEA+AM251 or ACEA+AM630. All drugs were purchased from Sigma except for AM630, which was purchased from Tocris (UK).

Passive avoidance (PA) learning and memory test

On day 4 post-surgery, the rats were underwent passive avoidance training. The passive avoidance (shuttle box) apparatus consisted of two equal sized, connected chambers that were separated by a guillotine door. On the third day post-surgery, the rats were individually allowed to be habituated to the apparatus prior to testing. The rats from the control and

each experimental group were placed individually in the lighted chamber facing away from the entrance to the dark side; 10 s later, the guillotine door was raised, and the latency to enter the dark chamber was recorded. If the rats did not enter the dark chamber, they were eliminated from the test. The habituation trial was repeated after 5 min for the same interval. For the learning trial, after 2 hrs, the third adaption trial was administered in which an electrical stimulation (0.5 mA, 50 Hz, 2 s once) was delivered to the feet through the stainless-steel floor after entering the dark chamber. After 20 s, the rats were removed from the dark compartment and returned to their own cage. If the rats did not enter the dark chamber within 60 s, they were eliminated from the test and replaced with a new rat. After 5 min, the same test was conducted again, and if the rats did not enter the dark chamber by 300 s, the successful acquisition of passive avoidance response was recorded.

In the retention trials, which were performed 1 day and 7 days after the learning trial (acquisition test), the rats were again individually placed in the illuminated chamber, but no foot shock was administered if they entered the dark chamber. The rats were allowed to step into the dark compartment, and then the latency to re-enter the dark chamber was recorded. In both the learning and retention trials, the time to enter the dark section was recorded as the step-through latency. The maximum cut-off time for the step-through latency was 300 s when the rat did not enter the dark chamber in the retention trials [12].

Molecular assessment: Western blot analysis

On the twelfth day post-surgery, for Western blot analysis, rats were sacrificed by decapitation, and the hippocampus was isolated from the brain, rapidly frozen in liquid nitrogen and stored at -80°C . Briefly, the hippocampi were homogenised and lysed mechanically by rapid passage through a 23-gauge syringe needle containing lysis buffer (Tris-HCl 50 mM, NaCl 150 mM, Triton X-100 0.1%, sodium deoxycholate 0.25%, SDS 0.1%, EDTA 1 mM and protease inhibitor cocktail 1%) several times, and then the protein extract was obtained by centrifugation for 45 min at 13,000 g at 4°C and re-centrifuged for 15 min. Next, the protein concentration was determined using the Bradford assay, and equal amounts of each sample were used for SDS-PAGE electrophoresis. Thereafter, the gels were transferred onto a PVDF membrane (Millipore) and incubated with one of the following antibodies: Calbindin rabbit monoclonal antibody (1:1000, Sigma), cleaved caspase-3 rabbit monoclonal antibody (1:1000 Sigma) or β -actin rabbit monoclonal antibody (1:1000, Sigma). Protein expression was quantified by band densitometry scanned off the radiographic (X-ray) films.

Whole-cell patch clamp recording in slice preparation

Transverse hippocampal slices (300 μm) were made from adult, male Wistar rats (150–200 g). The rats were anaesthetised with ether and then decapitated. The brains were removed and placed in ice-cold ACSF containing (in mM) 206 sucrose, 2.8 KCl, 1 CaCl_2 , 1 MgCl_2 , 2 MgSO_4 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , and 10 D-glucose and equilibrated to a pH of 7.4 (with 95% oxygen and 5% carbon dioxide); the osmolarity was adjusted to 295 mOsm. The hippocampus was dissected, and transverse slices (300 μm) were cut using a vibroslicer (752 M, Campden

Instruments Ltd, UK). Thereafter, the slices were incubated in ACSF containing (in mM) 124 NaCl, 2.8 KCl, 2 CaCl_2 , 2 MgSO_4 , 1.25 NaH_2PO_4 , 26 NaHCO_3 , and 10 D-glucose at pH 7.4 and adjusted to 295 mOsm for at least 1 h at 35°C ; afterwards, they maintained at room temperature ($22\text{--}24^{\circ}\text{C}$) before being transferred to a recording chamber.

