Neurochemical Research (2018) 43:1511–1518
https://doi.org/10.1007/s11064-018-2565-0

ORIGINAL PAPER

Systemic Injections of Cannabidiol Enhance Acetylcholine Levels from Basal Forebrain in Rats

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Received: 29 January 2018 / Revised: 9 May 2018 / Accepted: 28 May 2018 / Published online: 6 June 2018

Abstract

Cannabis sativa is a plant that contains more than 500 components, of which the most studied are Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD). Several studies have indicated that CBD displays neurobiological effects, including wake promotion. Moreover, experimental evidence has shown that injections of CBD enhance wake-related compounds, such as monoamines (dopamine, serotonin, epinephrine, and norepinephrine). However, no clear evidence is available regarding the effects of CBD on additional wake-related neurochemicals such as acetylcholine (ACh). Here, we demonstrate that systemic injections of CBD (0, 5, 10 or 30 mg/kg, i.p.) at the beginning of the lights-on period, increase the extracellular levels of ACh collected from the basal forebrain and measured by microdialysis and HPLC means. Moreover, the time course effects on the contents of ACh were present 5 h post-injection of CBD. Altogether, these data demonstrate that CBD increases ACh levels in a brain region related to wake control. This study is the first to show the effects of ACh levels in CBD-treated rats and suggests that the basal forebrain might be a site of action of CBD for wakefulness modulation.

Keywords Cannabis · Dopamine · Monoamines · Sleep · Wakefulness

Abbreviations

ACh	Acetylcholine
CBD	Cannabidiol
HPLC	High performance liquid chromatography
R^2	Linear regression analysis
Δ^9-THC	Δ^9-Tetrahydrocannabinol

Introduction

Among the 500 molecules present in *Cannabis sativa*, cannabidiol (CBD) is one of the most abundant along with Δ^9-tetrahydrocannabinol (Δ^9-THC), but unlike the latter, CBD displays no psychotropic activity [1–3]. Currently, CBD generates considerable attention due to its antiepileptic,

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anxiolytic, and antipsychotic properties, just to mention a few [4–10]. As one can assume, the interest of describing the mechanism of action of CBD has been addressed in multiple experimental paradigms aimed to understand the molecular targets engaged in CBD's therapeutical actions. Along this line, it has been suggested that CBD may be acting as an antagonist against CB_1 cannabinoid receptor [11] whereas others have suggested that CBD behaves as an agonist either to 5-HT_{1A} receptors [12] or to the transient receptor potential cation channel subfamily V member 1 [13]. In addition, current evidence has suggested that CBD blocks adenosine uptake [14] and antagonist the putative cannabinoid receptor G Protein-coupled Receptor 55 [15].

Whatever its exact mechanisms of action, several reports have shown that CBD exerts actions in neurobiological functions such as learning and memory [16, 17], pain perception [18, 19], and sleep [20–23]. In this regard, our laboratory reported that administrations of CBD in rats during the lights-on period increased wakefulness, but decreased sleep [20–22]. Remarkably, the effects of CBD on sleep has been found after systemic or central injections, as well as posterior to intracerebral perfusion [20–22, 24]. Moreover, CBD also caused a significant enhancement of the extracellular levels of dopamine (DA) collected from nucleus accumbens [20–22]. Altogether, the data suggest that CBD induces alertness by increasing contents of DA. However, later reports have indicated that CBD also modifies the levels of other wake-related compounds, such as adenosine [24] as well as monoamines [25, 26]. Thus, while it seems that CBD exerts effects on adenosine and monoamines contents, it is not clear whether this cannabinoid might also affect an additional neurochemical related to wakefulness, such as acetylcholine (ACh). Data regarding the role of CBD on ACh modulation are limited [27–29]. To determinate whether systemic administrations of CBD would enhance the extracellular contents of ACh, we first injected this phytocannabinoid (0, 5, 10 or 30 mg/kg, i.p.) at the beginning of the lights-on period of animals. Then, we analyzed the dialysates collected from the basal forebrain, a wake-related nuclei that contains cholinergic neurons [30–32], by measuring the extracellular contents ACh using HPLC means. Finally, we addressed the relationship between CBD dosage and ACh contents as well as the likely prediction of the enhancements on ACh levels caused by administration of different doses of CBD.

