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ORIGINAL RESEARCH

Modulatory Effects of "Minor" Cannabinoids in an in vitro Model of Neuronal Hypersensitivity

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Aim: Effective treatment for neuropathic pain remains an unmet clinical need. The therapeutic benefits of the Cannabis plant are well known, especially for pain relief. Here, we have assessed ten "minor" cannabinoids for their analgesic effects in an established model of neuronal hypersensitivity, a key mechanism which underlies neuropathic pain.

Methods: Adult rat DRG neurons were cultured in medium containing 100 ng/mL nerve growth factor (NGF) and 50 ng/mL glial cell-line derived neurotrophic factor (GDNF) for 48 hours to sensitize the neurons. Ca^{2+} imaging was used to measure the responses to pain stimulation using capsaicin, and to determine the modulatory effects of the cannabinoids, in individual neurons.

Results: Control neurons (nociceptors) showed robust responses of Ca^{2+} influx to capsaicin application, while neurons treated with ten minor cannabinoids tetrahydrocannabiorcol (THCC), cannabitriol (CBT), cannabidivarin (CBDV), cannabinol (CBN), cannabichromene (CBC), cannabichromevarin (CBCV), cannabicitran (CBCT), cannabigerol monomethyl ether (CBGM), tetrahydrocannabutol (THCB) or tetrahydrocannabiphorol (THCP), at concentrations of 0.001–100 μM, showed differential dose-related effects on the responses to capsaicin. Ca^{2+} influx in response to capsaicin application was completely inhibited for each compound in 35–78% capsaicin-sensitive neurons, while other neurons showed reduced responses. The opioid receptor agonist morphine and α2δ1- Ca^{2+} channel inhibitor gabapentin were also tested for comparison and showed similar results. All the cannabinoids tested here inhibited calcium influx in response to capsaicin, and two, namely, CBN and THCC elicited calcium influx at higher doses. Inhibition of Ca^{2+} influx due to cannabichromene (CBC) was reversed by the potassium channel inhibitor Tertiapin Q.

Conclusion: All the cannabinoids tested here inhibited TRPV1 signalling. CBC targeted K^+ channels to block TRPV1 mediated Ca^{2+} influx, demonstrating potential analgesic effects in vitro.

Plain Language Summary: Nerve damage due to disease or injury causes neuropathic pain which is very difficult to treat and affects the quality of life of affected individuals. The cannabis plant is known for its medicinal properties for millennia, especially for treating pain. This is due to its constituents such as THC which is psychoactive, limiting its usefulness. There are several other constituents also known as cannabinoids, which are known not to be psychoactive, and whose medicinal effects are unknown. In this study, we examined ten different cannabis constituents for their ability to block pain signals in nerve cells which sense pain in the body. This was done by growing nerve cells or sensory neurons that sense pain, in a dish and treating them with NGF and GDNF that increase the nerve signals which lead to neuropathic (nerve) pain. We then used capsaicin, the hot ingredient of chilli peppers, to stimulate these neurons (nerve cells) to generate pain signalling. These signals were measured by using a calcium indicator dye to identify which neurons were responding and how large their responses were to capsaicin. We observed that treating the neurons with individual cannabinoids either completely blocked or reduced the capsaicin responses to a very large extent, in most neurons. We have also shown that the cannabinoid cannabichromene blocked responses to capsaicin by activating the potassium channels in the neuron membrane, by using bee venom, which blocks potassium channels. As different cannabinoids inhibited capsaicin responses to a greater or lesser extent, we can identify which cannabinoids are likely to be most useful for blocking pain signalling to develop new drugs for providing pain relief without side effects.

Keywords: chronic pain, analgesia, cannabinoids, sensory neurons, tertiapin Q

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Introduction

The therapeutic benefits of the Cannabis plant are well known, especially for pain relief. These effects are highly variable, likely due to its several hundred constituents that vary between chemovars. Analgesia is a characteristic benefit of cannabinoids, the best known of which is delta 9 tetrahydrocannabinol (Δ^9THC) which is also psychoactive. There is emerging evidence for the analgesic effects of the other non-psychoactive minor cannabinoids.

Neuropathic pain is pain due to nerve damage or disease which involves enhanced sensitivity resulting from changes in nociceptive ion channel and receptor expression. There is no single effective treatment for neuropathic pain, which remains an unmet need. The therapeutic benefits of cannabinoids for neuropathic pain relief are variable and further studies are required to determine the long-term effects.[1] As side effects of somnolence, confusion and psychosis may limit the clinical usefulness of THC containing medicines, a clearer understanding of the efficacy and mechanism of action of the non-psychoactive "minor" cannabinoids is likely to identify other potent and more useful alternatives.

There is increasing clinical evidence for the efficacy of cannabinoids such as improved pain scores, range of motion and grip strength in a randomized controlled clinical trial for arthritis-related pain.[2] A more recent observational study found that medical marijuana was comparatively more effective than prescription medications for the treatment of chronic pain at 3 months.[3] The UK Medical Cannabis Registry (UKMCR) was established in December 2019 to prospectively collect data of patient outcomes for assessing the benefit-risk profile, long-term safety and efficacy of cannabis-based medicinal products (CBMPs) for several medical conditions. The analysis from chronic pain patients registered in the UKMCR suggests that treatment with CBMPs is associated with significant improvements in self-reported and general quality of life outcomes.[4]

These results are consistent with findings from several preclinical studies, showing potent analgesic and anti-inflammatory effects of cannabichromene (CBC) and cannabidiol (CBD) treatment.[5] CBC diminished nociceptive behaviour in mouse models of inflammatory,[6,7] and neuropathic pain.[8] Tetrahydrocannabiorcol (THCC) and other phytocannabinoids also exerted antinociceptive effects via cannabinoid receptor-independent targets.[9,10] Cannabigerol (CBG) alleviated mechanical hypersensitivity in a mouse model of cisplatin-induced peripheral neuropathy and reduced pain sensitivity by 60–70%, in male and female mice.[11,12] Cannabinol (CBN) was effective in relieving pain due to chemotherapy induced peripheral neuropathy (CIPN),[13] and inhibited voltage-dependent sodium channels in dorsal root ganglion (DRG) neurons via membrane hyperpolarization.[14] We have recently described neuronal desensitization by cannabidiol (CBD), cannabigerol (CBG) and THC, which had inhibitory effects on capsaicin responses when applied individually, and these were enhanced when applied in combination.[15]

The DRG contain cell bodies of primary sensory neurons, including the polymodal nociceptors which express different receptors to transduce noxious signals for the perception of pain. The transient receptor potential subtype vanilloid 1 (TRPV1) is a non-selective transmembrane cation channel expressed by polymodal nociceptors in the DRG that integrates noxious endogenous and exogenous stimuli such as capsaicin, heat and inflammatory ligands to mediate pain.[16–18] TRPV1 function and expression are regulated by the neurotrophic factors nerve growth factor (NGF), glial cell-line derived neurotrophic factor (GDNF),[19,20] and the levels of NGF, GDNF and its receptor Ret are increased in injured human peripheral nerves and DRG.[21] NGF and GDNF treatment induces increased expression of the TRPV1 receptor, neuronal sensitization and hyperalgesia in DRG neurons, that is reflected in the enhanced responses to noxious stimulation including capsaicin, providing a model of neuronal hypersensitivity.[22–24] We have recently reported potent inhibitory effects of cannabis plant-derived terpenes on capsaicin responses in cultured DRG neurons treated with NGF and GDNF using Ca^{2+} imaging in this model.[25]

There is little or no information available for the lesser-known or minor cannabinoids, in relation to pain signalling. There are several hundred cannabinoid components in the *C Sativa* plant, and we chose to study a few of those which have some information available.