Whole-cell patch clamp recording

Whole-cell recordings were made as described previously [26]. Slices were transferred to a submerged recording chamber on the stage of an upright microscope (Olympus; BX 51WI). CA1 pyramidal neurons were visualised by infrared video imaging (Hmamatsu, ORSA, Japan) with a 60x water immersion objective. The slices were continuously superfused at 1–2 ml/min with ACSF at room temperature ($22\text{--}24^{\circ}\text{C}$). Whole cell current clamp recordings were made from CA1 pyramidal neurons using Multiclamp 700B amplifiers (Axon Instruments, Foster City, CA) equipped with Digidata 1320 A/D converter (Axon Instruments, Foster City, CA). Electrophysiological recordings were filtered at 5 kHz, sampled at 10 kHz and stored on a personal computer for offline analysis. For the recordings, the patch pipettes were pulled with a PC10 two-stage vertical puller (Narishige, Japan) from borosilicate glass capillary (1.2 mm O.D., 0.95 mm I.D. with inner filament). The pipettes had a resistance of 3–6 $\text{M}\Omega$ when filled with internal solution containing (in mM) 135 potassium methylsulfate (KMeSO_4), 10 KCl, 10 HEPES, 1 MgCl_2 , 2 Na_2ATP and 0.4 Na_2GTP . The pH of the internal solution was set to 7.3 by KOH, and the osmolarity was adjusted to 290 mOsm. For whole-cell voltage-clamp recording from acute slices, the pipettes were filled with a solution containing (in mM) 130 CsCl, 4 NaCl, 10 HEPES, 0.9 EGTA, 4 Na_2ATP , 0.5 Na_2GTP , 5 QX-314, and 20 Cs_2MeSO_4 at pH 7.4 adjusted with CsOH, and the osmolarity of the pipette solution was 290 mOsm. The external solution to isolate Ca^{2+} currents was composed of (in mM) 106 NaCl, 20 TEA-Cl, 2 CaCl_2 , 1.5 MgCl_2 , 26 NaHCO_3 , 10 KH_2PO_4 , 10 glucose, and 5 4-AP (pH 7.4 adjusted with NaOH), and the osmolarity was 296 mOsm.

After the establishment of a $\text{G}\Omega$ seal, the whole-cell configuration was achieved simply by applying a brief suction, and recordings were only considered when seals of more than 1 $\text{G}\Omega$ resistance were established. The test seal function was constantly monitored throughout the recording to ensure that the seal was stable. In addition, the series resistance (typically $<17\text{ M}\Omega$) was checked for stability during the experiments, and only cells with stable resting membrane potentials and input resistance were included in the analysis. The following electrophysiological parameters were measured: resting membrane potential (RMP), action potential duration at half width, after hyperpolarization (AHP), action potential (AP) number and the first spike latency. The first spike latency was defined as the time between the offset of the hyperpolarizing current steps and the peak of the first spike. The AHP amplitude was measured from the RMP before the stimulus to the peak of the hyperpolarization. The spike frequency adaptation index was calculated using ratios of the final to initial discharge frequency elicited by depolarizing current pulses. The coefficient of variation (CV) of the interspike intervals was calculated as the ratio of the standard deviation to the mean.

Fig. 1. Neurotoxic effect of A β and neuroprotective action of cannabinoid receptor activation on passive avoidance learning and memory. Bilateral injection of A β alone into the prefrontal cortex significantly decreased 24h and 72h testing retention latencies, whereas activation of CB1 receptors by either ACEA or WIN significantly improved retention and recall capability in the passive avoidance test in rats receiving A β . Co-application of CB1 receptor agonists (WIN or ACEA) and antagonist (AM251) prevented the neuroprotective effect of cannabinoid receptor activation against A β toxicity, but injection of AM630 (a CB2 receptor antagonist) did not further block the neuroprotective effects of CB1 receptor activations.***,###, \$\$\$ and \$\$\$ represent significantly difference between A β -treated group versus control, vehicle, WIN and ACEA treated groups ($P<0.001$), respectively. †††, BBB, £££ and ‡‡‡ donate significant difference between control, vehicle, A β +WIN and A β +ACEA vs A β +WIN+AM251 ($P<0.001$), respectively. hhh,YYY, PPP, δδδ represent significantly differences between A β +WIN+AM251+AM630 and control, vehicle, A β +WIN, A β +ACEA, respectively. FFF, ooo, HHH and ØØØ show significantly differences between A β +ACEA+AM251 vs control, vehicle, A β +WIN and A β +ACEA, respectively. E shows significantly difference from A β +ACEA+AM630.

All recordings were obtained in the presence of kynurenic acid (1 mM), a selective blocker of ionotropic glutamate receptors [31], and picrotoxin (100 μ M), a known GABA_A blocker [32].

In voltage-clamp experiments, to isolate different calcium currents, 5 μ M diltiazem and 4 μ M mibefradil were added to the external recording solution. Ca²⁺ currents were elicited by voltage ramp and step protocols. The holding potential was -60 mV, and a ramp potential at 79 mVs⁻¹ between -80 and +20 mV for 1252 ms from the same holding potential of -60 mV was applied. Depolarizing voltage steps (820 ms duration) were also applied from -60 mV to +50 mV in 10 mV increments with 2 s intervals.

Statistical analyses

The values are presented as mean $\pm$ SEM. The PA latency results were initially analysed using the K-S (Kolmogorov-Smirnov) test and were then evaluated using one-way ANOVA. For the additional data, one-way ANOVA was used for multiple comparisons followed by Tukey's test. A probability of 0.05 was considered as the criterion for significance.

Results

Behavioural experiment

Impairments in learning and memory following bilateral injection of A β into the frontal cortex. The results of the passive avoidance assessment of learning and memory performance showed that the A β (1-42) peptide treatment did not alter the rate of learning acquisition (data not shown) but significantly disrupted memory retention compared to the control and sham-treated rats.