Materials and Methods

Animals

Male Wistar rats (n = 20; 250–300 g) were singly housed in polycarbonate cages under controlled humidity (60 ± 10%), constant temperature (21 ± 1 °C) and light–dark cycle (lights-on from 07:00–19:00 h). All rats had free access to Purina Rat Chow (Purina. México) as well as tap water. The whole experimental protocols were approved by the Research and Ethics Committee of our Institutions fulfilling the domestic and International Standards of Animal Welfare observed in the Mexican Standards Related to Use and Management of Laboratory Animals (DOF. NOM-062-Z00-1999) as well as the National Institutes of Health (NIH Publication No. 80-23, revised 1996). For ethical reasons, efforts to minimize animal suffering were considered during the whole experiment and a reduced number of animals were included in the current report.

Chemicals

CBD was provided by Prof. Raphael Mechoulam (Hebrew University of Jerusalem. Israel) and compound was prepared in vehicle (polyethylene glycol/saline; 5:95, v/v) as previously reported [20, 21, 25, 26]. All reagents, chemicals, and materials were purchased from Sigma-Aldrich (St Louis, MO, USA).

Microdialysis Surgeries

In each animal, a guide-cannula (IC guide; BioAnalytical Systems [BAS], West Lafayette, IN, USA) was placed stereotaxically into the basal forebrain (target coordinates: A = − 0.35; L = − 2.0; H = − 7.5 mm. [33]). The guide-cannula was fixed onto the skull with dental cement. Right after the microdialysis surgery, rats were placed individually into the microdialysis bowl (Raturn Microdialysis Stand-Alone System, MD-1404, BAS, West Lafayette, IN, USA) for recovery as well as habituation for the experimental conditions (7 days). On the day of surgery and 24 h post-surgery rats were given injections of an antibiotic (amikacin, 250 mg/mL, 0.1 mL, i.p.) and an analgesic (buprenorphine, 0.01 mg/kg/mL, i.p.). All surgical procedures of microdialysis probes were accomplished as previously reported [26].

Microdialysis Sampling Procedures

Once reaching the recovery and habituation time, animals were removed from microdialysis bowls and the stylet from guide-cannula was withdrawn. Next, the microdialysis probe (1 mm of length; polyacrylonitrile, MWCO = 30,000 Da; 340 µm OD; BAS, West Lafayette, IN, USA) was inserted at 07:00 h. Later, artificial cerebrospinal fluid (aCSF containing the following [in mM]: KCl (2.4), Na_2SO_4 (0.5), NaCl (126.5), $CaCl_2$ (1.2), $NaHCO_3$ (27.5), KH_2PO_4 (0.5), $MgCl_2$ (0.8), dextrose (5.0) and pH [6.8 ± 0.1]) was perfused through a minitube (0.65 mm OD × 0.12 mm ID; BAS. West

Lafayette, IN, USA) attached to a 2.5 mL syringe (BAS, West Lafayette, IN, USA) with a pump (flow rate: 2 µL/min; BAS Bee, West Lafayette, IN, USA). Before sampling time, microdialysis membrane was stabilized during 24 h as suggested from previous reports [34].

Pharmacological Challenge

We and others have previously reported that treatment with CBD modifies wake-related neurochemical levels [24, 26, 35, 36]. However, there is no data available regarding whether CBD administration might exert effects in additional wake-related neurochemicals, such as ACh. Moreover, since several papers have suggested that CBD behaves as a wake-promoting compound [20–22, 25, 26], we administered this phytocannabinoid at the beginning of the lights-on period (active phase of rodents). Furthermore, to avoid circadian influences in effects of CBD on contents of ACh, the injections were applied 1 h after the start of the lights-on period (08:00 h). Therefore, 7 days post-surgery and 24 h after finishing the stabilization period of microdialysis probes, rats received at 08:00 h either of the following treatments: vehicle (control group; n = 5) or CBD (5, 10 or 30 mg/kg, i.p [n = 5 each dose]). Right after the experimental challenges were applied, rats were reattached to the microdialysis system, and samples were collected every 20 min at the beginning of each hour across 4 h. Later, all samples were stored (− 80 °C) for further analysis. The microdialysis sampling procedure was developed as previously reported [24–26].

