Differential effects of cannabis extracts and pure plant cannabinoids on hippocampal neurones and glia

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Abstract

We have shown previously that the plant cannabinoid cannabidiol (CBD) elevates intracellular calcium levels in both cultured hippocampal neurones and glia. Here, we investigated whether the main psychotropic constituent of cannabis, Δ⁹-tetrahydrocannabinol (THC) alone or in combination with other cannabis constituents can cause similar responses, and whether THC affects the responses induced by CBD. Our experiments were performed with 1 μM pure THC (pTHC), with 1 μM pure CBD (pCBD), with a high-THC, low CBD cannabis extract (eTHC), with a high-CBD, low THC cannabis extract (eCBD), with a mixture of eTHC and eCBD (THC:CBD = 1:1) or with corresponding ‘mock extracts’ that contained only pTHC and pCBD mixed in the same proportion as in eTHC, eCBD or the 1:1 mixture of eTHC and eCBD.

We detected significant differences in neurones both between the effects of pTHC and eTHC and between the effects of pCBD and eCBD. There were also differences between the Ca²⁺ responses evoked in both neurones and glia by eTHC and mock eTHC, but not between eCBD and mock eCBD. A particularly striking observation was the much increased response size and maximal responder rates induced by the mixture of eTHC and eCBD than by the corresponding 1:1 mixture of pTHC and pCBD.

Our data suggest that THC shares the ability of CBD to elevate Ca²⁺ levels in neurones and glia, that THC and CBD interact synergistically and that the cannabis extracts have other constituents yet to be identified that can significantly modulate the ability of THC and CBD to raise Ca²⁺ levels.

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The major psychoactive component found in the marijuana plant (Cannabis sativa), Δ⁹-tetrahydrocannabinol (THC), is most renowned for its psychotropic effects, mediated via the body’s endogenous cannabinoid (endocannabinoid) system. This comprises at least two cannabinoid receptors (CB₁ and CB₂) [21,11], with increasing evidence for the existence of others [23,20]. CB₁ receptors are located, in the main, in the central nervous system (CNS) and assumed to be responsible for THC’s psychotropic actions (e.g. [26]). Distribution of the CB₁ receptor throughout the brain is not uniform, and most dense in areas such as the hippocampus, basal ganglia and cerebral cortex [10]. In contrast, CB₂ receptors were once thought to be found peripherally in cells of the immune system only [18]. However, expression of this receptor has now been shown in activated microglia [33,15,19], suggesting a possible involvement in CNS disease states. Moreover, recent evidence suggests neuronal CB₂ receptors in brainstem and cerebellum [29].

Caution regarding THC’s medicinal potential lies in its psychoactive nature, since high concentrations may acutely have dramatic behavioural side effects (e.g. learning and memory disturbance [24]), and cause precipitation of mental disorders after long-term use [9]. Thus, some of the non-psychotropic cannabinoids have been suggested for therapeutic considerations, and when applied in combination may be able to counteract some of the unwanted side-effects of CB₁ acting compounds [14,19]. The non-psychoactive phytocannabinoid, cannabidiol (CBD), has a weak affinity for CB₁ receptors and does not act as an agonist at this receptor, but displays a number of therapeutically useful characteristics, e.g. anti-inflammatory effects [7,4], neuroprotective effects [28] and anticonvulsant effects [32] (for review, see [20,22]). However, the precise cellular mechanisms of CBD’s actions, its interactions with THC, and the details of the involvement of the endocannabinoid system have yet to be fully demonstrated.
Recently, a number of clinical trials have been performed on cannabis-based compounds to test the so far mainly anecdotal evidence of their analgesic properties. For example, it was found that the cannabis-based drug Sativex® (GW Pharmaceuticals) was an effective analgesic in patients with central neuropathic pain [1]. This drug is a whole plant extract of Cannabis sativa L. containing both THC and CBD in approximately equal measure (see below). This and other cannabis extracts have undergone further clinical trials, for instance in patients with multiple sclerosis, with the assumption that they may offer additional benefits compared to the pure compounds [25,31,2]. Accordingly, in the present study, a comparison of a THC-rich (Tetranabinex®) and a CBD-rich (Nabidiolex®) extract with their pure counterparts was conducted on cultured hippocampal neurones. We focussed on the observation that pure CBD can modulate intracellular Ca2+ in hippocampal tissue [6], and conducted a comparative study for pure compounds, extracts and 1:1 mixtures of both. Primary hippocampal cultures were prepared from Lister-Hooded rat pups (1–3 days old), euthanised by cervical dislocation, in accordance with Home Office and institute regulations as described previously [6]. Briefly, the brain was quickly removed, the hippocampi dissected out and placed in ice-cold filtered HEPES buffered solution (HBS, composition in mM: NaCl 130; KCl 5.4; CaCl2 1.8; MgCl2 1; HEPES 10; glucose 25, compounds from Sigma, Pool, UK). Hippocampal tissue was treated with 1 mg/ml type X and XIV protease solution (Sigma, 40 min), washed and triturated. Following centrifugation and re-suspension in tissue culture medium (90% minimum essential medium (MEM) (Gibco, Paisley, UK) and 2 mM L-glutamine (Sigma)), cells were plated in culture dishes (Gibco). Cultures were kept in a humidified incubator (37°C; 5% CO2) and allowed to mature for 2 days before replacement of MEM with neurobasal medium (Gibco, Paisley, UK), supplemented with 2% B27, 2 mM L-glutamine (Sigma), and 25 μM L-glutamate. Culture dishes were used for imaging at 5–10 days in vitro.