As shown in Fig. 1A, the rats that received the bilateral A β injection 24 hours after the passive avoidance training trial (i.e., 4 day post-surgery) exhibited significantly shorter (123.16 $\pm$ 14.5 s) step-through latencies compared to the control group (258.16 $\pm$ 20.2 s, $P<0.001$) and sham-treated group (253.33 $\pm$ 21.07 s, $P<0.001$). When the testing was performed 7 days after the shock experience (12 days post-surgery), the step-through latency was

Fig. 2. Effects of *in vivo* A β (1-42) treatment and neuroprotection induced by CB1cannabinoid receptor activation against A β toxicity on the level of active Caspase-3. Western blot analysis revealed that A β treatment increased significantly the level of active Caspase-3, but combined treatment with CB1 receptor agonists (WIN or ACEA) prevented the Caspase-3 activation. The protective effects of CB1 receptors activation were significantly suppressed by CB1 receptor antagonist, AM251, but not CB2 receptor antagonist, AM630. The relative density is expressed as the ratio (Caspase-3/ β -actin). **,##, \$\$ and §§ represent significantly difference between A β -treated group vs control, vehicle, WIN and ACEA treated groups ($P < 0.01$), respectively. ††, BB, ££ and ‡‡ donate significant difference between control, vehicle, A β +WIN and A β +ACEA vs A β +WIN+AM251 ($P < 0.01$), respectively. hh YY PP, dd, significantly difference between A β +WIN+AM251+AM630 and control, vehicle, A β +WIN, A β +ACEA, respectively. oo, FF, HH and ØØ show significantly difference between A β +ACEA+AM251 vs control, vehicle, A β +WIN and A β +ACEA ($P < 0.01$).

significantly decreased in A β -treated rats (46.83 ± 19.06 s) compared to the control (224.83 ± 17.03 s, $P < 0.001$) and sham-treated (205.16 ± 3.84 s, $P < 0.001$) rats, and the latency was shorter (61.97%) than the latency obtained in the first retention test (i.e., testing performed at 24 h) in the same treatment group (Fig. 1B).

Combined treatment with cannabinoid CB1 receptor agonists prevented the amyloid beta induced memory impairment. The rats that were treated chronically with A β combined with either WIN or ACEA showed a significant memory improvement to animals treated with A β alone. The treatment with WIN or ACEA immediately after the passive avoidance training trial caused a significant increase (270.4 ± 15.36 s and 271.6 ± 25.04 s, respectively; $P < 0.001$) in the latencies to cross into the dark compartment (shock chamber) upon retesting 1 day later compared to A β -treated alone (Fig. 1A). Similarly, when these two groups of rats were retested 7 days after training, they showed significantly longer step-through latencies (Fig. 1B, 215.83 ± 17.45 s for WIN and 264.6 ± 18.17 s for ACEA; $P < 0.001$). However, there were no significant differences between the control and sham-treated rats with either combined treated rats.

Cannabinoid CB1, but not CB2, receptors may be directly involved in neuroprotection against A β -induced memory impairments. CB1 receptor inhibition by AM251 abolished the protective effect of both WIN and ACEA on A β -induced memory dysfunction. Rats co-treated with A β +WIN+AM251 or A β +ACEA+AM251 exhibited no protection from A β -induced memory loss (Figs. 1A, B). The inhibition of CB1 receptors significantly reduced step-through latency after 24 h (116.5 ± 10.89 s and 74.5 ± 25.41 s for the rats that received A β +WIN+AM251 and A β +ACEA+AM251, respectively; $P < 0.001$) or 7 days (70.83 ± 22.52 s and 35.16 ± 13.09 s for A β +WIN+AM251 and A β +ACEA+AM251 groups, respectively; $P < 0.001$), whereas the blockade of CB2 receptors by AM630 did not further significantly reduce the passive avoidance latencies when injected either alone or with AM251 (Figs. 1A, B).

Molecular study

The antibody used for Western blotting was able to detect both total and cleaved (activated) caspases-3. The density of cleaved caspase-3 was normalized to β -actin

Fig. 3. Changes in firing pattern of hippocampal pyramidal neurons following treatment with either A β or A β +ACEA. Somatic whole cell patch clamp recordings revealed that *in vivo* treatment with A β decreases the firing rate and converted the pattern of action potentials firing of pyramidal neurons from a regular pattern to a more irregular firing compared to control condition. Combined treatment with ACEA, a selective CB1 receptor agonist, however, restored the normal neuronal firing. The change in firing precision is shown on an expanded time scale (lower traces). Superimposed action potentials from the control, A β -treated and A β +ACEA-treated conditions. Note the larger AHP amplitude in A β -treated group compared to either control or A β +ACEA treated groups.

and then compared between the groups. A significant ($P<0.01$) increase in active caspase-3 (17 KD), in the hippocampus of A β treated rats was observed compared to either the control or sham groups (Fig. 2), whereas activation of CB1 receptors with the combined treatment of A β and ACEA (1 mg/kg) or WIN (3 mg/kg) significantly ($P<0.01$) suppressed the level of active caspase-3 in the hippocampus at 12 days after the last injection of either ACEA or WIN (Fig. 2).