In the present study, we have used DRG neurons treated with NGF and GDNF to examine the modulatory effects of the cannabinoids tetrahydrocannabiorcol (THCC), cannabidivarin (CBDV), cannabinol (CBN), cannabitriol (CBT), cannabichromene (CBC), cannabichromevarin (CBCV), cannabicitran (CBCT), cannabigerol monomethyl ether (CBGM), tetrahydrocannabutol (THCB) and tetrahydrocannabiphorol (THCP). Ca^{2+} influx in response to noxious pain

stimulation with the chilli pepper extract capsaicin, was measured in the presence of each of these cannabinoids. We report that of these ten minor cannabinoids, THCC and CBN elicited Ca^{2+} influx at high concentrations, while all were able to potently inhibit capsaicin responses over a wide range of concentrations. We also included morphine and gabapentin (GBP) in our studies, for comparison of inhibitory effects using the same protocol. Morphine is the gold standard for pain therapy especially in cases of treatment-resistant pain. GBP is effective in treating neuropathic pain due to post herpetic neuralgia by targeting the neuronal voltage-gated α2δ1 calcium channels to attenuate neuronal excitation.[26,27]

We observed that treating the neurons with individual cannabinoids either completely blocked or reduced the capsaicin responses to a very large extent, in most neurons. We have also shown that the cannabinoid cannabichromene blocked responses to capsaicin by activating the potassium channels in the neuron membrane, by using bee venom, which blocks potassium channels. As different cannabinoids inhibited capsaicin responses to a greater or lesser extent, we can identify which cannabinoids are likely to be most useful for blocking pain signalling to develop new drug combinations for providing pain relief.

Materials and Methods

Preparation of DRG Neurons

DRG neurons were prepared as described earlier[15] using adult female Wistar rats (Charles River, Harlow, UK), following approved procedures (by the Animal Welfare Ethical Review Body, Imperial College London, and in keeping with the 3Rs ARRIVE guidelines). Bilateral DRG from all levels were harvested in Ham's F12 medium containing penicillin/streptomycin (100 μg/mL each), and enzyme digested in 2 mL Ham's F12 medium containing antibiotics, collagenase (0.2%) and dispase (0.5%), at 37 °C for 3 h. The enzyme digested tissue was mechanically dissociated by pipetting in 1 mL BSF2 medium containing 100 ng/mL NGF and 50 ng/mL GDNF, soybean trypsin inhibitor and DNase, to obtain a cell suspension containing 150,000–200,000 neurons. This was diluted in BSF2 medium containing NGF and GDNF, and 8000–10,000 neurons were plated on each of 20 glass-bottom petri dishes (MatTek Corp., USA), precoated with 20 μg/mL each of poly-l-lysine and laminin. The culture dishes were incubated at 37 °C for 45 min for the cells to attach before adding 2 mL BSF2 medium containing NGF and GDNF to each dish. 5 μM cytosine arabinoside was added to all dishes after 24 h, to inhibit the growth of non-neuronal cells.

Calcium Imaging

Ca^{2+} imaging was performed between 2 and 4 days after plating the neurons. The neuron cultures were rinsed with warm 1 mL HEPES buffered HBSS (HHBSS) containing 10 mM HEPES and 0.1% BSA (pH 7.4). One mL HHBSS containing 2 μMol Fura2AM (Life Technologies, Paisley, UK) was added to each dish, and the petri dishes were incubated at 37 °C for 40 min in the dark. The medium containing Fura2AM was replaced with HHBSS for 20 min for de-esterification of Fura2AM. Fresh 2 mL HHBSS was added to each dish for each experiment. Twelve to fifteen healthy neurons i.e with phase bright cell bodies, absence of blebbing, and presence of neurites were selected using a 10× objective lens with brightfield illumination for each experiment, and a region of interest was highlighted using a different colour for measuring intracellular Ca^{2+} changes in each neuron (Figure 1A). The neuron culture was alternately excited at 340 and 380 nm (λex), 510 λem wavelengths to obtain the intracellular bound/unbound Ca^{2+} ratio. For vehicle treated controls, a stable baseline of the 340/380 nm ratio was recorded, and 0.1% vehicle was applied, followed by 1 μM capsaicin after 5 min. One image was captured every two seconds in each of 3 channels: brightfield, 340 nm and 380 nm λex, as previously described,[15] and the mean 340/380 nm λex ratio was recorded for each neuron to reveal intracellular Ca^{2+} changes due to vehicle, cannabinoid or capsaicin application for up to 10 minutes after capsaicin application. In each experiment, the cells demonstrating the largest Ca^{2+} responses were selected for analysis, with a minimum ratio change of 0.02 from the baseline. Responses were recorded as the difference between baseline (mean 340/380 nm λex ratio) just before adding the cannabinoid or capsaicin, and peak after addition, and normalized to the vehicle treated control average capsaicin response. In separate dishes, following baseline recording, individual cannabinoids or GBP or morphine were added at either 0.001, 0.01, 0.1, 1, 10 or 100 μM, followed 5 min later by 1 μM capsaicin. Cannabinoid

