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RESEARCH PAPER

Neural correlates of interactions between cannabidiol and Δ⁹-tetrahydrocannabinol in mice: implications for medical cannabis

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BACKGROUND AND PURPOSE

It has been proposed that medicinal strains of cannabis and therapeutic preparations would be safer with a more balanced concentration ratio of Δ⁹-tetrahydrocannabinol (THC) to cannabidiol (CBD), as CBD reduces the adverse psychotropic effects of THC. However, our understanding of CBD and THC interactions is limited and the brain circuitry mediating interactions between CBD and THC are unknown. The aim of this study was to investigate whether CBD modulated the functional effects and c-Fos expression induced by THC, using a 1:1 dose ratio that approximates therapeutic strains of cannabis and nabiximols.

EXPERIMENTAL APPROACH

Male C57BL/6 mice were treated with vehicle, CBD, THC or a combination of CBD and THC (10 mg·kg^{-1} i.p. for both cannabinoids) to examine effects on locomotor activity, anxiety-related behaviour, body temperature and brain c-Fos expression (a marker of neuronal activation).

KEY RESULTS

CBD potentiated THC-induced locomotor suppression but reduced the hypothermic and anxiogenic effects of THC. CBD alone had no effect on these measures. THC increased brain activation as measured by c-Fos expression in 11 of the 35 brain regions studied. CBD co-administration suppressed THC-induced c-Fos expression in six of these brain regions. This effect was most pronounced in the medial preoptic nucleus and lateral periaqueductal gray. Treatment with CBD alone diminished c-Fos expression only in the central nucleus of the amygdala compared with vehicle.

CONCLUSIONS AND IMPLICATIONS

These data confirm that CBD modulated the pharmacological actions of THC and provide new information regarding brain regions involved in the interaction between CBD and THC.

Abbreviations

BNST, bed nucleus of the stria terminalis; CBD, cannabidiol; PAG, periaqueductal gray; PVH, paraventricular hypothalamic nucleus; THC, Δ⁹-tetrahydrocannabinol

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[c]
CB1 receptors	FAAH
Ion Channels[b]	**Transporters**[d]
TRPV1 channels	ABC transporters

LIGANDS
CBD, cannabidiol
THC, Δ^9-tetrahydrocannabinol

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a b c d]Alexander *et al.*, 2013a, b, c, d).

Introduction

Cannabis is a complex mixture of approximately 100 different cannabinoids that may modulate the effects of Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of the plant (Elsohly and Slade, 2005; Radwan *et al.*, 2008). A number of studies now support the view that cannabidiol (CBD) may reduce the negative psychotropic effects of THC while enhancing its positive therapeutic actions (Russo, 2011; Niesink and van Laar, 2013). In naturalistic human studies where cannabinoid content in smoked material or the user's hair is compared with the subjective effects of cannabis, it has been inferred that CBD attenuates some effects of THC, such as memory impairment, attentional bias to drug-related stimuli, appetite stimulation, anxiety and psychotic-like states (Morgan and Curran, 2008; Niesink and van Laar, 2013). Controlled human laboratory studies administering known doses of CBD and THC largely agree with the results of the naturalistic studies, with CBD decreasing the psychoactive and physiological effects of THC. Specifically, CBD was shown to reduce the effects of THC on anxiety (Zuardi *et al.*, 1982), hippocampus-dependent episodic memory, psychotic-like symptoms (Englund *et al.*, 2013) and emotional processing (Hindocha *et al.*, 2014).

These findings have major therapeutic and public health implications. For recreational use, they suggest that the widespread consumption of cannabis strains high in THC but low in CBD may endanger users by shifting the balance toward the more detrimental psychotropic effects of THC (Swift *et al.*, 2013). Breeding CBD back into the plant may therefore be a wise public health strategy in locations where recreational cannabis is now legal. Further, for medicinal cannabis, this opens the possibility of utilizing plant strains with balanced CBD to THC concentrations that maximize therapeutic endpoints while minimizing side effects. The near 1:1 ratio of CBD and THC in nabiximols is thought to explain this preparation's favourable therapeutic and side-effect profile (Robson, 2014; Allsop *et al.*, 2014a). Likewise, companies and regulatory bodies such as the Office of Medicinal Cannabis in the Netherlands have made available cannabis strains that contain near equal amounts of CBD and THC. It is for this reason that the current study will focus on the interactive effects of CBD and THC in a 1:1 ratio.

Animal studies provide better experimental control in advancing our understanding of any neuropharmacological interactions between CBD and THC. These studies have revealed great complexity in the nature of these interactions with factors such as dose, the dose ratio of CBD to THC and the interval between CBD and THC injection, all influencing the experimental outcome (Zuardi *et al.*, 2012). While animal studies reproduce the effects observed in human research, where CBD inhibited the actions of THC, in many instances CBD also potentiated the effects of THC (see Arnold *et al.* (2012)). The mechanism of interaction between CBD and THC requires clarification, and no human or animal studies have addressed the question of which brain circuits are activated during such an interaction. In rodents, the expression of the transcriptional factor c-Fos is used as a marker of neuronal activation and is reliably induced by exposure to THC (McGregor *et al.*, 1998; Boucher *et al.*, 2007). Therefore, the aims of this study were to investigate in mice whether CBD was able to modulate the acute behavioural and physiological effects of THC, at a 1:1 THC/CBD dose ratio, and to observe whether CBD affected the characteristic brain activation pattern promoted by THC using the well-validated marker c-Fos.

Methods

Animals

All animal care and experimental procedures complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by University of Sydney's Animal Ethics Committee. This study was reported in accordance with ARRIVE guidelines for experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 51 animals were used in the experiments described here.

Male C57BL/6 wild-type mice were provided by the Animal Resources Centre (Perth, Australia) and kept in the animal facility of the Brain and Mind Centre (Sydney, Australia). The animals were housed in standard cages of six under a reversed 12h light : dark cycle with food and water freely available. Mice were provided with an enriched environment including mouse house igloo, tissue bedding, paper roll, climbing ring and sunflower seeds. Mice aged 14 weeks (N = 24, wt 24–30 g) were used for behavioural tests and immunohistochemistry, while mice aged 12 weeks were used for body temperature measurements (N = 27, wt 26–32 g).

Experimental procedures

All drugs were administered i.p. in a volume of 10 mL·kg^{-1}, at a dose of 10 mg·kg^{-1} (1 mg·mL^{-1} final drug concentration). A

dose of 10 mg·kg^{-1} THC was chosen as we have previously shown it to be a threshold dose for the induction of hypothermia, locomotor suppression and anxiety-related behaviour in C57BL/6 mice (Long *et al.*, 2010). Moreover, it is a dose that reliably induces significant c-Fos expression (Boucher *et al.*, 2007).