In order to investigate which type of cannabinoid receptor contributed to the neuroprotection against A β neurotoxicity, AM251, a potent CB1 antagonist, was concurrently injected with either WIN-55212 or ACEA to the rats receiving A β . AM251 significantly prevented the neuroprotective effect of CB1 receptor activation against A β toxicity, as shown by the increased level of active caspase-3 (Fig. 2). In contrast, in the presence of CB1 receptor inhibition, the inactivation of CB2 receptors by AM630 did not further affect the level of active caspase-3 (Fig. 2).

Whole-cell patch clamp assessment of neuroprotection against A β -induced electrophysiological alterations in CA1 pyramidal neurons. The present behavioural and molecular results suggest that CB1, but not CB2, receptors functioned in a potential neuroprotective role against A β -induced neurotoxicity. Therefore, to address whether these changes could be associated with electrophysiological dysfunction and how CB1 receptor activation could protect pyramidal neurons from A β neurotoxicity, alterations in the intrinsic electrophysiological characteristics of neurons were determined. In these experiments, four groups of rats were

used: the control ($n=8$) group and three separate groups of rats treated with A β alone ($n=8$) or either combined A β +ACEA ($n=7$), A β +ACEA+AM251 ($n=7$), or A β +ACEA+AM630 ($n=7$).

In vivo treatment with A β peptide (1-42) causes profound alterations in the intrinsic electrophysiological properties of CA1 hippocampal pyramidal cells. Following 12 days of A β treatment, pyramidal neurons exhibited significant differences in their intrinsic electrophysiological properties when compared to control cells. A β treatment had no effect on resting membrane potential (-62.38 ± 3.65 mV in control vs -60.26 ± 1.1 mV in A β -treated rats), but it significantly decreased the input resistance of the CA1 pyramidal neurons in compare with the control group (from 79.30 ± 4.9 M Ω to 58.8 ± 0.9 M Ω , $p<0.01$). The amplitude (91.303 ± 4.22 mV) and time to peak (1.59 ± 0.16 ms) of the action potential (AP) was also unchanged in A β -treated rats compared to the control rats (91.423 ± 2.24 mV and 1.23 ± 0.17 ms, respectively). However, as can be seen in Figs. 3, 4, the comparison of the AP characteristics between pyramidal neurons recorded from the control and A β -treated rats revealed significant differences. CA1 hippocampal pyramidal neurons from the A β -treated group showed a significant decrease in firing frequency (from 1.5 ± 0.36 Hz in control cells to 0.36 ± 0.03 Hz in cells from A β group; $P<0.001$, Figs. 3, 4A). This difference was accompanied by a disruption of spontaneous firing, as shown by the significant increase in the coefficient of variation from 0.28 ± 0.04 in the control condition to 0.62 ± 0.08 after A β treatment (Figs. 3, 4B, $P<0.01$). In addition, there was a significant

Fig. 4. Altered intrinsic firing properties and action potential characteristics following Aβ treatment and preservation of normal firing activity by combined treatment with Aβ+ACEA. *In vivo* Aβ treatment significantly decreased the firing frequency (A) which was associated with a significant increase in the coefficient of variation (B). It was caused also a significant increase in the AHP amplitude (C) and the action potential duration (D). The combination treatment with Aβ+ACEA significantly preserved the intrinsic neuronal firing properties. **,***, significantly different ($P<0.01$, $P<0.001$) from control; ##, ###, significant different ($P<0.01$, $P<0.001$) from Aβ+ACEA treated group. §§ represents significant difference between Aβ+ACEA+AM251 treated group and control; ££ donates significant difference between Aβ+ACEA+AM251 treated group and control and Aβ+ACEA. Y shows significant difference between Aβ+ACEA+AM251 and Aβ+ACEA+AM630.

increase in the amplitude of AHP (control: -2.93 ± 0.24 mV; Aβ-treated: -6.77 ± 0.4 mV; $P<0.001$, Figs. 3, 4C) and AP duration at half-amplitude (control: 1.61 ± 0.08 ms; Aβ-treated 2.8 ± 0.19 ms; $P<0.001$, Fig. 4D). Repetitive firing properties were also altered by treatment with Aβ. Pyramidal neurons from Aβ-treated rats showed a lower excitability that was reflected by the significant decrease in instantaneous firing frequency (from 24.24 ± 4.2 Hz in control to 7.5 ± 1.63 Hz in Aβ-treated group, $P<0.001$). Furthermore, the number of action potentials evoked either by depolarizing current pulses of increasing (Figs. 5A, B) or fixed (Figs. 5C, D) amplitudes were reduced in hippocampal pyramidal neurons from Aβ-treated rats in comparison to the control group. This reduction in firing rate was associated with increased spike frequency adaptation (Fig. 5E). Depolarizing voltage sag induced by hyperpolarizing current injections, which is shown to be due to activation of the hyperpolarization-activated cationic I_h current, was also significantly affected by Aβ treatment (Fig. 6). In addition, *in vivo* treatment with Aβ caused significant prolongation in the monotonic first-spike compared to the control group (Fig. 7).