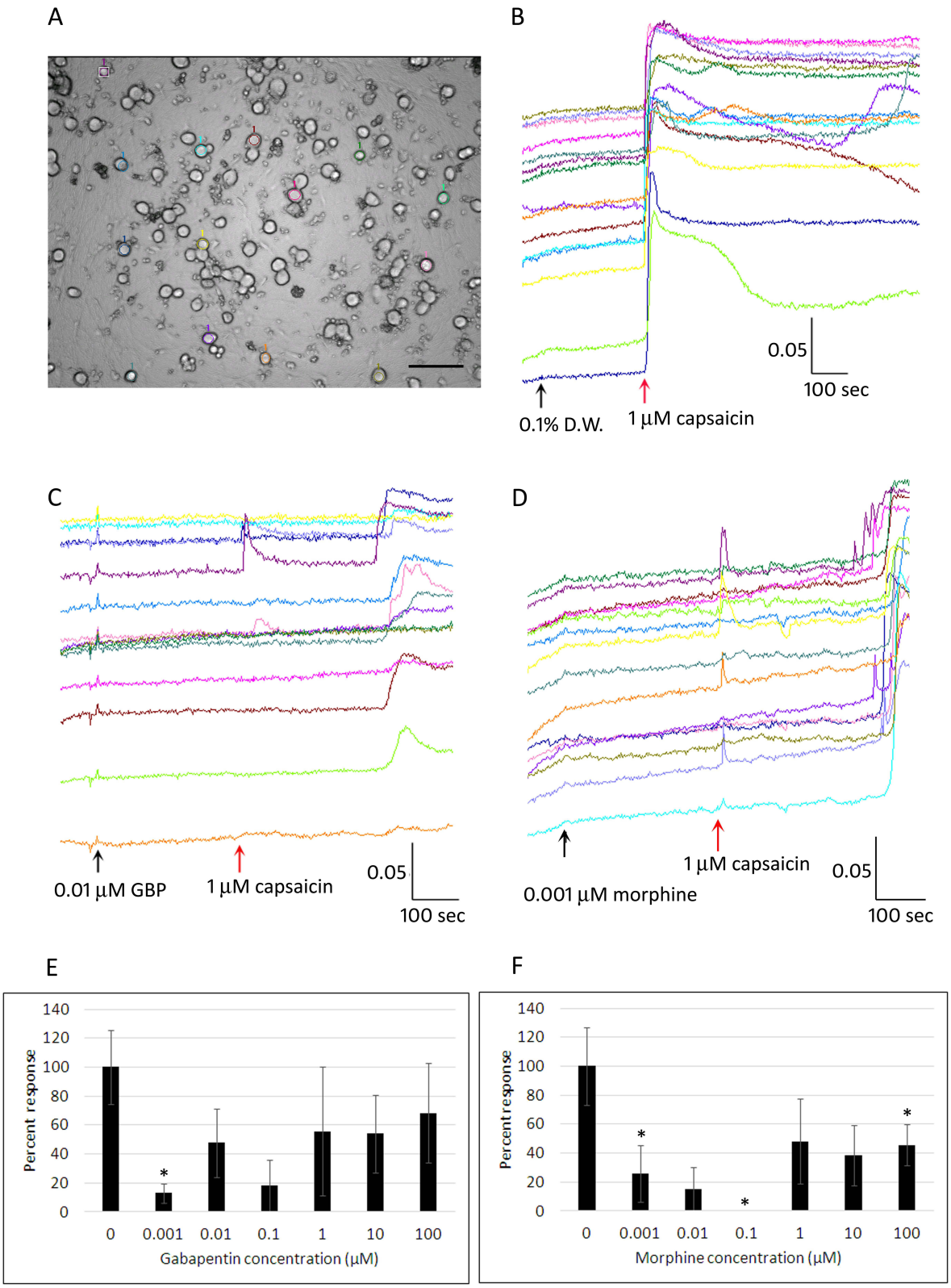

Figure 1 GBP and morphine inhibit Ca^{2+} influx. Representative brightfield image of DRG neurons showing different coloured highlighted regions of interest for measuring intracellular Ca^{2+} changes (**A**) (10x magnification). Sample traces showing intracellular calcium levels in individual neurons with stable baselines after applying vehicle (black arrow) and immediate responses to capsaicin application (red arrow) (**B**) Application of 0.01 µM GBP (**C**) and 0.001 µM morphine (**D**) did not affect the baseline but completely blocked capsaicin mediated Ca^{2+} influx, without affecting intracellular Ca^{2+} release. Graphs showing the dose related average capsaicin response (n=3 rats) in the presence of GBP (**E**) and morphine (**F**) *P<0.05. Scale bar in (**A**) 100 microns, in (**B**) (**C**) (**D**): x axis indicates time in seconds, and y axis indicates the 340/380 (bound/unbound Ca^{2+}) ratio.

effects were examined in the presence of CB_1 antagonist SR141716A or the Na^+/K^+-ATPase inhibitor ouabain or the potassium channel inhibitor tertiapin Q in the same neurons demonstrating capsaicin response inhibition, by change of medium followed 45 min later by the inhibitor and the cannabinoid. Vehicle treated neurons were similarly treated with capsaicin twice, following the same protocol, to control for desensitization due to repeated capsaicin stimulation.

The cannabinoids tetrahydrocannabiorcol (Cayman Chemical Company Ann Arbor, Michigan, USA), cannabinol, cannabitriol, cannabichromene, cannabidivarin, cannabichromevarin, cannabicitran and cannabigerol monomethyl ether, tetrahydrocannabutol and tetrahydrocannabiphorol (Medalchemy Ltd, UK), were freshly prepared in ethanol in amber glass vials immediately before use at 1000× final concentration. Stocks of Tertiapin Q (Cambridge Bioscience Ltd, Cambridge, UK), ouabain (100 mM, Abcam Ltd., Cambridge, UK) were prepared in ethanol, aliquoted and stored at −20 °C, until use. Aliquots of capsaicin stock solution (Sigma-Aldrich UK, 100 mM) prepared in ethanol were stored at −20 °C and used for preparing an intermediate stock solution of 500 µM prior to use. Morphine sulphate and Gabapentin (Abcam Ltd, Cambridge UK), were dissolved in distilled water. All chemicals were obtained from Merck (Gillingham, UK), unless indicated otherwise.

Data Analysis

Capsaicin responses were averaged from all the neurons for each concentration of each cannabinoid from at least n=3 rats, for measuring the latency (time taken to respond from applying capsaicin), and amplitude of response (peak minus baseline) at the point of capsaicin application. Average values of capsaicin response amplitudes (Ca^{2+} influx) for each cannabinoid concentration were compared to control using the Students *T*-test, and *p < 0.05 was considered statistically significant. **P<0.01, ***P<0.001.

Results

Effect of vehicle. Vehicle application (0.1% ethanol or 0.1% distilled water) in phase bright neurons (Figure 1A), had no effect on baseline intracellular Ca^{2+}. Application of 1 µM capsaicin elicited immediate and sustained Ca^{2+} influx, followed by a second response observed between 6 and 8 minutes after capsaicin application (Figure 1B). The second response was due to Ca^{2+} released from intracellular stores as it was previously shown to be thapsigargin sensitive and absent in Ca^{2+} free media.[25]

In control experiments, neurons treated with vehicle and 1 µM capsaicin had a change of medium and rest period of 45 minutes (n=4 rats, 40 neurons). This was followed by a second 1 µM capsaicin application in the presence of vehicle to control for desensitization due to repeat capsaicin stimulation and showed that the second capsaicin response was reduced to 26.9% of the first response. This control was essential for testing the cannabinoid twice in the same neurons, with and without tertiapin Q, to determine the mechanism.

Effect of Gabapentin

In the presence of gabapentin at concentrations between 0.001 and 100 µM, capsaicin responses were either reduced in some neurons or completely inhibited in others (Figure 1C). Of 257 capsaicin-sensitive neurons (n=3 rats), 51.36 ± 15.9% neurons were completely inhibited in the presence of GBP at concentrations between 0.001 and 100 µM. The average capsaicin response amplitude was reduced at all concentrations and significantly reduced in the presence of 0.001 µM GBP (*P<0.05, Figure 1C and E). Application of GBP at all concentrations tested was observed to have no effect on the baseline intracellular Ca^{2+} levels.

Effect of Morphine

Application of morphine at concentrations of 0.001–100 µM completely blocked Ca^{2+} influx in response to capsaicin in 59.2 ± 3.9% capsaicin sensitive DRG neurons, while intracellular Ca^{2+} release was present 6–8 minutes after capsaicin application (Figure 1D). At 0.001 µM, 0.1 µM and 100 µM concentrations, capsaicin responses were significantly reduced compared with control (Figure 1F). Morphine application did not affect the baseline intracellular Ca^{2+} levels at any of these concentrations.

Effect of Tetrahydrocannabiorcol (THCC)

Compared to control capsaicin responses (Figure 2A), Ca^{2+} influx in response to capsaicin application was completely inhibited in the presence of THCC at 0.001–38 μM concentration in 64.8 ± 2.2% neurons (Figure 2B), while intracellular Ca^{2+} release was present. Other capsaicin-sensitive neurons showed reduced responses in a bell-shaped distribution (Figure 3A). The highest concentration of THCC examined was 38 μM, due to solubility issues.