Animals were injected with vehicle, CBD, THC or THC/CBD in combination (n = 6 per group, N = 24) from 9 AM to 2 PM. Thirty minutes after injection, mice were tested for locomotor activity in the open field for 45 min. Animals were habituated to the open field for 3 min the day prior to experiment. Locomotor activity was measured in red plexiglass open-field chambers (43 cm × 43 cm × 25 cm) with the horizontal distance travelled (m) recorded with video tracking software (Motion Mensura, Cooks Hill, NSW, Australia) (Spencer *et al.*, 2012). Data from the first 10 min were used for the analysis of anxiety-related behaviour. Anxiety was additionally measured in this test using a distance ratio measure, as rodents tend to avoid exploration of the central zone of the open-field test in favour of peripheral areas. Distance travelled in the centre inner 50% of the arena compared with the distance travelled in the total area was calculated into a distance ratio percentage, with a lower distance ratio percentage indicating higher anxiety (Denenberg, 1969; Long *et al.*, 2010). The comparative distance, rather than time, travelled in these regions was used in order to distinguish from any THC-induced locomotor suppression. The open-field data of three animals for locomotion were excluded due to computer recording failure (one each for vehicle, CBD and THC/CBD groups for locomotion).

A separate cohort was used to measure rectal body temperature (n = 7 for vehicle, CBD and THC groups; n = 6 for the THC/CBD group; N = 27) with injections occurring between 9 and 11 AM. Following injection with vehicle, CBD, THC or the THC/CBD mixture, body temperature was measured 30 min, 90 min and 6 h after cannabinoid administration. Body temperature was measured using a mouse rectal temperature probe (ADInstruments, Sydney, Australia) attached to a thermocouple as previously described (Boucher *et al.*, 2011). Temperature readings were excluded from analysis if output was below 28°C as evidence of probe error (one from vehicle at 90 min; one from THC/CBD at 6 h).

Immunohistochemistry

c-Fos immunohistochemistry was used to assess changes in neuronal activity following acute cannabinoid exposure in mice that underwent the open-field test (N = 24). A detailed overview of the Fos immunohistochemistry protocol can be found in Boucher *et al.* (2007). Briefly, 2 h following injection, mice were euthanized with isoflurane and then transcardially perfused with 4% paraformaldehyde. This time of 2 h after the THC injection coincides with the period of maximal THC-induced c-Fos expression (Miyamoto *et al.*, 1996). Following 24 h post-fixing, the extracted brains were preserved in a sucrose and phosphate buffer solution, first at 15% concentration over 24 h and then 30% over 72 h. Brains were sliced using a cryostat at 40 μm and incubated with c-Fos antibody (1:10 000, Santa Cruz Biotechnology, Santa Cruz, USA) for 72 h. After 1 h incubation with a biotinylated anti-rabbit IgG secondary antibody (1:500, Vector Laboratories, Burlingame, USA), a peroxidase reaction was visualized with 10 min incubation with diaminobenzidine and glucose oxidase (all Sigma, Australia). Brain slices were mounted on gelatinized glass slides before they were dehydrated with ethanol, cleared with xylene and coverslipped.

Cell quantification

An observer, unaware of the treatment conditions, quantified cells with black or brown staining under a light microscope at 20× magnification according to the mouse brain atlas of Paxinos and Keith (2001). A 0.5 mm square graticule was positioned over each structure and counted by eye for absolute counts. The following brain regions were analyzed: the cingulate and prelimbic cortices at plate 14 (Bregma 1.98 mm); the infralimbic cortex at plate 16 (Bregma 1.78 mm); the lateral septum (ventral and dorsal), nucleus accumbens shell and core, piriform cortex, caudate putamen (central, dorsal and dorsomedial) and anterior cingulate cortex at plate 23 (Bregma 0.98 mm); the bed nucleus of the stria terminalis (BNST), medial preoptic nucleus and preoptic area (lateral and medial) at plate 30 (Bregma 0.14 mm); the anterior paraventricular thalamic nucleus at plate 35 (Bregma −0.46 mm); the paraventricular thalamic nucleus and paraventricular hypothalamic nucleus (PVH) at plate 39 (Bregma −0.94 mm); the medial amygdala (posteroventral and posterodorsal), basolateral amygdala, central nucleus of the amygdala and the lateral, dorsomedial and ventromedial hypothalamus at plate 44 (Bregma −1.58 mm); the CA1 and CA3 regions of the hippocampus and the dentate gyrus at plate 45 (Bregma −1.70 mm); the ventral tegmental area at plate 59 (Bregma −3.40 mm); the Edinger–Westphal nucleus at plate 62 (Bregma −3.80 mm); and the periaqueductal gray (PAG) (dorsomedial, dorsolateral, lateral and ventrolateral) at plate 69 (Bregma −4.60 mm). For a diagrammatic representation of the quantification locations used, see Figure 1.

Data analysis

All statistical tests were undertaken in PASW 21.0 for Macintosh (SPSS Inc., Chicago, USA). A one-way ANOVA was used for all comparisons with Student–Newman–Keuls test for *post hoc* analysis. If data failed to pass homogeneity of variance, it was transformed logarithmically; if this also failed homogeneity, non-parametric tests were used (Kruskal-Wallis, with Mann–Whitney plus Bonferroni correction as *post hoc*). A significance level of $P < 0.05$ was used for all analyses.

Materials

THC and CBD (THC Pharm, Frankfurt, Germany) were dissolved in a mixture of ethanol, Tween 80 and saline (1:1:18) (Spiro *et al.*, 2012; Long *et al.*, 2013; Spencer *et al.*, 2013). In the case of the co-administration of THC and CBD, CBD powder was first dissolved in THC stock ethanol solution.

Results

CBD transiently inhibited THC-induced hypothermia

A one-way ANOVA showed a significant difference between groups on body temperature at 30 min ($F_{3,23}$ = 73.96, $P <$ 0.001) and 90 min ($F_{3,22}$ = 106.77, $P <$ 0.001) following