In vivo co-treatment with ACEA, a CB1 agonist, can prevent the Aβ-induced changes in intrinsic electrophysiological properties of rat CA1 pyramidal neurons. Pyramidal neurons from the combined treated rats had a mean RMP of -60.2 ± 0.4 mV. After Aβ plus ACEA treatment, the input resistance of pyramidal neurons significantly decreased (66.97 ± 1.78 MΩ $P<0.5$) compared to the control value; however, it was significantly higher than Aβ-treated rats ($P<0.01$). Co-treatment with ACEA did not significantly change either the amplitude (91.44 ± 3.4 mV) or the time to peak (1.31 ± 0.96 ms) of AP but did significantly increase the firing frequency to 1.92 ± 0.39 Hz ($P<0.01$) when compared to the Aβ-treated group but not the control group (Fig. 4A). ACEA+Aβ treatment was also associated with an increase in the firing pattern regularity, as evidenced by a significant decrease in the coefficient of variation (0.27 ± 0.02 , $P<0.01$) compared to Aβ treatment alone (Figs. 3&4B). Pyramidal neurons from the combined treated rats demonstrated significantly lower AHP amplitudes (-3.63 ± 0.49 , $P<0.001$) and shortened action potential durations (1.90 ± 0.11 , $P<0.001$) compared to the Aβ-treated group (Figs. 4C, D).

Fig. 5. Alterations in the evoked firing responses of CA1 pyramidal neurones following treatment with A β alone or in combination with ACEA. (A) Soma of pyramidal neurones recorded in whole cell patch configuration were given depolarizing current injections in a stepwise manner. The traces depicted indicate the response to three current steps to 100, 300 and 500pA in control condition and following treatment either with neurotoxin A β alone or A β +ACEA. (B) Plot of action potential number/pulse versus applied depolarizing currents. *,# donate significant changes in the plot in control and A β +ACEA groups versus A β -treated group, respectively. (C) Representative traces showing the differences in the action potential firing rate recorded from pyramidal neuronal somata in response to five consecutive depolarizing pulses with fixed amplitude (200pA) and duration (200ms). (D) The relationship between firing rate and applied pulses of fixed amplitude. (E) Comparison of the spike frequency adaptation (SFA) index calculated in response to injection of 100pA and 500pA depolarizing pulses in the control, A β -treated and A β +ACEA-treated groups.

Analysis of the repetitive firing properties of CA1 pyramidal neurones of ACEA+A β -treated rats has also revealed the neuroprotective potential of CB1 receptor activation against A β -induced alterations in the active properties of hippocampal pyramidal neurones. The treatment of ACEA in combination with A β resulted in a significant increase in the instantaneous firing frequency (18.15 ± 1.1 Hz, $P < 0.001$) and in the number of evoked action potentials in response to depolarizing current pulses of various (Figs. 5A, B) or constant (Figs. 5C, D) amplitudes when compared to A β -treated

rats. The adaptation index remained unaltered by ACEA+A β treatment when compared to A β treatment alone; however, it was significantly higher than the control rats in response to a small depolarizing pulse (Fig. 5E). In addition, this treatment did not prevent the significant enhancement of the depolarizing sag voltage observed in A β -treated rats (Fig. 6) but restored the first-spike latency to its control value (Fig. 7). We next examined the properties of inward currents in pyramidal neurones through voltage clamp conditions.

Fig. 6. Effect of A β and A β +ACEA on depolarizing “sag” voltage. In current clamp, the voltage responses to hyperpolarizing current pulses (-100pA and -500pA) were recorded from pyramidal neurons in control condition and after A β and A β +ACEA treatment. Moving the membrane potential to more negative levels resulted in an increase activation of depolarizing “sag”. Peak (*) and steady state (•) voltages were measured and the plot of “sag” amplitude (steady-state voltage minus peak hyperpolarization voltage, inset) was shown.

The activation of cannabinoid CB1, but not CB2, receptors may contribute to the neuroprotection against A β -induced electrophysiological changes in CA1 pyramidal neurons. In order to assess whether the electrophysiological neuroprotection induced by combined treatment of ACEA and A β is possibly due to the activation of CB1 but not CB2 receptors, AM251 and AM630, two CB1 and CB2 receptor antagonists respectively, were intraperitoneally administered to the rats which had been treated also with A β plus ACEA. The neuroprotection observed with combination treatment of A β and ACEA were almost fully prevented by AM251, but not AM630, as evidenced also by electrophysiological findings. In those rats which were given AM251, the mean firing frequency (0.48 ± 0.01 Hz) of CA1 pyramidal neurons was significantly lower than control ($P < 0.05$) and

A β +ACEA-treated rats ($P < 0.01$), while it was similar to the A β -treated rats (Fig. 4A). However, administration of AM630 did not significantly change the firing frequency when compared to the control and A β +ACEA-treated groups, but, compare to A β -treated alone, it significantly increased the cell firing frequency (1.8 ± 0.2 Hz, $P < 0.01$). These treatments were also associated with changes in the coefficient of variation (Fig. 4B). Blockade of CB1 receptors resulted in a significant increase in the firing irregularity as evidenced by an increase in the CV when compared to control and A β +ACEA-treated rats, but not A β -treated alone. In contrary, blockade of CB2 receptors did not significantly change the firing discharge regularity as compared to either control or A β +ACEA-treated rats. Moreover, treatment with AM251, similar to A β , significantly increased both the