Neurochemical Analysis of ACh

Once dialysates were collected, they were automatically injected (SIL-20A HT Prominence HPLC, Shimadzu, Japan) into high performance liquid chromatography (HPLC; Modular Prominence, Shimadzu, Japan) for ACh measurement with electrochemical detection. In addition, 100 nM neostigmine bromide was added to the aCSF to facilitate reliable detection of ACh contents. Analysis of this neurotransmitter included the following procedure: Using HPLC and acetylcholine-choline assay kit (MF-8910; BAS, West Lafayette, IN, USA), ACh was separated at a flow rate of 1 mL/min, with temperature of 28 °C (oven CTO-20A, Shimadzu, Japan), on 10 cm analytical column (MF-6150, BAS, West Lafayette, IN, USA) by using a mobile phase (Na_2HPO_4 [35 nM], EDTA [0.1 nM], and 0.005% ProClin 150 preservative. BAS. West Lafayette, IN, USA), and adjusted to pH 8.5 with phosphoric acid. By electrochemical means (LC-4C; BAS, West Lafayette, IN, USA) and maintaining the potential of + 0.5 V, ACh was detected in dialysates. Chromatographic data were quantified through comparison with external known standard concentrations using chromatograph report software (LC Solution, Shimadzu, Japan). Total values of ACh contents consisted in samples summed from 6 h continuously. Finally, the hourly extracellular contents of ACh consisted in the separate analysis of dialysates collected every hour across 6 h. The analytical procedure to determinate ACh was developed as previously reported [37–40].

Histological Verification of Probe Location

Microdialysis probe localization was confirmed in all rats after experiments, by perfusing them via intracardiac perfusion. The brain was removed and post-fixed overnight in formaldehyde (4%) followed by sucrose immersion (10, 20 or 30% sucrose/0.1 M PBS for 24 h each concentration). Later, brains were cut in coronal sections (20 µm) using a Portable Bench-top Cryostat (Leica CM1100, Germany) and collected in 1:5 serial order. One serial was used for probe location and it was identified by plotting using rat brain atlas [33]. All histological procedures were developed as previously reported [34].

Statistical Analysis

Chromatographic data were represented as mean ± standard error of the mean. Statistical differences among the experimental groups were determined by one-way ANOVA followed by Scheffé's post hoc test for multiple comparisons. All statistical analyses were performed using the StatView (version 5.0.0, SAS Institute, USA) and statistical differences among groups were determined if $P < 0.05$. For investigating the relationship between CBD doses and ACh contents, Pearson's correlation coefficient (r) was used (StatView; version 5.0.0, SAS Institute, USA). Strength of association between these variables was stablished if $r \geq 0.6$ and $P < 0.05$. In addition, linear regression analysis (R^2) was used to test if the doses of CBD significantly would predict enhancements in ACh levels. Significant statistical values for R^2 were determined within the range of 0–1 and $P < 0.05$.

Results

Effects of CBD Injection on the Levels of ACh Determined by Microdialysis and HPLC Means

As expected, CBD-treated rats exhibited a dose-dependent enhancement in extracellular levels of ACh collected from basal forebrain and determined by HPLC means. We found a significant increase in total value of contents of ACh across 6 h after the systemic injections of different doses of CBD (5, 10, 30 mg/kg, i.p.; Fig. 1a, $F_{(3,16)} = 1220.710$, $P < 0.0001$). The post hoc statistical analysis among the experimental

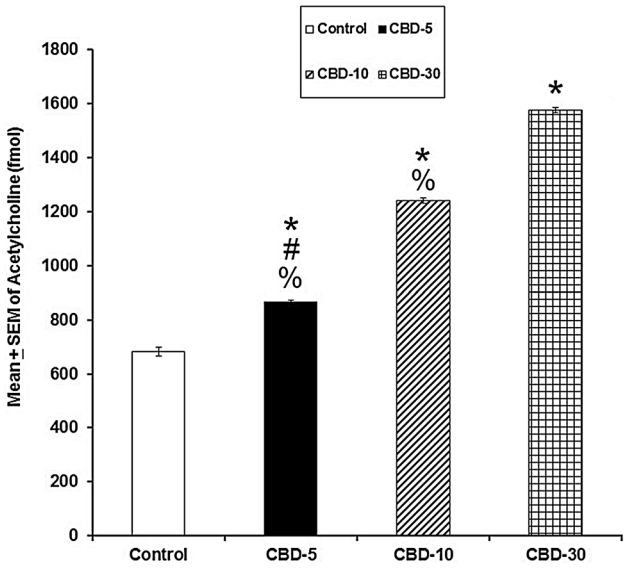

Fig. 1 Extracellular levels of acetylcholine (ACh) collected from basal forebrain in CBD-treated rats during 6 h of the lights-on period. The graph represents the total value of ACh concentration (mean ± SEM) from rats that received either vehicle or CBD (5, 10 or 30 mg/kg, i.p.; * vs. control, # vs. CBD-10, % vs. CBD-30, $P < 0.0001$)