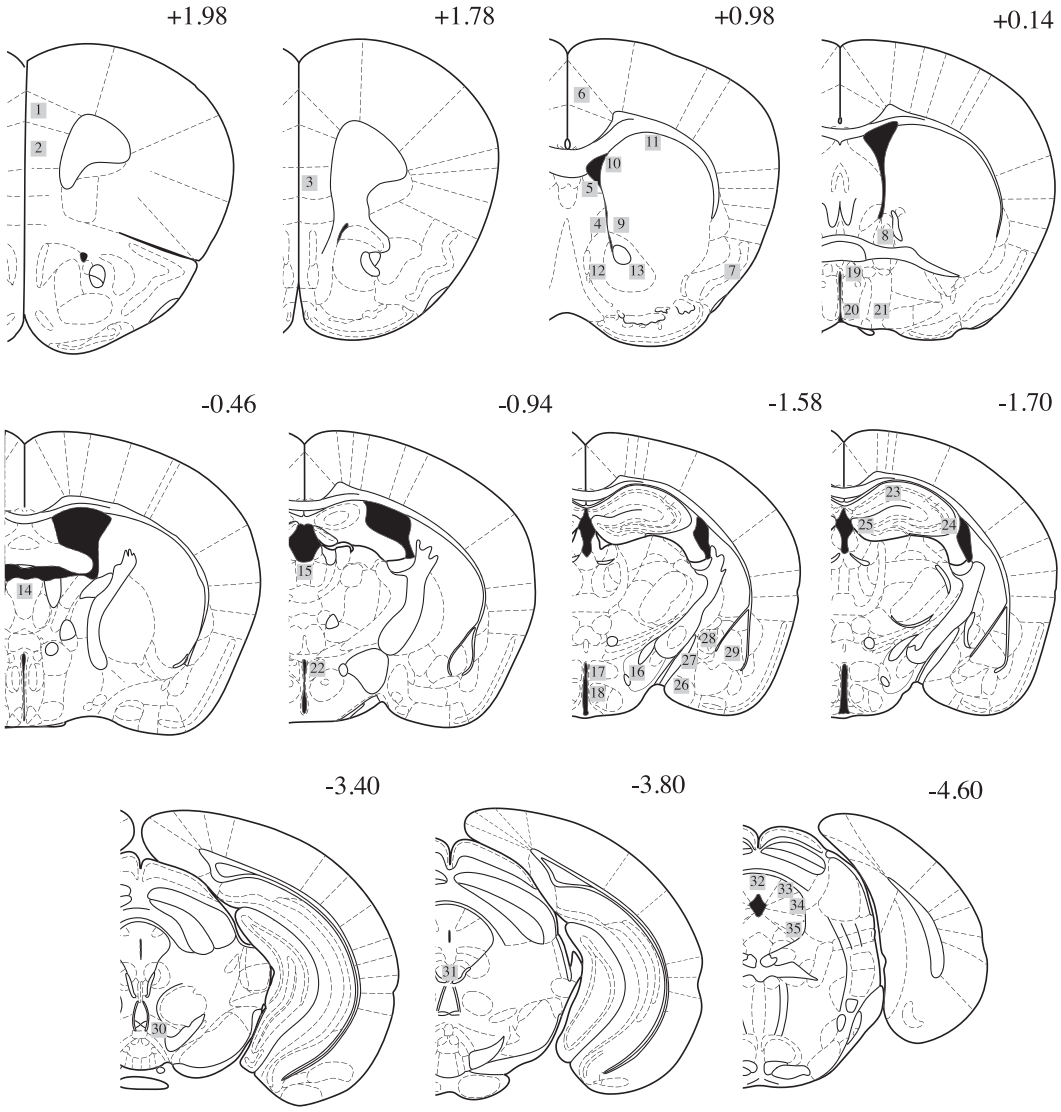

Figure 1

Bregma locations of coronal sections in mouse brain (adapted from Paxinos and Keith, 2001). c-Fos positive cells were counted within labelled regions and correspond to those listed in Table 1.

injection (Figure 2). CBD on its own had no effect on body temperature at any time point. THC caused significant hypothermia compared with vehicle control mice at 30 min ($P < 0.001$). Although mice in the THC/CBD group still displayed significant hypothermia ($P < 0.001$ compared with vehicle), the THC/CBD group had significantly less hypothermia than the THC-alone group at 30 min ($P < 0.05$). At 90 min, however, the CBD/THC group was not different from the THC group, with both groups inducing equivalent hypothermia ($P < 0.001$). By 6 h after injection, no significant differences were found between groups.

CBD potentiated THC-induced hypolocomotion

A one-way ANOVA indicated significant differences between the groups on total locomotor activity ($F_{3,17} = 51.23$, $P < 0.001$) (Figure 3A). *Post hoc* analysis showed no significant effects of CBD on locomotor activity compared with vehicle, while THC significantly suppressed locomotion in mice ($P < 0.001$). CBD potentiated the locomotor suppressant effects of THC as the THC/CBD group showed significantly less locomotor activity compared with THC alone ($P < 0.05$).

We additionally analyzed locomotion over the 45 min testing period in the open field (Figure 3B). A significant difference in locomotion between groups was found in all time bins measured: 0–5 min ($F_{3,17} = 5.39$, $P < 0.01$), 5–10 min ($F_{3,17} = 39.28$, $P < 0.001$), 10–15 min ($\chi^2(3) = 16.13$, $P < 0.01$), 15–20 min ($\chi^2(3) = 16.87$, $P < 0.01$), 20–25 min ($\chi^2(3) = 16.30$, $P < 0.01$), 25–30 min ($\chi^2(3) = 17.25$, $P < 0.01$), 30–35 min ($\chi^2(3) = 16.90$, $P < 0.01$), 35–40 min ($\chi^2(3) = 9.07$, $P < 0.05$) and 40–45 min ($\chi^2(3) = 9.89$, $P < 0.05$). Effects of CBD were not significantly different to those of vehicle at any time point. Locomotion in the THC group was

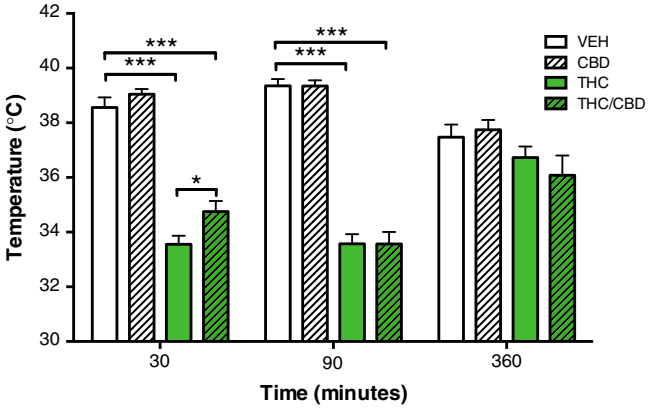

Figure 2

CBD reduced THC-induced hypothermia at 30 min following injection but was not different from THC at 90 min after injection ($n = 7$ for vehicle, CBD and THC; $n = 6$ for THC/CBD; $N = 27$). No significant cannabinoid-induced hypothermia remained 6 h after injection. VEH, vehicle control group; CBD, cannabidiol alone; THC, Δ^9-tetrahydrocannabinol alone; and THC/CBD = THC + CBD combination dose in a 1:1 ratio. CBD and THC were all administered at 10 mg·kg^{-1} i.p. in mice. Data represent means + SEM. analyses, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different as indicated; one-way ANOVA with *post hoc* Student–Newman–Keuls test.

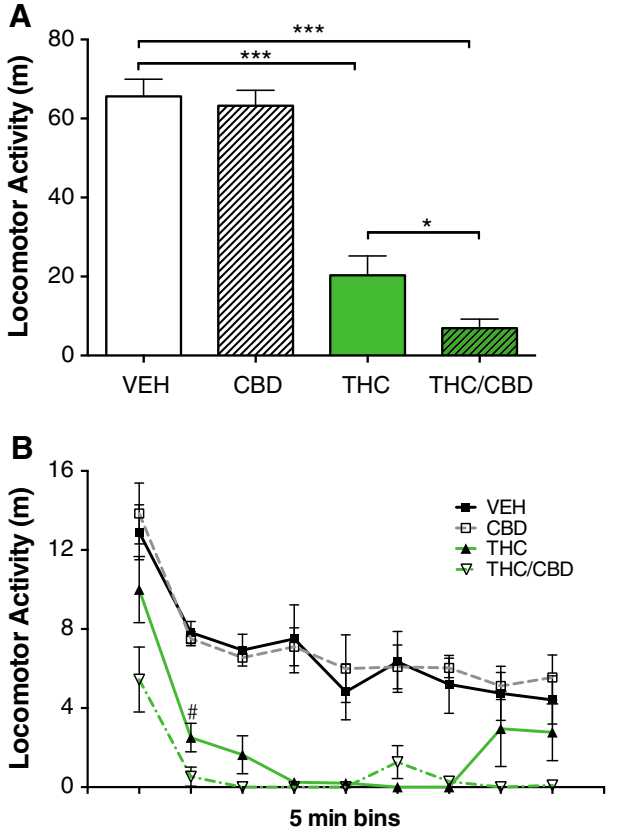

Figure 3

(A) CBD potentiated the locomotor suppressant effects of THC in the open-field test. (B) Locomotion in 5 min bins in the 45 min open-field test ($n = 6$ for THC group; $n = 5$ for vehicle, CBD and THC/CBD groups; $N = 23$). VEH, vehicle control group; CBD, cannabidiol alone; THC, Δ^9-tetrahydrocannabinol alone; and THC/CBD, THC + CBD combination dose in a 1:1 ratio. CBD and THC were all administered at 10 mg·kg^{-1} i.p. in mice. Data represent means + SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different as indicated. $^{\#}P < 0.05$; THC significantly different from THC/CBD. one-way ANOVA with *post hoc* Student–Newman–Keuls test.