For calcium imaging experiments, cultures were washed with HBS and loaded with the cell-permeable fluorescent calcium indicator Fura-2-AM (10 μM, Molecular Probes, USA) for 1 h in the dark. The sodium channel blocker tetrodotoxin (TTX, indicator Fura-2-AM (10 μM, Molecular Probes, USA) for 1 h of each)). Cultures were perfused with low Mg2+ HBS (composition in mM: NaCl 130; KCl 5.4; CaCl2 1.8; MgCl2 0.1; HEPES 10; glucose 25, compounds from Sigma, UK), at a rate of 1–2 ml/min, using a gravity perfusion system.

Ratiometric imaging was conducted using two different imaging systems fitted onto Olympus microscopes. The first system utilised a monochromator illumination system (Spectromaster 1, Perkin Elmer), controlled by the Oracal software (version 1.85, Life Sciences Resources Ltd.). The second system was controlled via the Improvision software package Openlab (version 4.03) using a DG-4 illumination system (Sutter Instruments, USA) and Hamamatsu Orca-ER CCD camera. Reproducibility of results was confirmed by testing all cannabinoid compounds on both systems.

During experimentation, a greyscale transmission image was initially captured. The ratio of the fluorescence excited at 380 and 340 nm was plotted over time after background fluorescence subtraction for all regions of interest (ROIs), with frame capture rates set at 5 s.

Pure CBD and THC (stored as 1mg/ml ethanolic stock solution) as well as cannabis extracts were a gift of GW Pharmaceuticals (UK). The 50 mg/ml ethanolic stock solution of the THC-rich extract (Tetranabinex®) contained 72.6% THC and 2.5% CBD, whilst the CBD-rich extract (Nabidiolex®) contained 64.6% CBD and 2.5% THC. The remaining percentage of both extracts contained minor cannabinoids (5–6%), terpenoids (6–7%), sterols (6%), triglycerides, alkanes, squalene, tocopherol, carotenoids and other minor, plant-derived components [27].

In each case, ethanol was evaporated off and the cannabinoids re-suspended in DMSO (at a cannabinoid concentration of 1 mM). The concentration of extracts applied was calculated to yield equimolar concentrations (1 μM) of pure THC (for eTHC) and pure CBD (for eCBD). Mock samples of extracts were also made, using only the ratio of THC to CBD. Thus, for mock-THC-rich extract, pure THC and CBD were added together in a ratio of 29:1:1 (i.e. 1 μM THC:0.034 μM CBD, as in Tetranabinex®) and for the mock CBD-rich extract, a ratio of CBD to THC of 25:9:1 (i.e. 1 μM CBD: 0.039 μM THC, as in Nabidiolex®). In addition to this, a 1:1 mixture of both pure THC and pure CBD was used to test if combining the two cannabinoids would yield additive effects. This contained 1 μM of both compounds (i.e. a 1:1 ratio). Similarly, a 1:1 ratio of the two genuine extract compounds was tested (again, containing ~1 μM of both THC and CBD extracts).