groups showed significant differences (Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30, $P < 0.0001$; CBD-5 vs. CBD-10 and CBD-30, $P < 0.0001$; CBD-10 vs. CBD-30, $P < 0.0001$).

Effects Hour by Hour of CBD Administration on the Contents of ACh Determined by Microdialysis and HPLC Means

The hourly effects of injection of CBD in ACh levels are shown in Fig. 2. The systemic administrations of the phytocannabinoid caused a dose-dependent effect that was observed 1 h after the injection and persisted across 4 h. The lower dose of CBD (5 mg/kg) increased ACh levels in the first hour after administration and the effects last 3 h post-injection whereas the highest dose (30 mg/kg) enhanced ACh contents in the first hour post-administration of CBD and the effects remained until the fifth hour [first hour (A): $F_{(3,16)} = 0.289$, $P > 0.01$; second hour (B): $F_{(3,16)} = 3149.994$, $P < 0.0001$, Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30 ($P < 0.0001$); CBD-5 vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); third hour (C): $F_{(3,16)} = 614.44$, $P < 0.0001$, Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30 ($P < 0.0001$); CBD-5 vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); fourth hour (D): $F_{(3,16)} = 875.835$, $P < 0.0001$, Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30 ($P < 0.0001$); CBD-5 vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); fifth hour (E): $F_{(3,16)} = 217.914$, $P < 0.0001$, Scheffé's test: control vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); sixth hour (F): $F_{(3,16)} = 0.871$, $P > 0.01$)].

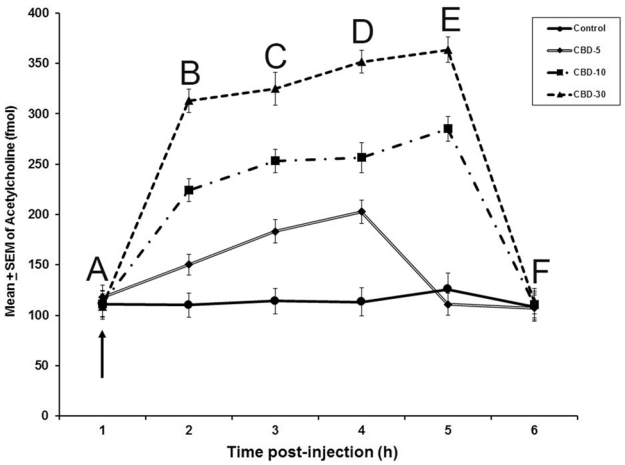

Fig. 2 Hourly effects of ACh levels (mean ± SEM) collected from basal forebrain during the lights-on period in CBD-treated rats. Samples were collected hour by hour during 6 h using microdialysis means and measured via HPLC procedures [first hour (*A*): $F_{(3,16)} = 0.289$, $P > 0.01$; second hour (*B*): $F_{(3,16)} = 3149.994$, $P < 0.0001$, Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30 ($P < 0.0001$); CBD-5 vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); third hour (*C*): $F_{(3,16)} = 614.44$, $P < 0.0001$, Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30 ($P < 0.0001$); CBD-5 vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); fourth hour (*D*): $F_{(3,16)} = 875.835$, $P < 0.0001$, Scheffé's test: control vs. CBD-5, CBD-10 and CBD-30 ($P < 0.0001$); CBD-5 vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); fifth hour (*E*): $F_{(3,16)} = 217.914$, $P < 0.0001$, Scheffé's test: control vs. CBD-10 and CBD-30 ($P < 0.0001$); CBD-10 vs. CBD-30 ($P < 0.0001$); sixth hour (*F*): $F_{(3,16)} = 0.871$, $P < 0.01$)]. The arrow represents the time of treatments. As noted, CBD induced a dose-dependent enhancement of ACh contents in 4 h

Correlation Between Doses Injected of CBD and the Levels of ACh

The Pearson's correlation coefficient analysis showed a significant and positive relationship between CBD doses administered (0, 5, 10 or 30 mg/kg) and ACh contents (Fig. 3; $r = 0.94$, $P < 0.05$). Data suggest that significant interactions between CBD doses and ACh levels were present. Then, we conclude that higher doses of CBD are linked with enhancements in extracellular contents of ACh as showed.