Fig. 7. Hippocampal CA1 pyramidal neurones in A β -treated rats exhibited long delay to first spike than either control or A β +ACEA-treated groups. Recordings of the latency to the first spike in a representative cell in control, A β -treated and A β +ACEA- treated conditions. The current injection protocols are shown at the bottom of the representative traces. (A) Conditioning with a small (-100pA) hyperpolarizing prepulse resulted in a significant prolonged delay to the onset of first spike in A β -treated group compared to control and A β +ACEA groups, whereas the delay in spike onset induced by a fixed depolarization test pulse (300pA) did not alter between all group by increasing intensities of prepulse currents (B). The latency to the first spike was plotted as a function of hyperpolarizing prepulse current injections of varying amplitudes (C).

AHP amplitude and duration of action potential compared to control and A β +ACEA-treated, whereas CB2 receptor antagonist AM630 failed to produce further significant changes in the amplitude of AHP and the action potential duration as compared to control and A β +ACEA-treated rats (Figs. 4C, D).

Furthermore, the intrinsic electrophysiological responses of CA1 pyramidal neurons evoked by current injections were also altered following treatment with A β +ACEA+AM251, but not A β +ACEA+AM630. Administration of AM251 caused a significant decrease in the number of evoked action potentials in response to 100pA step depolarization (Figs. 6A, B) compared to all groups except A β -treated rats, whereas following treatment with A β +ACEA+AM630 the neuronal evoked activity did not significantly change and remained almost the same as either control or A β +ACEA treated rats. In addition, the action of these cannabinoid receptor antagonists on repetitive firing responses elicited by injecting depolarizing pulses of fixed intensity (200pA) were similar to those evoked by current pulses of increasing amplitude (Figs. 6C, D). CB1 but not CB2 receptor blockade significantly increased the latency of the first action potentials evoked by current steps (Fig. 7).

Augmented Ca²⁺ inward currents may contribute to the A β -induced alterations in the intrinsic electrophysiological properties in rat hippocampal CA1 pyramidal neurons. During a voltage ramp in whole-cell recording of pyramidal neurons from control hippocampal slices after blocking the Na⁺ and K⁺ currents, an inward Ca²⁺ current was activated at -20.78 ± 3.57 mV and reached a mean peak amplitude of -309 ± 108.64 pA ($n = 8$, Fig. 8). However, *in vivo* treatment with A β induced a strong inward Ca²⁺ current with a threshold voltage of -36.86 ± 0.72 mV ($n = 8$) at -12.5 ± 3.7 mV, which was significantly shifted towards a more hyperpolarized voltage compared with the control group ($P < 0.01$). In A β -treated rats, pyramidal cells displayed significantly larger currents of -504.13 ± 49.94 pA ($P < 0.01$) at -25.78 ± 2.76 mV (Fig. 8). ACEA treatment with A β caused an activation of an inward current at -26.53 ± 3.1 mV ($n = 6$) with a mean peak amplitude of -188.4 ± 27.42 pA at -17.22 ± 3.99 mV, which was significantly lower compared to the control ($P < 0.01$) and A β -treated ($P < 0.001$) groups (Fig. 8). When voltage steps were applied, a similar augmentation of the inward current induced by the A β treatment was also observed compared to the control and the group treated with A β +ACEA (Fig. 9). In A β -treated rats, CA1

Fig. 8. A β -induced potentiation of Ca²⁺ currents was suppressed by combined treatment with A β plus ACEA, a selective CB1 receptor agonist. (A) Intrinsic somatic Ca²⁺ current (left column) recorded after blocking the synaptic currents and voltage dependent Na⁺ and K⁺ currents in control, A β and A β +ACEA- treated groups with voltage clamp ramp protocol (lower left). The effect of Ca²⁺ current blockade by diltiazem (middle column) and mibefradil (right column). (B) Mean amplitude of Ca²⁺ currents recorded before and after application of 1 μ M of diltiazem (Dilt) and 2 μ M of mibefradil (Mib) from pyramidal neurons obtained in control, A β and A β +ACEA-treated rats. *, **,***, significantly different ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from the control; ##, ###, significantly difference ($P < 0.01$, $P < 0.001$, respectively) from the A β +ACEA-treated group.

pyramidal cells had a significantly greater mean peak amplitude of the depolarization-activated Ca²⁺ current compared to either the control or A β +ACEA groups (Figs. 9A, B). This current was mediated by voltage-dependent Ca²⁺ channels because it was sensitive to the extracellular application of diltiazem, an L-type channel inhibitor, and mibefradil, a T-type channel blocker (Fig. 9C). The suppressive effect of mibefradil on the Ca²⁺ current was more effective in the A β -treated group than in the other groups (Fig. 9D); however, a large fraction of inward current still remained even after blocking T- and L-type Ca²⁺ currents (Fig. 9C).