Effect of Cannabinol (CBN)

In the presence of CBN, Ca^{2+} influx in response to capsaicin application was completely inhibited in 71 ± 12% capsaicin-sensitive neurons (Figure 2C), while reduced responses were observed in other neurons. This inhibition was observed at all concentrations between 0.001 and 100 μM and was significantly less than control between 0.01 and 100 μM (Figure 3C).

Effect of Cannabichromene

In the presence of CBC (0.001–100 μM), Ca^{2+} influx in response to capsaicin application was completely inhibited in 48.2 ± 20.9% capsaicin-sensitive neurons (n=3 rats, 216 neurons), while intracellular Ca^{2+} release was intact. Very few neurons showed immediate responses to capsaicin ie Ca^{2+} influx (Figure 2D). The dose-related distribution of response amplitude was also bell shaped (Figure 3D).

Effect of Cannabitriol (CBT)

Application of CBT did not elicit Ca^{2+} influx at any concentration. Ca^{2+} influx in response to capsaicin was completely inhibited in 70.5 ± 13.5% capsaicin-sensitive neurons (n=3 rats, 181/252 neurons), but delayed responses indicating intracellular Ca^{2+} release, were present at all concentrations of CBT from 0.001 to 100 μM (Figures 2E and 3E). A smaller proportion of capsaicin-sensitive neurons showed immediate but reduced Ca^{2+} influx in response to capsaicin.

Effect of Cannabidivarin (CBDV)

Application of CBDV did not elicit Ca^{2+} influx at any concentration from 0.001 to 100 μM. Few neurons showed immediate responses to capsaicin, but Ca^{2+} influx was completely inhibited in 39.9 ± 15.9% capsaicin-sensitive neurons and intracellular Ca^{2+} release was observed 6–8 minutes after applying capsaicin (Figure 2F). The dose-related distribution showed significant inhibition at 1 and 100 μM. (Figure 3B).

Effect of Cannabigerol Monomethyl Ether (CBGM)

Application of cannabigerol monomethyl ether at all concentrations from 0.001 to 100 μM did not elicit Ca^{2+} influx, but completely blocked Ca^{2+} influx in response to capsaicin in approximately 66.3 ± 10.9% capsaicin sensitive DRG neurons, while intracellular Ca^{2+} release was present 6–8 minutes after capsaicin application (Figure 2G). Highly significant inhibition was observed at 0.1 and 10 μM concentrations (Figure 3H).

Effect of Cannabicitran (CBCT)

Application of cannabicitran at all concentrations from 0.001 to 100 μM did not elicit Ca^{2+} influx, but completely blocked Ca^{2+} influx in response to capsaicin in approximately 35.2 ± 16.5% capsaicin sensitive DRG neurons. Intracellular Ca^{2+} release was present 6–8 minutes after capsaicin application (Figure 2I). Ca^{2+} influx was significantly reduced in the presence of 1 and 10 μM CBCT (Figure 3F).

Effect of Cannabichromevarin (CBCV)

Application of cannabichromevarin at all concentrations from 0.001 to 100 μM did not elicit Ca^{2+} influx. Ca^{2+} influx in response to capsaicin was completely blocked in approximately 66.6 ± 6.3% DRG neurons, while intracellular Ca^{2+} release was present 6–8 minutes after capsaicin application (Figure 2H). The dose-related distribution of capsaicin response amplitude was bell shaped and significantly reduced at 0.001, 0.1, 1, 10 and 100 μM concentrations (Figure 3G).

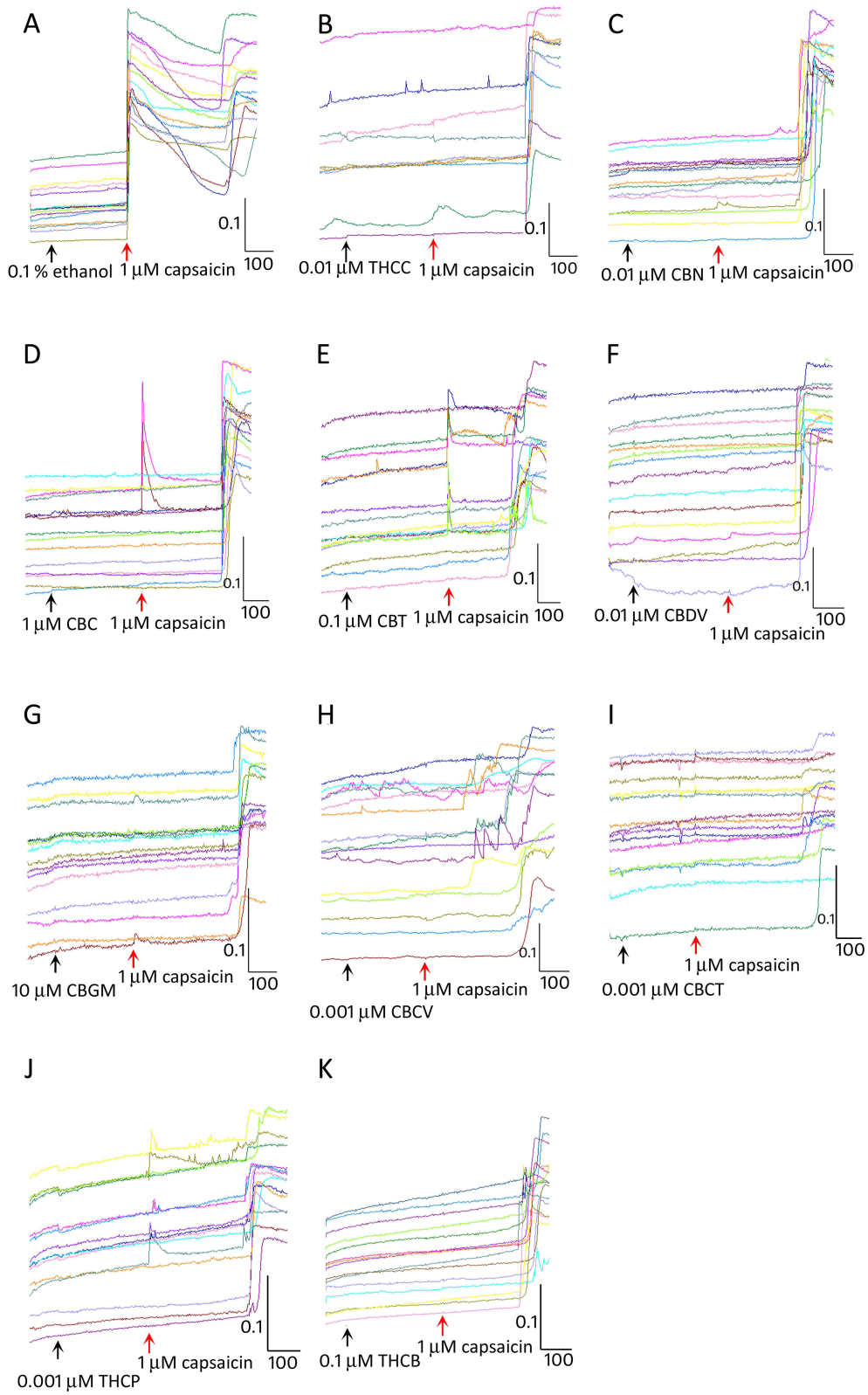

Figure 2 Minor cannabinoids inhibit Ca^{2+} influx in response to capsaicin. Sample traces of intracellular Ca^{2+} levels in individual control neurons represented by different colours, show stable baselines after vehicle application (0.1% ethanol, black arrow), while capsaicin application (red arrow) elicited immediate and sustained Ca^{2+} influx, followed 8 minutes later by Ca^{2+} release from intracellular stores (**A**) In the presence of THCC (**B**) CBN (**C**) CBC (**D**) CBT (**E**) CBDV (**F**) CBGM (**G**) CBCV (**H**) CBCT (**I**) THCP (**J**) and THCB (**K**) Ca^{2+} influx was either completely inhibited or highly reduced, followed by intracellular Ca^{2+} release. Scale bars: x-axis shows time in seconds and y-axis shows intracellular 340/380 (bound/unbound Ca^{2+}) ratio.