Injection of CBD Predicts the Levels of ACh

Finally, the linear regression analysis was used to test if doses of CBD (0, 5, 10 or 30 mg/kg) significantly would predict the enhancements on ACh levels. As expected, CBD injected at different doses predicted the increases in

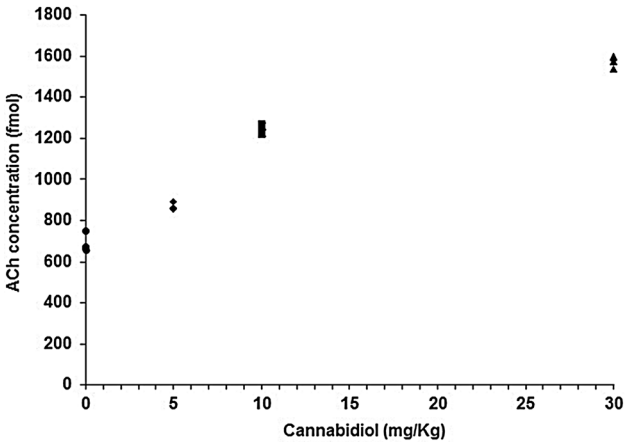

Fig. 3 The Pearson's correlation coefficient (r) between CBD doses administered (0, 5, 10 or 30 mg/kg) and changes in ACh contents collected across 6 h of the lights-on period. As noted, strength of association between these variables was statistically significant ($r \geq 0.94$, $P < 0.05$)

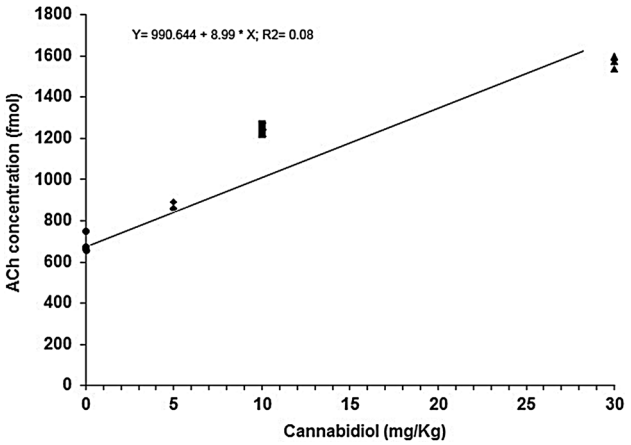

Fig. 4 The linear regression analysis used to test if doses of CBD (0, 5, 10 or 30 mg/kg) would predict the increase on ACh levels measured during 6 h of the lights-on period. The graph shows that CBD administered at different dosage predicted the enhancement in extracellular contents of ACh. The statistical analysis allow us to assume that higher doses of CBD promotes higher concentrations of ACh ($R^2 = 0.89$, $P < 0.05$)

extracellular contents of ACh (Fig. 4; $R^2 = 0.89$, $P < 0.05$). Therefore, we conclude that as higher doses of CBD were applied, higher concentrations of ACh were found by HPLC means.

Discussion

Over the last years, an accumulating body of evidence regarding the beneficial properties of CBD in health is available in the literature [4, 41–43]. Due to its promising therapeutic role, several studies describing the effects of CBD on neurochemical function have been published, including the characterization of the influence of this phytocannabinoid on wake-related compounds, such as adenosine [24] as well as monoamines [25, 26]. Although significant discoveries in neurochemical changes after CBD injection have been described, limited evidence is available regarding the relationship between this phytocannabinoid and additional wake-related compounds, such as ACh [27–29]. Here, we demonstrated that CBD-treated rats showed an enhancement on ACh contents collected from the basal forebrain, a brain area linked to wake control [30, 44, 45]. In addition, a significant dose–response increase was found in CBD-treated animals. Furthermore, we found a strong positive correlation between CBD tested doses (0, 5, 10 or 30 mg/kg) and levels of ACh. In addition, linear regression analysis showed prediction of CBD doses on contents of ACh. These last analyses allowed us to assume that higher doses of CBD promoted higher concentrations of ACh. Finally, the tacit findings in our time course effects analysis, showed that ACh contents in CBD-treated rats were higher 1 h post-injection reaching the highest peak of concentration 5 h after administration of compound. We did not observe a steady state, and the effect decreased at the sixth hour post-injection. The immediate effects observed in ACh levels in CBD-treated rats suggest that pharmacokinetic and pharmacodynamic factors could be involved, such as absorption, distribution and transport, among many others. Further studies are needed to fully describe the pharmacokinetic and pharmacodynamics in ACh modulation under CBD influence.