Discussion

There is considerable evidence that the prefrontal cortex is involved in memory processing [33-35] and

previous studies have shown that lesions of the prefrontal cortex in rodents result in deficits in memory function [34, 36-38]. Anatomical and functional evidence also suggest direct and indirect interplay between prefrontal cortex and hippocampus [39-41]. Several studies have provided evidence that intrafrontal injection of A β induced neuronal loss [42, 43] so subsequently this could affect the pyramidal neuronal function because of the anatomical connection between prefrontal cortex and hippocampus. van der Stelt et al. has demonstrated that 12 days after the injection of A β 1-42 into the frontal cortex, neuronal damage was observed in the CA1, CA2 and CA3 regions of hippocampus that were far from the injection site [12]. They concluded that A β 1-42 is capable of diffusing into the brain tissue.

Therefore, on the basis of the above studies, here a rat model of Alzheimer's disease was used by bilateral intraprefrontal injection of A β 1-42. Using behavioural,

Fig. 9. Comparison of Ca^{2+} currents of CA1 pyramidal neurones from normal, $\text{A}\beta$ and $\text{A}\beta$ +ACEA-treated rats. (A) Examples of Ca^{2+} current traces from a family of depolarizing voltage steps recorded between -40 to +50mV from a holding potential of -60mV in the absence (Upper) and in the presence (Lower) of Ca^{2+} channel blockers (diltiazem and mibefradil). The overly compares the amplitude-time course of the peak Ca^{2+} currents recorded in normal, $\text{A}\beta$ and $\text{A}\beta$ +ACEA-treated rats. Current-voltage relation of Ca^{2+} current derived from step depolarization from -40 to +50mV before (B) and after (C) blockade of Ca^{2+} currents by diltiazem (1 μM) and mibefradil (2 μM). (D) Comparison of the peak amplitude of Ca^{2+} current (% of change) after application of diltiazem, an L-type calcium channel blocker, and mibefradil, a T-type calcium channel blocker. *and # significantly different from control and $\text{A}\beta$ +ACEA, respectively.

molecular and electrophysiological techniques, we demonstrated that the activation of CB1 receptors restores normal memory function and the intrinsic electrophysiological properties of CA1 pyramidal neurones in an $\text{A}\beta$ -induced rat model of Alzheimer's disease. To our knowledge, we were the first to provide information about the *in vivo* effects of bilateral injections of $\text{A}\beta$ into the prefrontal cortex on intrinsic neuronal excitability of CA1 pyramidal neurones. In this study, the neuroprotective potential of CB1 receptor activation against $\text{A}\beta$ -induced alterations in intrinsic neuronal electrical activity was also assessed. *In vivo* treatment of rats with $\text{A}\beta$ caused memory impairment. Consistent with this finding, the accumulation of soluble $\text{A}\beta$, particularly $\text{A}\beta_{42}$, in the brain of patients and animal models of AD has been shown to

be associated with impairments of cognition and learning and memory [44-47]. Memory loss observed in early Alzheimer's disease has been reported to be related to a reduction in the integrated activity within a neural network that includes the prefrontal cortex and hippocampus [48]. $\text{A}\beta$ neurotoxicity, which is thought to be the main cause of memory loss in AD [49, 50] has been demonstrated to induce neurodegeneration through the activation of caspase-3 [51, 52]. Our molecular findings showed that $\text{A}\beta$ treatment caused an increase in the level of active caspase-3, but *in vivo* CB1 cannabinoid receptor agonist treatment in combination with $\text{A}\beta$ prevented the memory loss and caspase-3 activation that were associated with $\text{A}\beta$ treatment alone. This was consistent with several studies suggesting that cannabinoid receptor activation in

the brain participates in neuroprotection [53-55]; however, the cellular mechanisms underlying this neuroprotective effect are not yet well defined. It was reported that the lack of the CB1 receptors in knockout mice was associated with increased caspase-3 activation [56], indicating the neuroprotective potential of these receptors. A β neurotoxicity could be mediated by glutamate excitotoxicity [57] which could result in neuronal cell death [58]. However, CB1 receptor activation in the hippocampus inhibited the presynaptic release of glutamate, which has been shown to prevent excitotoxicity, leading to cell death [59].

To understand the functional impact of A β neurotoxicity on neuronal cells, several studies have been directed toward A β -induced alterations in synaptic activity [60, 61]. It was suggested that A β may be part of a regulatory mechanism of synaptic activity by acting as a positive presynaptic or a negative postsynaptic regulator [62]. Although there is increasing evidence that the initial synaptic and cellular changes resulted from the presence of A β peptide rather than the plaque formation itself [63] and that the accumulation of A β potentially disrupted the neuronal synaptic plasticity [64], there is little information about the effect of A β toxicity on the neuronal intrinsic properties. Neural excitability is determined based on the interplay between synaptic inputs and intrinsic membrane characteristics [65, 66]. Changes in either synaptic activities or membrane intrinsic properties affected the output of the neuron [67].