The following abbreviations are used throughout this manuscript: pCBD = pure CBD, eCBD = CBD-rich extract, M-eCBD = mock CBD-rich extract, pTHC = pure THC, eTHC = THC-rich extract, M-eTHC = mock THC-rich extract, pure mix (1:1) = pCBD + pTHC (1 μM of each) & extract mix (1:1) = eCBD + eTHC (1 μM of each).

N-Methyl-d-aspartate (NMDA, 50 μM with 100 μM glycine; stock: 10 mM in double-distilled water) was applied in every experiment to distinguish between neuronal and glial cells (for sample, see Fig. 1).

For data analysis, fluorescent units were converted into %ΔF/F, with F defined as an average of five baseline values before drug application. All experiments were performed a minimum of three times on different cultures. Data were exported to Excel and statistical analysis performed using Prism (version 4). As in previous studies, normality tests confirmed absence of normal distribution of data. Therefore, a Kruskal-Wallis test with a Dunn’s post test (multiple drug groups vs. control) was used for multiple group comparisons. Percentage responders were calculated based on morphological criteria and NMDA responses, as previously described [6]. Significance was set at P < 0.05 = significant; P < 0.01 = highly significant; P < 0.001 = very highly significant.

As in our previous study [6], acute application of CBD, but also of all other cannabinoid compounds tested, evoked Ca2+ responses in both neurones (Fig. 1) and glia (Fig. 2).
Fig. 1. Neuronal responses in hippocampal cultures to acute applications of cannabinoid compounds (1 μM). (A) Sample time course depicting the acute application of the CBD-rich extract (eCBD). NMDA (50 μM) was applied at the end of each experiment to distinguish between neurones and glia. (B) Summary of neuronal responses for all CB applications, selected statistical differences are given in the figure. For a full comparison and abbreviations, see Table 1. *P < 0.05, **P < 0.01 and ***P < 0.001.

In neurones, despite their established differences in pharmacological profiles, the Ca²⁺ response to pTHC (58 ± 8% ΔF/F, n = 49) was not found to be significantly different from the pCBD response (45 ± 4% ΔF/F, n = 73), with almost identical numbers of cells responding (48% and 51%, respectively). A comparison between responses to pTHC and eTHC revealed significantly lower response sizes (28 ± 3% ΔF/F, n = 34, P < 0.001) with a reduced rate of responders (36%) for eTHC. This pattern was also observed when comparing responses of eCBD to pCBD (eCBD: 25 ± 5% ΔF/F, n = 28, 35% of neurones responded) thus indicating that both extracts differ from their pure counterparts, with the latter inducing larger responses.

The differences between responses to the pure cannabinoids and their extracts led to the consideration that this could be due solely to the ratio of CBD:THC in the extracts. Thus, ‘mock’ extracts were tested which did not contain any additional phytocannabinoids, save THC and CBD. Interestingly, the M-eCBD response (36 ± 9% ΔF/F, n = 10) was not significantly different from the eCBD response, with similar numbers of neurones responding (31%). However, significant differences between M-eTHC and eTHC were found, with M-eTHC causing a markedly greater increase in [Ca²⁺], (134 ± 23% ΔF/F, n = 17) than eTHC (P < 0.001), though the numbers of neurones responding (52%) was again relatively unchanged.

Interestingly, glia cells also responded to all cannabinoid compounds applied. There were again no significant differences between responses to pTHC (65% ± 13% ΔF/F, n = 27) and pCBD (43 ± 3% ΔF/F, n = 75); however, a greater number of glia responded to pCBD than pTHC (77% vs. 48%). In contrast to neurones, there was no difference between glial responses to pCBD and eCBD or between pTHC and eTHC though a marked difference in the number of cells responding was observed, with 100% of glia responding to eCBD and only a small number of cells (20%) responding to eTHC (Fig. 2). The glial response to M-eCBD vs. eCBD was also identical, though maximal responder rates were seen for both extracts. The M-eTHC increased [Ca²⁺] more prominently compared to eTHC (P < 0.01; 170% ± 31% ΔF/F; n = 17), with responder rates (55%) resembling that for pTHC.

When responses to cannabinoids were compared between neurones and glia, no evidence for cell-type specificity was indicated (all P’s > 0.05), though responder rates tended to be higher in glia for all CBD-based compounds.