significantly lower than that in the vehicle controls in the 5–10 ($P < 0.001$) and all 5 min bins thereafter up to and including the 30–35 min time bin (*P*s < 0.05). THC/CBD was significantly lower than vehicle in the first 5 min ($P < 0.01$), at 5–10 min ($P < 0.001$) and at all remaining time bins (*P*s < 0.05). The THC/CBD mixture group had significantly lower locomotor activity than THC alone at the 5–10 min bin ($P < 0.05$).

CBD inhibited THC-induced anxiety-related behaviour

A one-way ANOVA demonstrated a significant difference between groups ($F_{3,20} = 6.98$, $P < 0.01$) (Figure 4) on the percentage ratio of distance travelled in the central region to total distance travelled (distance ratio). Student–Newman–Keuls *post hoc* analysis indicated that THC significantly decreased the distance ratio compared with the vehicle control group ($P < 0.05$). However, treatment with CBD abolished this THC-induced reduction in distance ratio, with the THC/CBD co-administration group displaying an equivalent distance ratio to the vehicle control group and a significantly higher distance ratio than the THC-alone group ($P < 0.05$).

CBD reversed THC-induced c-Fos expression in regions involved in the cognitive impairing, anxiogenic and hypothermic actions of THC

The effects of THC and CBD alone and in combination on c-Fos expression are displayed in Table 1 along with the associated ANOVA *F*-values or Kruskal–Wallis chi-square values. CBD alone did not affect c-Fos expression, except in the central nucleus of the amygdala where it decreased expression compared with the vehicle control group ($P < 0.05$). THC significantly increased c-Fos expression compared with vehicle in 11 of the 35 brain regions examined, that is, in the ventrolateral septum ($P < 0.05$), BNST ($P < 0.001$), paraventricular thalamic nucleus ($P < 0.01$), anterior paraventricular thalamic nucleus ($P < 0.05$), ventromedial hypothalamus ($P < 0.001$), medial preoptic nucleus and medial preoptic area (both $P < 0.001$), PVH ($P < 0.01$), medial dorsolateral and central nucleus of the amygdala ($P < 0.05$; $P < 0.001$) and the lateral PAG ($P < 0.05$).

CBD co-administration tended to suppress THC-induced c-Fos expression in six of the 11 brain regions. The effects of CBD on THC-induced c-Fos expression were most pronounced in the medial preoptic nucleus and lateral PAG (*P*s < 0.05). Additionally, in the dentate gyrus, CBD co-administered with THC significantly reduced c-Fos expression compared with THC alone. However, this was in the absence of THC significantly increasing c-Fos compared with vehicle in this region. This may be a false negative due to a lack of power, as earlier studies have found THC to increase c-Fos in this brain region in mice at a lower dose of 5 mg·kg^{-1} (Valjent *et al.*, 2002). Representative photomicrographs of

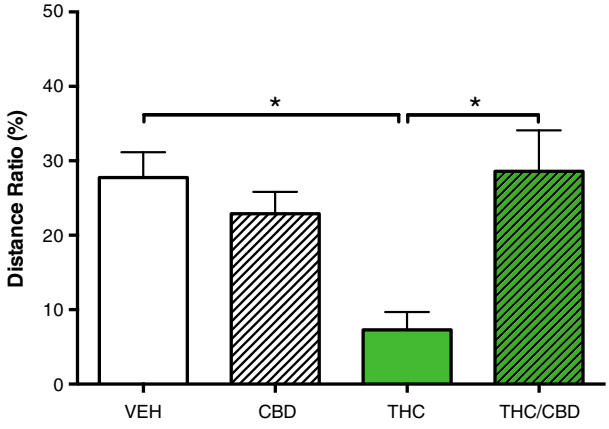

Figure 4

CBD reversed THC-induced avoidance of the centre region of the open field as measured by distance ratio ($n = 6$, $N = 24$). VEH, vehicle control group; CBD, cannabidiol alone; THC, Δ^9-tetrahydrocannabinol alone; and THC/CBD = THC + CBD combination dose in a 1:1 ratio. CBD and THC were all administered at 10 mg·kg^{-1} i.p. in mice. Data represent means + SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different as indicated; one-way ANOVA with *post hoc* Student–Newman–Keuls test.

these regions are displayed in Figures 5–7. While THC significantly increased c-Fos expression in the ventrolateral septum, the anterior part of the paraventricular thalamic nucleus and the PVH, results from the THC/CBD group failed to reach significance compared with the vehicle group with lower mean c-Fos positive neurons in these areas.

Discussion

This study reports for the first time the brain regions involved in CBD's modulation of the pharmacological actions of THC, using a 1:1 mixture, approximating that found in medicinal cannabis strains and nabiximols. Our results also demonstrate CBD may potentiate or hinder the effects of THC, depending on the effect being examined. We found that CBD enhanced the locomotor suppressant effects of THC, while reducing its hypothermic and anxiogenic actions. This was associated with CBD reversing THC-induced c-Fos expression in the medial preoptic nucleus, the lateral PAG and the dentate gyrus, the areas of the brain implicated in the hypothermic, anxiogenic and memory-impairing effects of THC. Our observations that CBD diminished the actions of THC could not be explained by physiological antagonism, as CBD alone was largely ineffective and there were no instances of CBD or THC alone promoting opposing actions on our measures.

We demonstrated here that CBD alone did not alter locomotor activity, anxiety-related behaviour and body temperature. However, CBD did decrease the number of c-Fos positive neurons in the central nucleus of the amygdala. The central nucleus of the amygdala is thought to have a role in the expression of fear and anxiety (Tye *et al.*, 2011; Penzo *et al.*, 2014), and direct CBD injection into the central nucleus of the amygdala can reduce anxiety behaviours (Hsiao *et al.*, 2012). We did not observe any effect of CBD on baseline anxiety-related behaviour, although it may be that the anxiolytic effects of CBD require application of an explicit stressor. While our results showed that CBD alone had a limited effect on the brain and behaviour of mice at 10 mg·kg^{-1}, this does not preclude effects at higher doses. For example, 120 mg·kg^{-1} CBD in rats increased c-Fos expression in the nucleus accumbens (Guimaraes *et al.*, 2004). We did however show that acute THC administration promoted robust hypolocomotion, anxiogenesis and hypothermia. Further, THC increased c-Fos expression in 11 of the 35 brain regions examined. This confirmed earlier findings that THC and its synthetic analogues increased c-Fos expression in various brain regions (Miyamoto *et al.*, 1996; McGregor *et al.*, 1998; Arnold *et al.*, 2001; Boucher *et al.*, 2007). These results provide a clear background to discern modulatory effects of CBD on THC.