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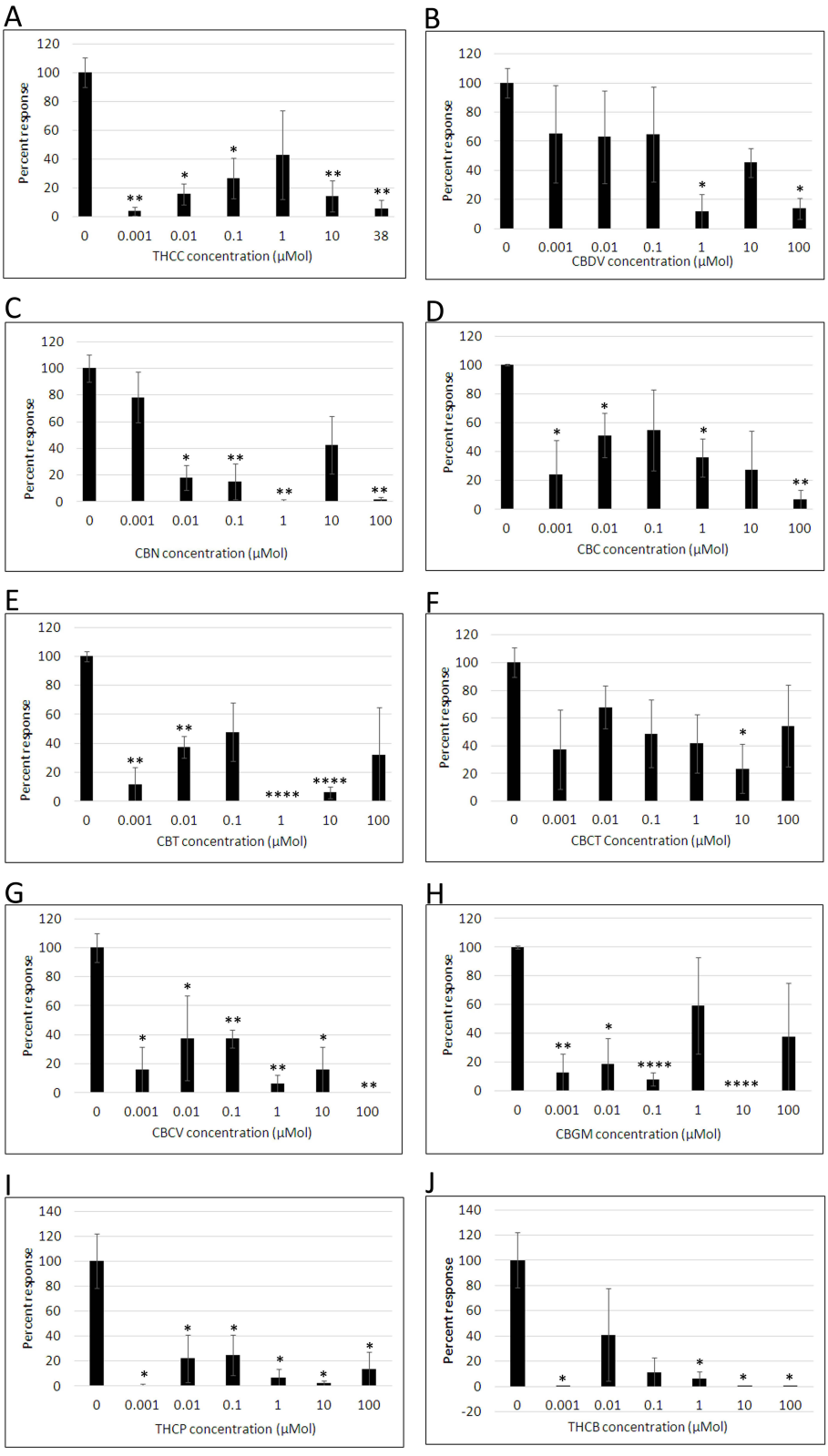

Figure 3 Dose related effects of minor cannabinoids on Ca^{2+} influx in response to capsaicin. Graphs showing the average capsaicin response at different doses of THCC (**A**) CBDV (**B**) CBN (**C**) CBC (**D**) CBT (**E**) CBCT (**F**) CBCV (**G**) CBGM (**H**) THCP (**I**) and THCB (**J**). *P<0.05, **P<0.01, ****P<0.0001.

Effect of Tetrahydrocannabiphorol (THCP)

Application of THCP at all concentrations from 0.001 to 100 µM did not elicit Ca^{2+} influx. A large proportion of capsaicin-sensitive neurons (68.36 ± 6.8%) showed no Ca^{2+} influx in response to 1 µM capsaicin, while others showed reduced responses (Figure 3I).

Effect of Tetrahydrocannabutol (THCB)

Application of THCB did not elicit Ca^{2+} influx, but capsaicin responses were completely inhibited in 78.14 ± 3.5% capsaicin-sensitive neurons. Data showing average dose-related distribution of the individual cannabinoids are presented in Figure 3J.

Table 1 provides a summary of the proportion of neurons showing complete inhibition of Ca^{2+} influx in response to capsaicin in the presence of the individual cannabinoids (n=3 rats for each cannabinoid).

Excitatory Effects of Cannabinoids

In phase bright neurons (Figure 4A), vehicle application had no effect on the baseline Ca^{2+} levels (Figure 4B), two of the ten cannabinoids described above, namely THCC and CBN were observed to elicit dose related Ca^{2+} influx following their application (Figure 4C and D). THCC application elicited Ca^{2+} influx at the higher concentrations of 10 and 38 µM (Figure 4E). In comparison, Ca^{2+} influx in response to 1 µM capsaicin in vehicle treated controls was significantly greater than influx due to 10 µM THCC (*P<0.05), and slightly greater than 38 µM THCC (Figure 4F). Although THCC application at 10 and 38 µM elicited Ca^{2+} influx in some neurons, Ca^{2+} influx in response to capsaicin was inhibited in

Table 1 Data Showing the Proportion of Total Capsaicin Sensitive Neurons That Were Completely Inhibited by the Different Cannabinoids Between 0.001 and 100 µm Concentrations. Morphine and Gabapentin Effects Have Been Included for Comparison and Show That Some Cannabinoids Appear to Be More Effective Than Morphine and Gabapentin While Others Were Less so in This in vitro Assay.