The electrophysiological findings of this study demonstrated that *in vivo* treatment with A β (1-42) led to profound alterations in the intrinsic membrane properties of rat CA1 pyramidal neurons. A β (1-42) caused a significant reduction in the firing frequency and a significant increase in the AHP amplitude, the amplitude of AP and the coefficient of variation, whereas treatment with A β +ACEA preserved the normal intrinsic electrophysiological properties of pyramidal neurons. Under voltage clamp, A β treatment induced a large inward Ca²⁺ current that was partially sensitive to L- and T-type Ca²⁺ channels blockers (diltiazem and mibefradil, respectively). A β (1-42) application was reported to decrease neuronal excitability associated with a decrease in the rate of AP firing in response to current injection and an increase in the AHP amplitude in hippocampal granule cells [14]. However, in rat prefrontal cortex, opposing effects of low and high doses of A β 42 on neuronal excitability have been reported, whereas, in contrast to the high dose of A β 42, the low dose significantly increased the AHP and reduced neuronal excitability [68].

Electroencephalography of many patients that suffer from dementia was also characterised by an increase in low frequency field potential oscillations [69]. Changes in neuronal excitability, which are often related with learning [70, 71], could be determined by modulating the amplitude and duration of AHP that follows the action potentials in nerve cells. An inverse relationship between the AHP and long-term potentiation (LTP) was reported, where an enlarged AHP prevented LTP and a small AHP facilitated LTP [72, 73]. In hippocampal pyramidal neurons, BK type Ca²⁺-activated K⁺ channels were responsible for a fast AHP occurring immediately after APs that helped repolarize somatic APs [74, 75]. However, A-type K⁺ channels, which have been implicated in learning and memory [76], could also influence the amplitude of AHP in rat hippocampal CA1 pyramidal neurons [77]. The activation of A-type K⁺ channels has been shown to regulate neuronal firing by affecting the AP onset time, threshold, repolarization and interspike intervals [74, 78]. In current clamp recordings in slices prepared from A β -treated rats, we found that, unlike control and ACEA+A β -treated conditions, A β treatment significantly increased first spike latency. In many central neurons, including hippocampal pyramidal neurons, D-type current or slowly inactivating A type K⁺ channels plays a role in slowly developing subthreshold ramp potential induced by depolarizing current injections and thereby slows repetitive firing activity [78, 79, 80]. These findings were consistent with other studies that have shown A β -induced I_A activation [81, 82]. In contrast, CB1 receptor activation has been demonstrated to attenuate the voltage-activated K⁺ currents [83, 84].

Another candidate that could be modulated with either A β or CB1 receptor agonist treatment are Ca²⁺ channels which, in turn, influence neuronal excitability. Under whole cell voltage clamp conditions, our results demonstrated that A β treatment induced a strong inward Ca²⁺ current, which was partially suppressed by extracellular application of diltiazem and mibefradil (L- and T-type calcium channel blockers, respectively), whereas co-treatment with ACEA prevented the potentiation of the inward Ca²⁺ current by A β .

Several reports have suggested that A β disrupted the calcium homeostasis [85] by forming transmembrane cation permeable channels [86] or through the potentiation of L- and N-type Ca²⁺ currents [6, 7, 87, 88]. The resulting increase in Ca²⁺ currents induced by A β has been shown to be accompanied by a large K⁺ conductance due to an increase in the intracellular Ca²⁺ concentration, which causes a chronic loss of cytoplasmic K⁺, thus re-

ducing neural excitability [89]. This was consistent with our findings that *in vivo* treatment with A β resulted in a significant decrease in neuronal excitability due to significant enhancement of AHP amplitude. However, combined treatment with both A β and ACEA preserved neuronal intrinsic electrophysiological properties against A β toxicity. Previous reports have shown that CB1 receptor activation reduced Ca²⁺ influx by blocking voltage-gated N-, P/Q- and L-type Ca²⁺ channels [90-92]. In this study, under voltage-clamp conditions, the peak inward Ca²⁺ current was significantly decreased in the A β +ACEA treated group compared with A β -treated alone. Based on these findings, another possible explanation for the reduction in neuronal firing rate which was observed in A β -treated rats, but not in control or A β +ACEA treated rats, is that A β treatment alone led to an increase in the intracellular Ca²⁺ level that in turn causes activation of calcium - dependent K⁺ channels underlying the AHP. Therefore, this increase in AHP amplitude prolongs the interspike intervals, as evidenced by a significant decrease in firing rate, and produces a substantial delay in the occurrence of the first action potential.

In conclusion, these results suggest that memory impairment induced by A β toxicity could result from significant alterations in the intrinsic electrophysiological properties of hippocampal pyramidal neurons, and the activation of CB1 cannabinoid receptors exerted a strong neuroprotective action against A β -induced neurotoxicity. However, some of this neuroprotection may be a result of inhibition of voltage-dependent Ca²⁺ channels and either direct or indirect suppression of Ca²⁺-activated K⁺ channels. The present study provides detailed information about neuroprotective effects of cannabinoids against neurotoxic A β , which may have potential therapeutic relevance in AD.

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