Our study showed that CBD inhibited THC-induced hypothermia in mice. The effect of CBD was modest, only reversing the profound hypothermic effects of 10 mg·kg^{-1} THC by about 24%. This inhibition of THC-induced hypothermia was also transient in nature, as although the effect was observed at 30 min, it disappeared by 90 min. This inhibition is consistent with the results of Borgen and Davis (1974), where rats were pretreated with 25 mg·kg^{-1} CBD 1 h before a 10 mg·kg^{-1} THC challenge injection. As in our study, repeated rectal probe measures were taken, and CBD reduced THC-induced hypothermia by 47% and decreased the duration of the hypothermia. However, other studies have been unable to replicate this effect or have found potentiation (Varvel *et al.*, 2006; Hayakawa *et al.*, 2008; Taffe *et al.*, 2015). In particular, Taffe *et al.* (2015) used a 1:1 THC/CBD dose ratio in rats at a higher dose of 30 mg·kg^{-1} and found CBD potentiated the hypothermic effects of THC. This study utilized biotelemetry, raising the possibility that the method of body temperature sampling might influence the result observed. However, Hayakawa *et al.* (2008) found that 50 mg·kg^{-1} CBD potentiated the hypothermic effects of 1 mg·kg^{-1} THC i.p. in mice using the same rectal probe methodology as we have used. It may be that it is impossible for CBD to modulate saturating doses of THC (e.g. 30 mg·kg^{-1} in a rat) and that only threshold doses of THC, as was used here, may be sensitive to modulation. More studies are needed to reconcile the diverse findings of the interactions between CBD and THC on body temperature, with species, mode of drug delivery, the endpoint being measured and dose ratio and specific doses all needing to be considered.

Our findings suggested that CBD inhibition of THC actions involved the medial preoptic nucleus, a structure that expresses CB_1 receptors and is known to mediate the temperature regulatory actions of THC (Fitton and Pertwee, 1982; Hrabovszky *et al.*, 2012). Body temperature is regulated by thermogenesis controlled by reciprocal connections between the brain and brown fat tissue. THC induces c-Fos expression in the medial preoptic nucleus, medial preoptic area, PVH and lateral PAG, all of which are key brain regions involved in the innervation of brown fat tissue (Ryu *et al.*, 2015). It is of interest then that CBD also significantly lowered THC-induced c-Fos expression in the lateral PAG and tended to do so in the PVH.

Table 1

Mean number (±SEM) of Fos-labelled cells in mouse brain following drug treatment and behavioural testing in the open field ($n = 6$ per condition)

Regions	Bregma	Groups				
		Vehicle	CBD (10 mg·kg^{-1})	THC (10 mg·kg^{-1})	THC + CBD (10 mg·kg^{-1})	Statistics (F/X, P)
Frontal						
1. Cingulate cortex	+1.98	2.0 ± 0.7	1.8 ± 0.8	1.5 ± 0.6	1.7 ± 0.5	0.10, >0.05
2. Prelimbic cortex	+1.98	1.5 ± 0.3	2.2 ± 0.8	2.3 ± 1.0	2.2 ± 0.7	0.25, >0.05
3. Infralimbic cortex	+1.78	1.2 ± 0.5	1.2 ± 0.6	1.8 ± 1.1	2.0 ± 0.8	0.33, >0.05
4. Ventrolateral septum	+0.98	7.3 ± 3.3	11.8 ± 3.3	31.8 ± 6.4$^{\#}$	27.0 ± 9.0	4.42, <0.05
5. Dorsolateral septum	+0.98	1.2 ± 0.7	1.0 ± 0.5	10.0 ± 4.8	10.40 ± 3.5$^{\#}$	5.16, <0.01
6. Anterior cingulate cortex	+0.98	1.8 ± 0.9	3.3 ± 1.8	2.2 ± 0.7	2.6 ± 1.1	0.30, >0.05
7. Piriform cortex	+0.98	5.7 ± 2.6	5.7 ± 1.9	5.2 ± 1.5	5.2 ± 1.2	0.02, >0.05
8. BNST	+0.14	1.2 ± 0.4	2.7 ± 1.1	54.7 ± 9.8$^{\#\#\#}$	40.3 ± 9.4$^{\#\#\#}$	60.01, <0.001
Striatum						
9. Central caudate putamen	+0.98	1.3 ± 0.4	1.5 ± 0.6	2.3 ± 0.8	1.8 ± 0.7	0.48, >0.05
10. Dorsal caudate putamen	+0.98	1.3 ± 0.5	0.7 ± 0.2	2.2 ± 0.3	1.3 ± 0.5	2.40, >0.05
11. Dorsomedial caudate putamen	+0.98	4.8 ± 1.8	4.5 ± 1.4	8.3 ± 1.9	7.5 ± 2.2	1.10, >0.05
12. Nucleus accumbens, shell	+0.98	3.5 ± 1.4	1.0 ± 0.4	5.0 ± 2.3	3.7 ± 1.4	1.02, >0.05
13. Nucleus accumbens, core	+0.98	0.8 ± 0.7	0.5 ± 0.3	1.0 ± 0.4	0.3 ± 0.2	0.51, >0.05
Thalamus						
14. Paraventricular thalamic nucleus, anterior	−0.46	18.0 ± 2.9	16.0 ± 5.4	60.0 ± 10.0$^{\#}$	39.0 ± 10.6	6.69, <0.01
15. Paraventricular thalamic nucleus	−0.94	4.8 ± 1.9	8.0 ± 3.2	18.5 ± 3.6$^{\#}$	18.3 ± 2.4$^{\#}$	6.05, <0.01
Hypothalamus/preoptic						
16. Lateral	−1.58	2.7 ± 1.1	3.0 ± 0.7	6.3 ± 2.0	5.8 ± 2.3	1.16, >0.05
17. Dorsomedial	−1.58	5.2 ± 2.3	6.7 ± 1.9	15.3 ± 3.0	12.3 ± 4.4	2.40, >0.05
18. Ventromedial	−1.58	1.2 ± 0.7	2.7 ± 1.1	28.0 ± 5.6$^{\#\#\#}$	29.8 ± 7.8$^{\#\#\#}$	25.98, <0.001
19. Medial preoptic nucleus	+0.14	0.7 ± 0.3	1.0 ± 0.8	10.8 ± 1.7$^{\#\#\#}$	5.8 ± 2.0$^{\#\#\ \$}$	16.30, <0.001
20. Medial preoptic area	+0.14	1.2 ± 0.5	1.8 ± 0.6	8.0 ± 1.1$^{\#\#\#}$	5.8 ± 1.5$^{\#}$	10.42, <0.001
21. Lateral preoptic area	+0.14	0.8 ± 0.3	1.0 ± 0.4	1.5 ± 0.4	1.0 ± 0.3	0.70, >0.05
22. Paraventricular hypothalamic nucleus	−0.94	5.3 ± 4.5	2.2 ± 1.6	26.3 ± 5.1$^{\#\#}$	16.0 ± 4.0	7.33, <0.01
Hippocampus						
23. CA1	−1.70	0.3 ± 0.2	0.3 ± 0.3	0.8 ± 0.4	0.3 ± 0.2	0.69, >0.05
24. CA3	−1.70	0.8 ± 0.3	0.8 ± 0.5	1.0 ± 0.5	0.0 ± 0.0	[4.72, >0.05]
25. Dentate gyrus	−1.70	4.0 ± 1.1	5.0 ± 1.6	8.5 ± 1.8	2.5 ± 1.2$^{\$}$	3.08, 0.051
Amygdala						
26. Medial, posteroventral	−1.58	2.3 ± 1.2	1.0 ± 0.5	5.8 ± 2.1	4.3 ± 1.2	2.45, >0.05
27. Medial, posterodorsal	−1.58	0.8 ± 0.3	0.2 ± 0.2	3.5 ± 0.9$^{\#}$	6.7 ± 2.2	[14.30, <0.01]
28. Central nucleus	−1.58	3.3 ± 1.8	0.3 ± 0.2$^{\#}$	44.0 ± 5.8$^{\#\#\#}$	37.8 ± 6.6$^{\#\#\#}$	51.35, <0.001
29. Basolateral	−1.58	1.3 ± 0.5	2.2 ± 1.0	3.7 ± 1.0	1.7 ± 0.7	1.54, >0.05
Midbrain						
30. Ventral tegmental area	−3.40	1.0 ± 0.5	0.2 ± 0.2	1.5 ± 0.4	0.3 ± 0.2	2.96, >0.05
31. Edinger–Westphal nucleus	−3.80	5.3 ± 3.2	3.3 ± 1.3	10.0 ± 2.7	10.8 ± 4.4	1.52, >0.05
Periaqueductal gray						
32. Dorsomedial	−4.60	0.3 ± 0.2	1.3 ± 0.8	3.5 ± 1.0	2.0 ± 1.2	2.35, >0.05
33. Dorsolateral	−4.60	0.2 ± 0.2	0.5 ± 0.5	1.7 ± 0.3	0.7 ± 0.5	2.63, >0.05
34. Lateral	−4.60	1.2 ± 0.5	1.0 ± 0.5	4.7 ± 1.4$^{\#}$	1.7 ± 0.7$^{\$}$	4.35, <0.05
35. Ventrolateral	−4.60	1.7 ± 0.7	3.7 ± 2.0	6.8 ± 1.2	6.7 ± 2.2	2.36, >0.05