Cannabinoid	Percent Neurons Completely Inhibited	Total Neurons (n=3 Rats)
CBCT	35.2 ± 16.5	171
CBDV	39.9 ± 15.9	272
CBC	48.2 ± 20.9	216
Gabapentin	51.36 ± 15.9	257
Morphine	59.2 ± 3.9	280
THCC	64.8 ± 2.2	268
CBGM	66.3 ± 10	244
CBCV	66.6 ± 6.3	346
THCP	68.36 ± 6.8	269
CBT	70.5 ± 13.5	252
CBN	71.0 ± 12.7	233
THCB	78.14 ± 3.5	270

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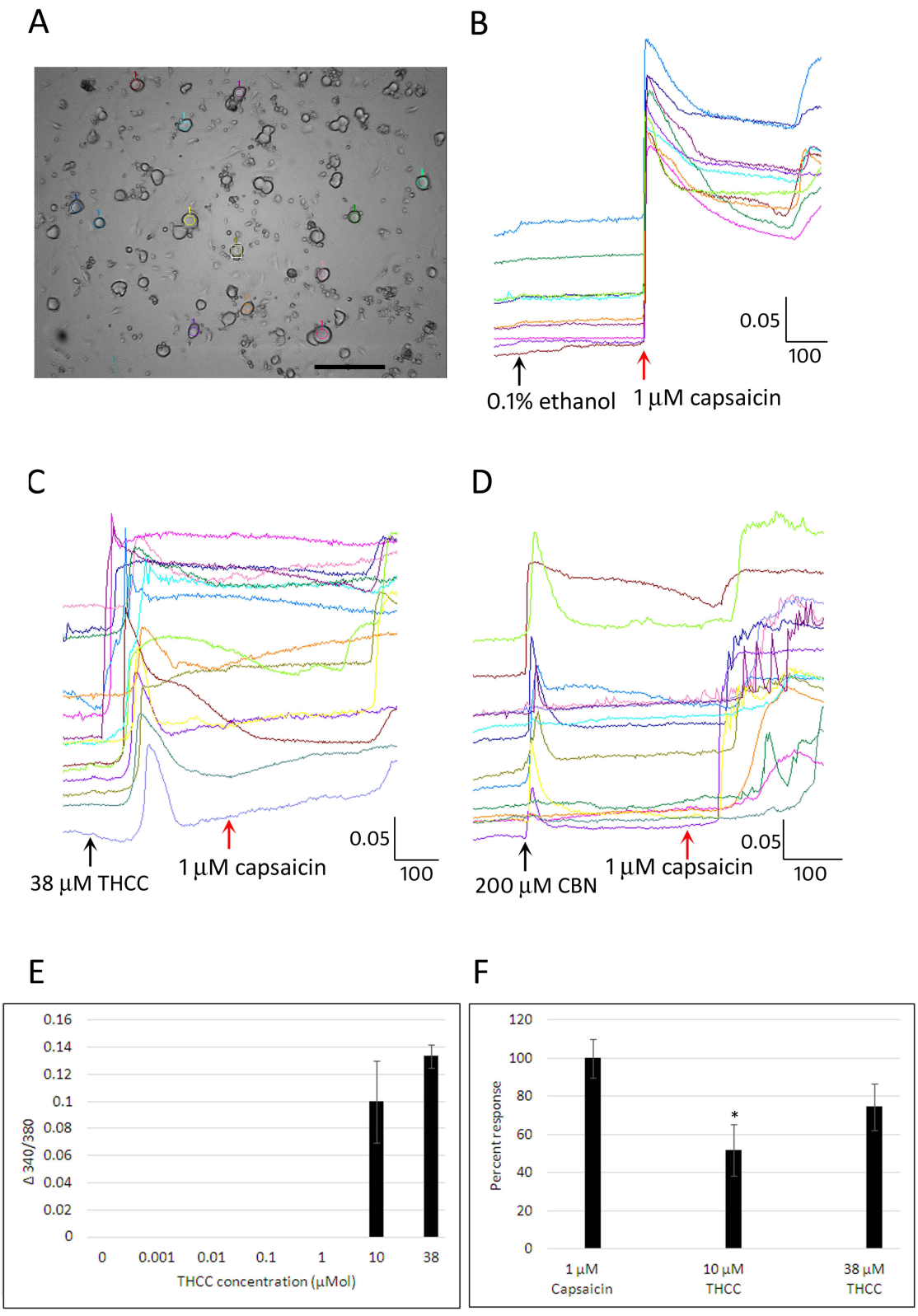

Figure 4 Excitatory effects of THCC and CBN. Sample brightfield image of DRG neurons with highlighted regions of interest for measuring intracellular Ca^{2+} levels in individual neurons represented by different colours (**A**). Traces showing vehicle application (0.1% ethanol, black arrow) did not affect baseline intracellular Ca^{2+} levels, but capsaicin application (red arrow) elicited immediate Ca^{2+} influx (**B**). 38 µM THCC (black arrow) elicited Ca^{2+} influx with subsequent inhibition of capsaicin responses (**C**). Similarly, 200 µM CBN (black arrow) elicited Ca^{2+} influx and inhibited capsaicin responses in others (**D**). Graph showing dose related amplitude of responses to 10 µM and 38 µM THCC application (**E**), and comparison of responses elicited by capsaicin and THCC (**F**). *P<0.05. Scale bars in (**B**–**D**): x-axis shows time in seconds and y-axis shows intracellular 340/380 (bound/unbound Ca^{2+}) ratio.

both groups of neurons, while intracellular Ca^{2+} release was not affected. No calcium influx was observed in response to CBN application at concentrations of 0.001–100 μM (n=3 rats), but application of 200 μM CBN elicited Ca^{2+} influx (Figure 4D), equivalent to 50% of 1 μM capsaicin response. Ca^{2+} influx in response to THCC and CBN was observed to be either transient or sustained elevation of intracellular Ca^{2+} in different neurons (Figure 4C and D).

Mechanism of Ca^{2+} Influx Inhibition by the Minor Cannabinoid CBC

To determine the mechanism of action underlying CBC-mediated inhibition of Ca^{2+} influx in response to capsaicin, we examined the effects of CBC in the presence of the K^+ channel inhibitor, tertiapin Q. Neurons in which capsaicin mediated Ca^{2+} influx was inhibited due to CBC, had a change of medium and rest period of 45 minutes to overcome desensitization due to prior capsaicin stimulation. This was followed by reapplication of CBC and capsaicin in the presence of the K^+ channel inhibitor 45 minutes later. Vehicle-treated control neurons (Figure 5A) also had a change of medium, rest period of 45 minutes, followed by vehicle and the second capsaicin stimulus. The second capsaicin response amplitude in the presence of vehicle was reduced to 26.9% of the first capsaicin response (Figure 5B and **E**). Neurons that showed complete inhibition of Ca^{2+} influx in the presence of 0.01μM CBC (Figure 5C), showed immediate responses to capsaicin in the presence of 1 μM K^+ channel inhibitor Tertiapin Q in 66/90 capsaicin-sensitive neurons (73.3% neurons n=4 rats) (Figure 5D). The percent response amplitude of 25.08 ± 7.5 was similar to the second response to capsaicin in vehicle-treated neurons (Figure 5E).

Discussion

We have used an in vitro model of neuronal hypersensitivity using DRG neurons sensitized by the presence of NGF and GDNF, to test the potential analgesic efficacy of the lesser known or "minor" cannabinoids. Ca^{2+} imaging was used to determine the effects of ten "minor" cannabinoids, namely tetrahydrocannabiorcol (THCC), cannabidivarin (CBDV), cannabinol (CBN), cannabitriol (CBT), cannabicitran (CBCT), cannabichromevarin (CBCV), cannabigerol monomethyl ether (CBGM), cannabichromene (CBC), tetrahydrocannabutol (THCB) and tetrahydrocannabiphorol (THCP). These were compared with the gold standards gabapentin (GBP) and morphine, used for treating clinical chronic pain. All the cannabinoids tested individually showed inhibition of capsaicin mediated calcium influx.