To further elaborate on the comparison between pure mixtures and extracts containing additional cannabinoids, a 1:1
mixture of pure compounds was compared with a 1:1 mixture of eCBD and eTHC (~1 μM of each), as this is identical to the ratio of the two extracts used in the drug Sativex®. This extract mixture caused a large, super-additive increase in [Ca^{2+}]i when applied acutely in neurones (314% ± 21% ∆F/F; n = 31, Fig. 1) and also glia (152% ± 20% ∆F/F; n = 28, Fig. 2) with maximal (100% neurones and 93% glia) response rates. The 1:1 mixture containing pCBD and pTHC led to a [Ca^{2+}]i with maximal (100% neurones and 93% glia) response rates.

<table>
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<th>pCBD</th>
<th>cCBD</th>
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<th>pTHC</th>
<th>eTHC</th>
<th>M-eTHC</th>
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Overall comparison between CB-induced (1 μM) Ca^{2+} responses in cultured hippocampal neurones and glia. Indicated are differences between compounds for both neurones (N) and glia (G): *P < 0.05, **P < 0.01, ***P < 0.001, NS = not statistically significant. pCBD = pure CBD, eCBD = CBD-rich extract, M-eCBD = mock CBD-rich extract, pTHC = pure THC, eTHC = THC-rich extract, M-eTHC = mock THC-rich extract, pure mix (1:1) = pCBD + pTHC (1 μM of each) & extract mix (1:1) = eCBD + eTHC (1 μM of each).

Table 1

Comparison of CB-induced Ca^{2+} responses

Though both neurones and glia may potentially express CB1 receptors [3], we have recently shown that CBD-induced responses are not CB1-mediated [6], as indicated by the lack of inhibition with AM281 and in agreement with its low affinity for CB1 receptors. Further studies will have to be conducted to establish whether there is a contribution of known CB/vanilloid receptors to the responses observed here. Alternatively, non-receptor mediated processes (e.g. interaction with membrane fluidity or anti-oxidant related activities [17,16]), or a direct action on intracellular targets relevant to Ca^{2+} regulation, as suggested by recent studies by ourselves and others [12,6,5], should be considered. It can however be proposed that THC and CBD are likely to raise [Ca^{2+}]i via different but synergistic mechanisms, despite the similarities in responses: when applied together (1:1 pure mixture) response were super-additive in terms of the actual response size, and responder rates were also dramatically enhanced, indicative of the activation of additional cell types. Modulation between THC and CBD has been shown previously [13,14,34], although in these studies CBD was found to reduce THC’s action. Recently, some evidence has however been presented that indicates a potentiation of THC’s anti-nociceptive effects by high concentrations of CBD [30].

The most important results from the current study come from the comparison between pure THC and CBD vs. THC- and CBD-containing extracts currently used or considered for medicinal purposes. Significant differences between the genuine extracts and their main pure components were seen for both THC and CBD, but the genuine extract differed from the ‘mock’ counterpart only for THC, while eCBD’s action was similar to the mock eCBD. Moreover, while eTHC was less potent than pTHC, the mock eTHC caused a stronger response. Strikingly, even though the extracts applied alone were less potent than pure THC and CBD, the combination of the two extracts (1:1 mix) led to a massive response enhancement. It is therefore proposed that pTHC and pCBD are acting synergistically (as indicated by enhanced responses seen for the mock extracts and the 1:1 pure...
CBD:THC mix), an action that is counteracted by compounds present in the extract at low concentrations (see extract vs. pure responses), but facilitated at higher concentrations, i.e. when the extract is combined at a 1:1 ratio (see Table 1).

Differences between pure cannabinoids and their extracts were not determined by the ratio of CBD:THC, providing strong evidence for complex interactions with other compound(s) present in these extracts. The differences between the extract and the mock extracts, and even more so between the pure and extract mix clearly indicate that additional compounds are present in extracts that can strongly influence the behaviour of the principal phytocannabinoids. The super-additivity of the response size for the extract mix alongside maximal responder rates indicates that separate subsets of cells were responding to specific cannabinoid combinations. Recently, evidence has been provided that the presence of some of the lesser-known compounds in cannabinoid extracts can induce effects independent of the main phytocannabinoids [35,36]. This is however in contrast to some recent in vivo work that suggested THC- and CBD-rich extracts had similar effects on learning and memory.

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References