All ANOVA F-values represent (3,20) d.f. [Kruskal-Wallis Test, d.f. = 3]. *Post hoc* Student–Newman–Keuls. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; significantly different from vehicle. $^{\$}P < 0.05$ significantly different from THC.

The PVH receives projections from the preoptic hypothalamus (Nakamura, 2011), and CB_1 receptors are critical to PVH-mediated thermogenesis in brown fat (Monge-Roffarello *et al.*, 2014). Taken together, our results suggest that the medial preoptic nucleus, PVH and lateral PAG could be further explored in studies examining the molecular mechanisms of the inhibition by CBD of THC-induced hypothermia.

We found that CBD potentiated THC's locomotor suppressant effects, as has been documented previously in mice and rats at both high and low CBD: THC dose ratios (Hayakawa *et al.*, 2008; Malone *et al.*, 2009; Klein *et al.*, 2011; Taffe *et al.*, 2015). Taken together with our findings on body temperature and anxiety-related behaviour, this reinforces the premise that both inhibiting and potentiating interactions between THC and CBD may be simultaneously observed. This is promising as it provides potential for therapeutic outcomes to be maximized while minimizing deleterious effects of THC. It could be that enhanced motor suppression explains the beneficial therapeutic effects of nabiximols in treating movement dysfunction such as spasticity in multiple sclerosis and

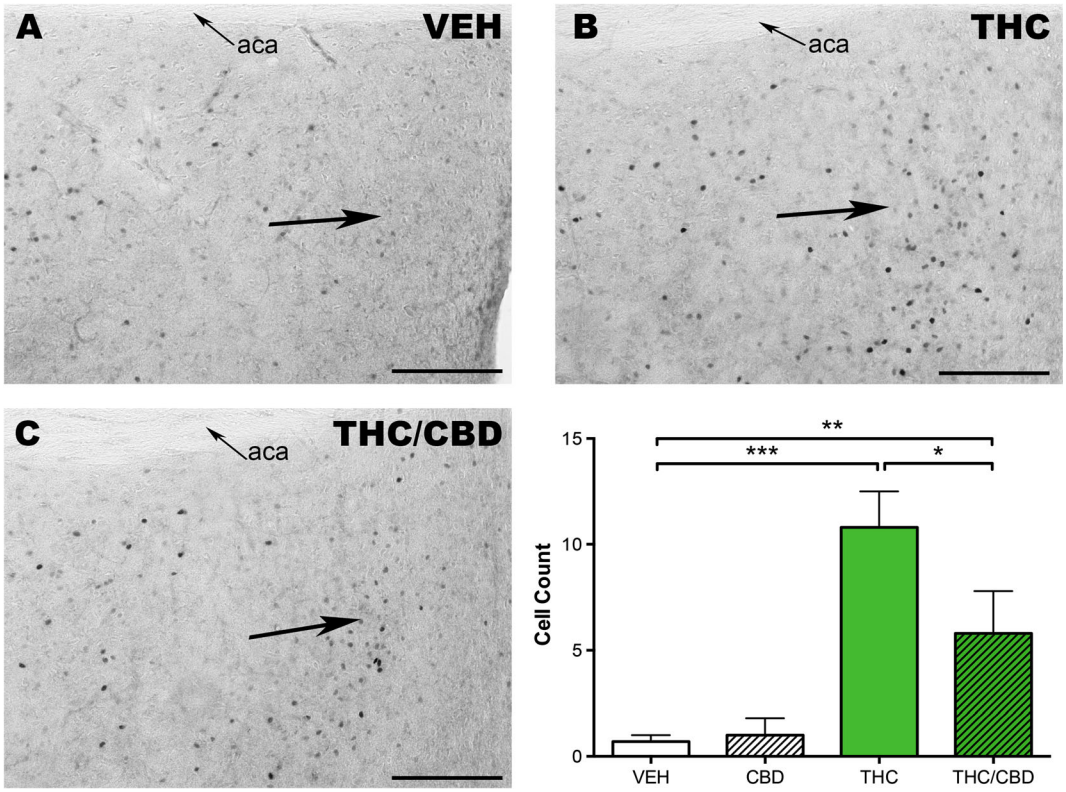

Figure 5

Representative photomicrographs of c-Fos positive neurons in the medial preoptic nucleus at +0.14 Bregma in (A) vehicle, (B) THC and (C) THC/CBD groups. The anterior commissure (aca) is also indicated. Scale bar = 150 μm. Mean c-Fos counts across groups are displayed in (D). VEH, vehicle control group; CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol; and THC/CBD, THC + CBD combination dose in a 1:1 ratio. CBD and THC were all administered at 10 mg·kg^{-1} i.p. in mice (n = 6 per group, N = 24). Data represent means + SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different as indicated; one-way ANOVA with *post hoc* Student–Newman–Keuls test.