The current report suggests that it is plausible to consider that cholinergic neurons in basal forebrain could be target of CBD. If injection of CBD elevates the levels of ACh from the mentioned brain area, what might the mechanism of action? Despite that we did not develop in the current report a mechanistic experiments, we would like to propose the following hypothesis: CBD might influence activity of the CB_1 cannabinoid receptor placed in cholinergic brain areas (such as basal forebrain) and in turn, enhance ACh levels. This assumption is based on: (a) Despite that CBD does not bind to CB_1/CB_2 cannabinoid receptors [46], recent data have demonstrated that this phytocannabinoid may influence the CB_1/CB_2 cannabinoid receptor neurotransmission [47]; (b) The CB_1 cannabinoid receptor has been mapped in several areas of the central nervous system, including the basal forebrain [48, 49]; (c) The CB_1 cannabinoid receptor is also present in cholinergic neurons [50, 51]; (d) The activation of the CB_1 cannabinoid receptor promotes waking [52, 53].

An alternative explanation for our findings may involve other neurobiological pathways. It is likely that enzymes involved in the synthesis/degradation of ACh could be inhibited or stimulated by CBD as reported for other neurotransmitters [54, 55]. For example, previous studies have shown

that CBD enhances extracellular levels of DA but decreases contents of L-DOPA [20], suggesting that the activity of tyrosine hydroxylase might be under inhibition whereas DOPA decarboxylase could be stimulated by CBD.

Another putative mechanism of action of CBD on ACh levels might engage nicotinic receptors. Recent data have suggested that CBD suppresses the activity of the α7-nicotinic acetylcholine (α7-nACh) receptor [56]. Interestingly, Papouin et al. [57] demonstrated the wakefulness-dependent activity of septal cholinergic fibers through the α7nAChR [57]. It would be worthy to explore the neurobiological role of nicotinic receptors in CBD's effects on ACh levels.

Due to the wide spectrum of action of CBD, additional neurobiological routes should be taken in count. In this regard, it has been demonstrated that CBD exerts influence in sleep–wake cycle modulators placed in basal forebrain, such as adenosine [58, 59]. It is known that adenosine A_2 receptor-mediated component of the inflammatory response is suppressed by action of CBD [60] while adenosine A_1 receptor is activated by this phytocannabinoid [61]. As demonstrated by others, adenosine A_1 and A_2 receptors are involved in the sleep–wake cycle modulation. Whereas the A_2 receptor has been linked with sleep generation, activation of A_1 receptor seems to be responsible of wakefulness promotion [58, 59, 62]. Thus, the alternative hypothesis that CBD might mediate the activity of adenosine receptors, as previously has been suggested by others [63, 64] seems to be plausible as well.

Currently, CBD generates interest due to its medical potential benefits. For instance, CBD behaves as a promising agent for controlling cholinergic-related diseases, such as schizophrenia [5, 35, 65]. Therefore, it is needed the clarification of the mechanism of action of this phytocannabinoid in neurobiological processes that involves ACh activity. Moreover, future studies should extend the current findings to other brain sites of action of CBD and measure additional endogenous compounds such as peptides, hormones, lipids, etc.

Conclusions

This research has provided evidence that systemic injections of CBD (0, 5, 10 or 30 mg/kg, i.p.) enhanced the extracellular contents of ACh measured by microdialysis and HPLC means. The current data strengthen our understanding regarding the effects of CBD in wake-related neurochemicals modulation.

Acknowledgements This work was supported by The University of California Institute for Mexico and the United States (UC MEXUS) and Consejo Nacional de Ciencia y Tecnología (CONACyT; Grant: CN-17-19) and Escuela de Medicina, Universidad Anáhuac Mayab (Grant: PresInvEMR2017) given to E.M-R.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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