Vehicle-treated neurons showed immediate Ca^{2+} influx in response to capsaicin application, followed by calcium release from intracellular stores 6–8 minutes after applying capsaicin. In the presence of minor cannabinoids, calcium influx in response to capsaicin was completely inhibited in majority of capsaicin-sensitive neurons, but intracellular calcium release was observed 6–8 minutes after applying capsaicin. These results are similar to the effects of terpenes in our previous study which also inhibited Ca^{2+} influx in response to capsaicin using the same model, and where capsaicin responses appeared to be similarly delayed.[25] This study showed that delayed capsaicin responses observed in the presence of terpenes were thapsigargin sensitive and were eliminated in Ca^{2+}-magnesium free media, indicating that the delayed responses were due to Ca^{2+} release by intracellular stores. It further showed that calcium influx was completely inhibited in the presence of terpenes. TRPV1 receptors are present in the plasma membrane and respond to capsaicin application with immediate Ca^{2+} influx, while TRPV1 receptors in the membranes of intracellular organelles respond 6–8 minutes after applying capsaicin, by releasing stored Ca^{2+}. The capsaicin sensitivity of these neurons was indicated by the delayed response, distinguishing them from capsaicin-insensitive neurons.

In the present study, neurons treated with minor cannabinoids, GBP and morphine also potently inhibited Ca^{2+} influx in response to 1 μM capsaicin application, across a wide range of concentrations in majority of neurons, while few neurons showed reduced responses. Inhibitory effects were assessed from response amplitudes observed across a wide range of concentrations and depicted as the dose related percent average responses for the individual cannabinoids. As a large majority of neurons were completely inhibited in the presence of all the compounds tested, this proportion of the total capsaicin-sensitive neurons was used as a measure of their efficacy which is indicated in Table 1. Accordingly, THCB > CBN > CBT > THCP > CBCV > CBGM > THCC > morphine > gabapentin > CBC > CBDV > CBCT based on the proportion of capsaicin-sensitive neurons that were completely inhibited in the presence of each compound. These results show that individual cannabinoids are potent inhibitors of nociceptor activation over a wide range of concentrations in sensitized sensory neurons.

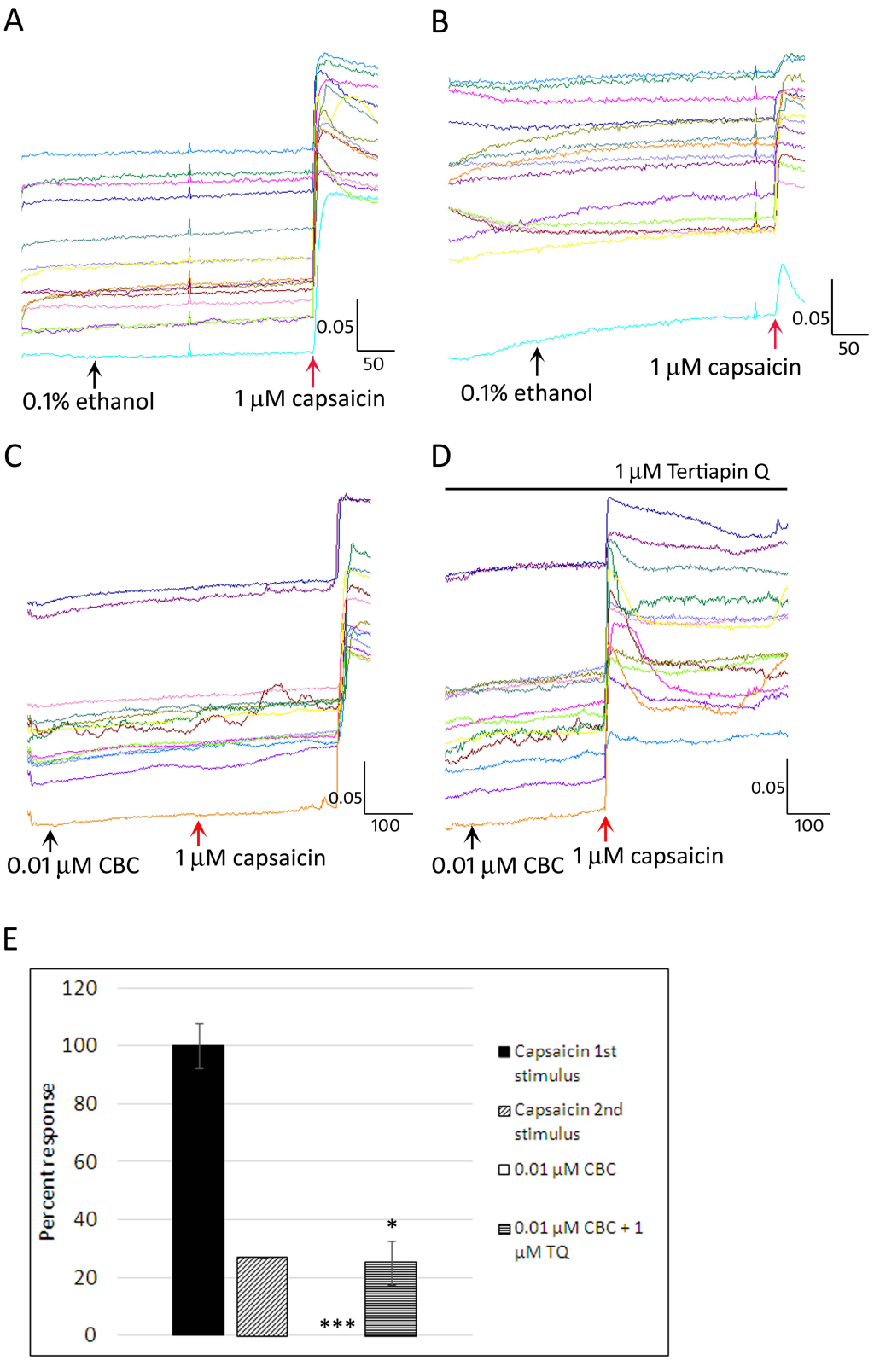

Figure 5 Effect of tertiapin Q. Sample traces with each colour representing intracellular Ca^{2+} levels in individual neurons, showing vehicle application (0.1% ethanol, black arrow) followed by immediate Ca^{2+} influx in response to capsaicin application (red arrow, **A**). After change of medium and 45-minute rest period, the same neurons (in "A") were treated with vehicle and second capsaicin application, which showed reduced responses (**B**). In the presence of 0.01 µM CBC (black arrow), Ca^{2+} influx in response to capsaicin application (red arrow), was completely blocked without affecting intracellular Ca^{2+} release (**C**). After change of medium and 45-minute rest period, the same neurons (in "C") were treated with the potassium channel inhibitor tertiapin Q, and 0.01 µM CBC resulting in immediate robust responses to capsaicin (**D**). Graph showing reduced amplitude of second capsaicin responses in vehicle treated control neurons. CBC inhibition of capsaicin mediated calcium influx was reversed in the presence of Tertiapin Q (**E**). Scale bars in (**A**–**D**): x-axis shows time in seconds and y-axis shows intracellular 340/380 (bound/unbound Ca^{2+}) ratio.