hyperkinesis in Huntington's disorder (Sagredo *et al.*, 2012; Moreno Torres *et al.*, 2014). Unfortunately, we could not demonstrate a neural correlate of CBD and THC interaction on locomotor activity. Cannabinoids affect a number of regions involved in locomotion including the prefrontal cortex, nucleus accumbens, caudate putamen, substantia nigra and globus pallidus. However, THC did not increase c-Fos expression in any of these brain regions, as c-Fos is a poor marker for cannabinoid action on motor function (McGregor *et al.*, 1998; Arnold *et al.*, 2001; Boucher *et al.*, 2007)

CBD reversed the anxiogenic effects of THC in the open-field test. We used a ratio of distance travelled in the centre (anxiety-related behaviour) to the total distance travelled (locomotor activity) to control for the locomotor suppressant effects of THC. However, we cannot completely rule out the confounding influence of locomotor suppression and our results on anxiety should be interpreted with caution. Arguing in favour of a selective effect on anxiety is that our results replicate numerous findings that CBD reduced the anxiogenic and aversive effects of THC in rodents and humans. CBD reversed THC-induced conditioned emotional responses in rats (10 mg·kg^{-1} CBD and 2 mg·kg^{-1} THC) (Zuardi and Karniol, 1983), social withdrawal in rats (20 mg·kg^{-1} CBD and 1 mg·kg^{-1} CBD) (Malone *et al.*, 2009) and conditioned place aversion in mice (Vann *et al.*, 2008). It is of note that the latter finding also used a 1:1 THC/CBD dose ratio at 10 mg·kg^{-1} of both drugs. Moreover, in humans, CBD reduced the anxiety-provoking effects of THC (Zuardi *et al.*, 1982; Bergamaschi *et al.*, 2011). Further arguing in favour of a selective effect of CBD on THC-induced anxiety is that CBD reduced c-Fos expression induced by THC, in anxiety-related regions of the brain.

THC exposure robustly increased c-Fos expression in the lateral septum, the BNST, the PVH, the paraventricular thalamic nucleus, the central nucleus of the amygdala and the lateral PAG, regions all involved in stress and anxiety circuits (Luthi and Luscher, 2014; Allsop *et al.*, 2014b). CBD significantly reduced THC-induced c-Fos in the dentate gyrus and lateral PAG and tended to reduce it in the ventrolateral septum, the anterior part of the paraventricular thalamic nucleus and the PVH. Electrical stimulation of the dorsolateral and lateral PAG elicits defensive behaviours characterized by vigilance, freezing and escape (Borelli *et al.*, 2004). Direct infusion of synthetic cannabinoid agonists such as HU210 into the dorsal PAG is anxiolytic, which is surprising given that cannabinoids have been shown to exert anxiogenic effects in rodents (Finn *et al.*, 2003; Arnold *et al.*, 2010). However, the concentrations infused into the PAG may reflect that found in low systemic doses that are anxiolytic (Moreira and

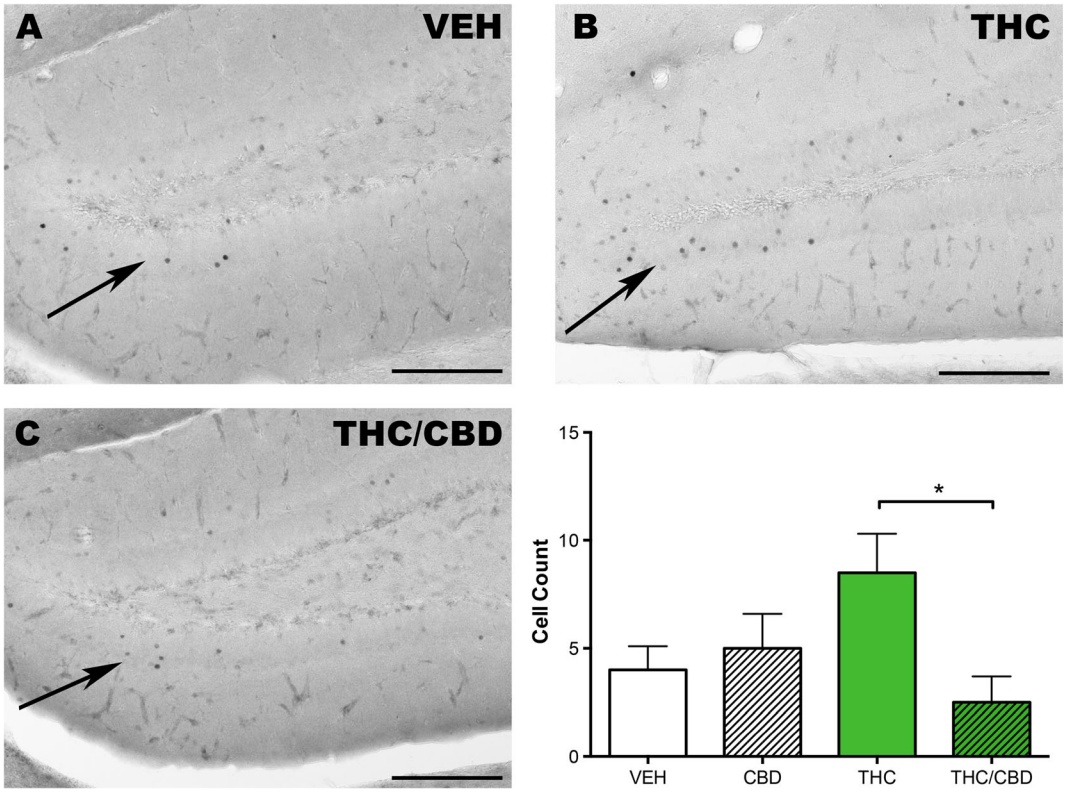

Figure 6

Representative photomicrographs of c-Fos positive neurons in the dentate gyrus at −1.70 Bregma in (A) vehicle, (B) THC and (C) THC/CBD groups. Scale bar = 150 μm. Mean c-Fos counts across groups are also displayed (D). VEH, vehicle control group; CBD, cannabidiol alone; THC, Δ^9-tetrahydrocannabinol alone; and THC/CBD, THC + CBD combination dose in a 1:1 ratio. CBD and THC were all administered at 10 mg·kg^{-1} i.p. in mice (n = 6 per group, N = 24). Data represent means + SEM. *$P < 0.05$; significantly different as indicated; one-way ANOVA with *post hoc* Student–Newman–Keuls test.

Wotjak, 2010). CBD administered into the PAG also promoted anxiolytic effects (Campos *et al.*, 2013). It would be of interest to examine if the reversal by CBD of THC anxiogenesis, as observed here, could be reproduced via a direct infusion of these drugs into the lateral PAG. The ability of CBD to reduce the effects of THC on brain regions that mediate anxiety supports the viewpoint that the psychotropic effects of 1:1 THC/CBD medicinal cannabis strains may be better tolerated than those of street cannabis, which contains high THC but low CBD concentrations (Swift *et al.*, 2013).