We observed similar inhibitory effects of morphine and GBP, as the cannabinoids, though some cannabinoids inhibited greater number of neurons than morphine or gabapentin in this in vitro assay. Morphine is known to provide analgesia via opioid receptor activation, with hyperpolarization effects in the CNS and other tissues.[28–30] Our findings of complete Ca^{2+} influx inhibition in the presence of morphine are consistent with these results of neuronal inactivation.

Our preliminary results showed that inhibition of capsaicin mediated calcium influx by CBC was not reversed by the presence of the Na^{+}/K^{+}-ATPase inhibitor ouabain, unlike inhibition by terpenes in our previous studies.[25] However, pretreatment with the K^{+} channel inhibitor tertiapin Q reversed the inhibitory effects of CBC and restored Ca^{2+} influx immediately after applying capsaicin, indicating that CBC had the effect of K^{+} channel activation. This observation is consistent with a previous study showing CBD activation of neuronal Kv7 channels that resulted in reduced neuronal excitability.[31] Similarly synthetic cannabinoid activation of G protein-gated inwardly rectifying potassium^{+} (GIRK) channels at submicromolar concentrations resulted in membrane hyperpolarization.[32,33] Potassium channel activation results in hyperpolarization of sensory neurons in vitro and reduced nocifensive behaviour in vivo, by blocking action potential propagation.[34] Another study showed that CBC activated CB_2 receptors resulting in hyperpolarization of AtT20 cells.[35]

The capsaicin response amplitude in the presence of tertiapin Q was similar to the corresponding second capsaicin response in vehicle-treated controls. Increased intracellular Ca^{2+} due to Ca^{2+} influx following capsaicin application or intracellular Ca^{2+} release results in desensitization. This results in reduced subsequent responses to repeat stimulation, depending on the concentration and duration of application.[36–38] In our study, the neurons were exposed to capsaicin for up to 10 minutes which led to significant desensitization and reduced second capsaicin response.

The restoration of capsaicin evoked Ca^{2+} influx in the presence of the K^{+} channel inhibitor tertiapin Q, indicates that CBC activated K^{+} channels leading to neuronal inhibition. Thus, the minor cannabinoids have a similar effect of inhibiting capsaicin evoked Ca^{2+} influx as cannabinoid terpenes but via different targets. While terpenes activated the ouabain-sensitive plasma membrane Na^{+}/K^{+} ATPase, cannabinoids activated the tertiapin Q sensitive K^{+} channels. The inhibitory effects of the other cannabinoids tested here may involve similar mechanisms and need to be confirmed in future studies.

Four types of K^{+} channels are expressed in DRG neurons.[39] Nerve injury in rodent models of neuropathic pain is associated with reduced expression of K^{+} channels and hyperalgesia,[40–42] that is reversed by K^{+} channel openers.[43] The Ca^{2+} activated potassium channels SK (small conductance) and IK (intermediate conductance) are expressed in human DRG neurons and injured peripheral nerves, and their expression is reduced after injury, which enhances nerve excitability.[44,45] Further, K^{+} channel blockers are known to excite sensory nerve endings[46] while K^{+} channel activation has an inhibitory effect via membrane hyperpolarization.[32,33]

Tertiapin Q is a peptide derived from honeybee venom, that blocks GIRK channels[47] and large conductance potassium (BK) channels[48] increasing activity in DRG neurons. The reversal of CBC mediated inhibition of capsaicin responses in the presence of Tertiapin Q indicates that CBC had the effect of K^{+} channel activation.

GBP treatment resulted in complete inhibition of capsaicin mediated Ca^{2+} influx in 51.36 ± 15.9% of capsaicin-sensitive neurons. GBP alleviates neuropathic pain by binding to the voltage gated Ca-α2δ1 channels in sensory neurons, preventing their trafficking to presynaptic terminals[26] and diminishing action potential frequency in injured but not normal DRG neurons.[49] There is further evidence that BK potassium channels are co-expressed and interact with Cavα2δ1 channels in DRG neurons, to reduce their cell surface expression, and inhibit neuropathic pain.[50] GBP was also reported to block TRPA1 activation in peripheral sensory neurons.[51]

Of the ten cannabinoids tested here, THCC and CBN demonstrated a dual effect of dose-related inhibitory or excitatory effects. THCC application at the higher concentrations of 10 and 38 µM, elicited Ca^{2+} influx, but not at lower concentrations. This finding agrees with previous reports of THCC eliciting Ca^{2+} influx, by activating the noxious sensor TRPA1 in DRG neurons at high micromolar concentrations.[9] There is a strong overlap of TRPA1 and TRPV1 expression in rodent and human DRG neurons leading to cross-desensitization.[52] Like THCC, CBN application also elicited Ca^{2+} influx in DRG neurons at the higher concentration of 200 µM, but inhibited Ca^{2+} influx between 0.001 and 100 µM concentration. Similar inhibition of Ca^{2+} uptake by submicromolar concentrations of THC, CBD and CBN, was described in brain synaptosomes, related to membrane potential,[53] at concentrations observed in human plasma levels

after smoking marijuana.[54] CBN evoked Ca^{2+} influx has been reported in previous studies and has the potential to cause desensitization. Some cannabinoids have previously been shown to elicit Ca^{2+} influx at high micromolar concentrations, followed by desensitization to subsequent capsaicin stimulation in TRPV1 transfected HEK cells,[55] and DRG neurons.[15] Accordingly, our present study also showed Ca^{2+} influx with 10 and 38 μM THCC and 200 μM CBN, followed by inhibition of responses to capsaicin applied subsequently. Similar dose-related effects of capsaicin response inhibition by CBG, CBD and THC were previously reported by us; this study also showed excitatory effects of eliciting calcium influx by these cannabinoids at higher concentrations.[15] Thus cannabinoids appear to exert biphasic dose-related effects that are inhibitory at low concentrations and excitatory at high concentrations in DRG neurons.

While the inhibitory effects of THC, CBD and CBG were previously observed above 1 μM concentration, the minor cannabinoids tested here demonstrated potent inhibition at lower concentrations including 0.001 μM. As the effects are additive, this feature may underlie the enhanced efficacy of full-spectrum cannabinoid combinations.

Cannabinoids have inhibitory effects on inflammatory cells including macrophages[56,57] which may be present in DRG neuron cultures. Our findings are unlikely to represent effects of macrophage derived inflammatory mediators, as all the cultures in our study were treated with cytosine arabinoside, a well-established antimitotic agent commonly used to inhibit non-neuronal cell proliferation in vitro,[20] and for antineoplastic therapy in the clinic.[58,59]

Conclusion

In conclusion, our results show that the minor cannabinoids potently inhibit TRPV1 signaling in sensitized DRG neurons, and for CBC by blocking Ca^{2+} influx via K^+ channel activation. This conclusion is based on the reversal of CBC-mediated inhibition in the presence of the K^+ channel inhibitor Tertiapin Q. Further studies are necessary to confirm the mechanism, pathways and targets involved in the observed inhibitory effects of the other minor cannabinoids. This will facilitate the identification of cannabinoid combinations likely to have the maximum effect in providing analgesia for inhibiting neuronal sensitization that underlies chronic pain.

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Disclosure

M.H.S. is a consultant hepatopancreatobiliary surgeon, a director at Sapphire Medical Clinics and a consultant at Imperial College NHS Trust, London. He is a senior clinical lecturer at Imperial College London and Chief Medical Officer at Curaleaf International. The authors declare no other conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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