The molecular mechanisms responsible for the interaction of CBD and THC in discrete brain regions need to be elucidated. CBD has a multimodal pharmacology affecting numerous drug targets that might interfere with the actions of THC including the orphan GPCR GPR55, 5-HT$_{1A}$ receptors, voltage-dependent anion-selective channel protein 1 and glycine receptors (Devinsky *et al.*, 2014). In the absence of physiological antagonism, our results accord with the view that CBD might modulate the effects of THC at cannabinoid receptors. This could be achieved through indirect competition, where CBD inhibits FAAH, which increases anandamide concentrations that then compete with THC for CB$_1$ receptor binding (Pertwee, 2008; McPartland *et al.*, 2015). Alternatively, a new finding suggests that CBD inhibits the effects of THC through allosteric modulation. In cultured cells heterologously and endogenously expressing CB$_1$ receptors, CBD behaved as a negative allosteric modulator of the CB$_1$ receptor and decreased the effects of THC at the orthosteric binding site (Laprairie *et al.*, 2015). However, neither theory can explain why CBD inhibited THC-induced c-Fos expression in a restricted set of brain regions.

One explanation for the anatomical specificity of CBD–THC interactions involves TRPV1 receptors, as CBD activates TRPV1 receptors (Iannotti *et al.*, 2014). The regional localization of TRPV1 receptors in the brain is still a matter of debate and appears to be restricted to some of the brain regions where we observed CBD to reduce THC-induced c-Fos expression, that is, the medial preoptic nucleus of the hypothalamus, the dentate gyrus of the hippocampus and the periaqueductal gray (Karlsson *et al.*, 2005; Chavez *et al.*, 2010; Cavanaugh *et al.*, 2011; Fan-xin *et al.*, 2012; Tsurugizawa *et al.*, 2013; Puente *et al.*, 2015). CB$_1$ and TRPVI receptors colocalize (Cristino *et al.*, 2006), and TRPV1 receptors oppose the effects of CB$_1$ receptor activation in brain circuits (Xing and Li, 2007; Lisboa and Guimaraes, 2012). For example, elevation of anandamide in the PAG may simultaneously inhibit or excite glutamatergic synaptic transmission mediated by CB$_1$ and

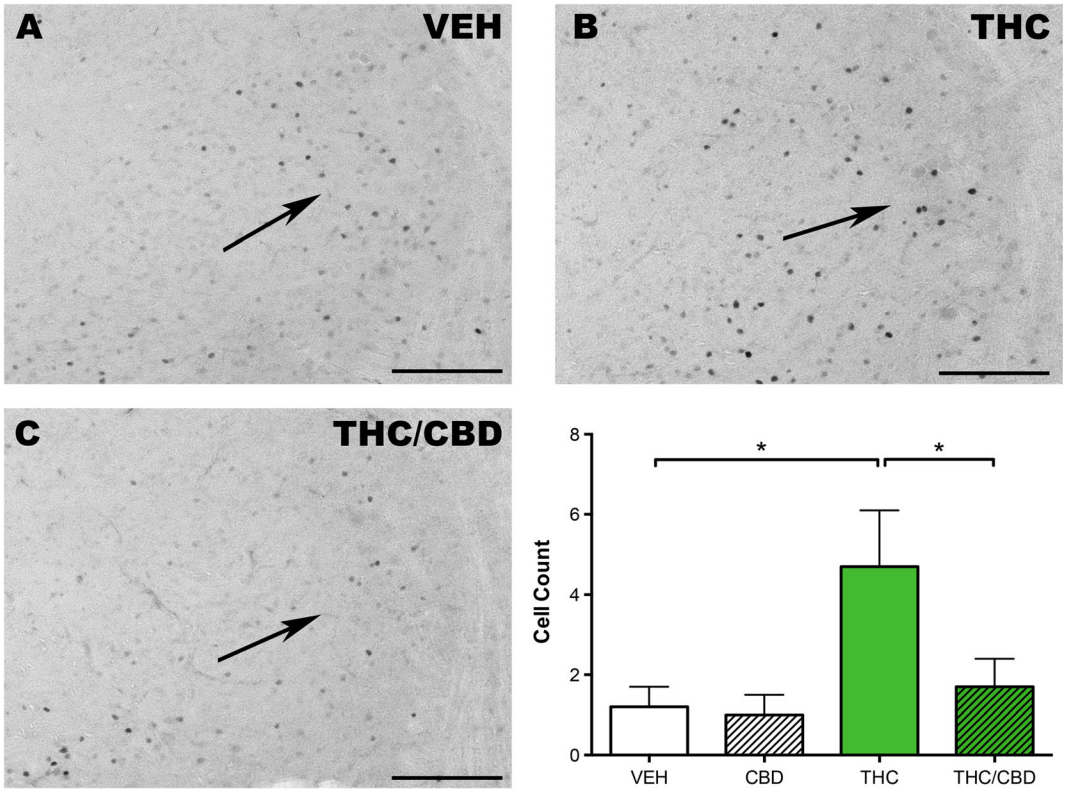

Figure 7

Representative photomicrographs of c-Fos positive neurons in the lateral periaqueductal gray at −4.60 Bregma in (A) vehicle, (B) THC and (C) THC/CBD groups. Scale bar = 150 μm. Mean c-Fos counts across groups are also displayed (D). VEH, vehicle control group; CBD, cannabidiol alone; THC, Δ^9-tetrahydrocannabinol alone; and THC/CBD = THC + CBD combination dose in a 1:1 ratio. CBD and THC were all administered at 10 mg·kg^{-1} i.p. in mice ($n = 6$ per group, $N = 24$). Data represent means + SEM. *$P < 0.05$; significantly different as indicated; one-way ANOVA with *post hoc* Student–Newman–Keuls test.

TRPV1 receptors respectively (Kawahara *et al.*, 2011). In addition, CB_1 and TRPVI receptor activation in the PAG has opposing effects on panic-like responses (Casarotto *et al.*, 2012). Therefore, it may be that CBD only inhibits the effects of THC in brain regions that co-express both CB_1 and TRPV1 receptors.

The mechanism responsible for CBD potentiation of THC effects also requires clarification. Pharmacokinetic mechanisms may play a role as CBD treatment increased brain concentrations of THC (Klein *et al.*, 2011). One proposed mechanism for this involves ABC transporters, which regulate the brain uptake of drugs due to their localization at the blood brain barrier (Arnold *et al.*, 2012). CBD inhibits these transporters, and THC is an ABC transporter substrate (Zhu *et al.*, 2006; Holland *et al.*, 2007; Spiro *et al.*, 2012); therefore, it is possible that CBD reduces THC efflux from the brain, thereby enhancing its brain concentration.

This study showed that the behavioural and physiological interactions of 1:1 ratios of THC and CBD were associated with CBD inhibiting THC-induced c-Fos expression in various regions, with robust inhibition in the hypothalamus and the PAG. This suggests that the mechanism of THC/CBD interactions is complex, as CBD simultaneously potentiated the locomotor suppressant effects of THC while inhibiting its anxiogenic and hypothermic actions. Our results are consistent with the notion that cannabis plant strains that contain THC and CBD at 1:1 ratios may be preferable to street cannabis for medicinal applications because they maximize therapeutic efficacy while minimizing the adverse effects of THC.

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Author contributions

S. M. T. and J. C. A. designed the research study. S. M. T. performed behavioural experiments, immunohistochemistry and cell quantification. J. C. A. performed perfusions. S. M. T. conducted statistical analysis of data and created figures with significant input from J. C. A. S. M. T. and J. C. A. wrote and revised the manuscript for submission.

Conflict of interest

The authors report no conflicts of interest.

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