Evaluating naturally-derived cannabis combinations and conopeptides in a rat SCI pain model

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Introduction

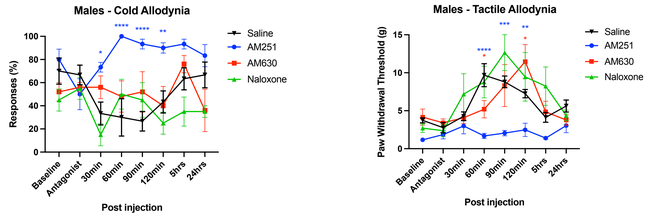
Chronic neuropathic pain resulting from spinal cord injury (SCI) is often difficult to treat, as most available therapies are associated with undesirable side effects or are only modestly effective. Activation of cannabinoid receptors have been known to regulate pain responses. Cannabinoid receptor 1 (CB1) is found throughout the CNS and is a promising target for attenuating neuropathic pain. As such, we looked at two sources of CB receptor agonists, plant-derived cannabis constituents and Conus venoms, for SCI pain. For phytocannabanoids, we explored combinations of cannabidiol (CBD) and β-caryophyllene (BCP), as neither is associated with psychoactive side effects. To test these, the analgesic properties of CBD, BCP and their combination in a rat SCI clip compression pain model were tested, and their activity at CB1 and CB2 receptors in vitro were evaluated. In addition, the venom of marine snail genus Conus is a natural source of various peptides with potent antinociceptive activity; thus we screened these as a potential alternative sources of CB1 receptor active peptides that may hold promise for pain-reducing therapies. The long-term goal of this work is to develop more effective and safe treatments for the management of chronic SCI pain.

Methods

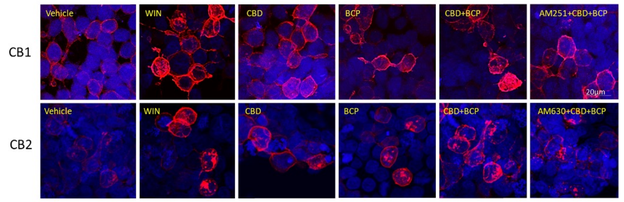
Animals: Male and female Sprague Dawley rats, 140g initially. **Spinal cord injury (SCI):** 60 second clip compression at T6-T8. **Behavioral evaluation:** Animals were evaluated for the presence of tactile and cold allodynia. Von Frey test: Animals were placed into testing cages with mesh floor, with the access to hind paws. Von Frey filaments were applied gently to plantar area of the hind paw and the response (paw withdrawal, licking, shaking, head turning to stimulus) was recorded. Acetone test: Animals were placed in the same testing cage as above. 100uL of acetone was gently squirted onto plantar surface and the response was recorded. **Drug Injection:** Our pre-determined A50 CBD/BCP dose was coadministred with one of the following antagonists: AM251 (3mg/kg, sc), AM630 (1mg/kg, sc), or Naloxone (5mg/kg, sc) and assessed by Von Frey test and Acetone evaporation test. Rats were tested at baseline and every 30 min for up to 2 hrs post- injection, then again at 5 and 24 hours post-drug administration. **Cell Stimulation:** Cells in 12 well plates were treated for 20 minutes with 600 µL of vehicle or drugs at following concentrations: Synthetic mixed CB agonist WIN55,212-2 (3µM), CBD (0.5mg/ml), BCP (4mg/ml), CBD/BCP (0.5mg/ml:4mg/ml). Cells were washed, fixed with 4% paraformaldehyde and stained. **Dose Response:** The "low" dose consisted of 0.25mg/mL of CBD, 2mg/mL of BCP, and 0.25:2 mg/mL of CBD:BCP. The "medium" dose consisted of 0.5mg/mL of CBD, 4mg/mL of BCP and 0.5:4 mg/mL of CBD:BCP. The "high" dose consisted of 1mg/mL of CBD, 8mg/mL of BCP, and 1:8mg/mL of CBD:BCP. **Fractions:** HPLC fractions of Conus venoms were evaluated for CB1 internalization capacity. C.Tex fractions were selected, active peptide isolated by immunoprecipitation and characterized by Mass Spectrometry. **Recombinant AAV2/8 particles:** Plasmids with identified sequences of two C.Tex fractions were designed and generated by Vector Builder, AAV2/8 particles were synthetized by the Viral Vector core. **Immunostaining and analysis:** Cells were stained using standard immunocytochemistry with hemagglutinin as primary antibody (1:200) and Alexa Fluor 598 as secondary antibody. Cells were observed under 40x magnification and the proportion of cells with visually identified internalization was counted out of 20-30 examined cells at random location within the slide. Internalization of CB1 receptor was evaluated by stereologic analysis (Neurolucida, MBF Bioscience) and ImageJ. **In vivo AAV injections:** AAV2/8 encoding the two selected C. Tex fractions (L914-195 and L914-185) were injected intrathecally at 4 weeks post-SCI and tested over time for changes in tactile, cold, and heat hypersensitivity. CSF samples from these animals were collected and assayed in vitro for retention of CB1 agonist activity.

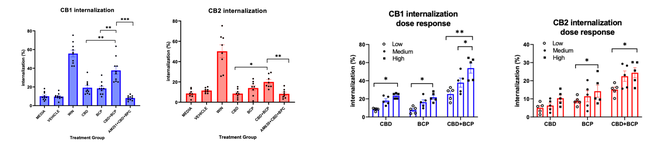
Results

Behavioral evaluations and internalization of CB1 and CB2 receptors



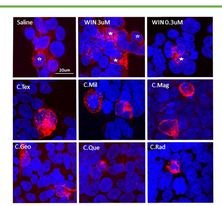
CB2 receptor antagonist AM630 and μ-opioid receptor antagonist naloxone did not affect the anti-allodynic effects of CBD:BCP coadministration. However, CB1 receptor antagonist AM251 did, which differs from the existing body of literature that suggests neither CBD nor BCP act via CB1 receptors. This suggests that the mechanism of action underlying CBD:BCP's combined analgesic effect may be partially mediated by CB1 receptors upregulated in SCI. Another possibility is that coadministration indirectly activates CB1 pathways via a cascade effect from the activation of upstream pathways. *, **, ***, **** denote p < 0.05, 0.01, 0.001 and 0.0001 compared to vehicle for each treatment group.



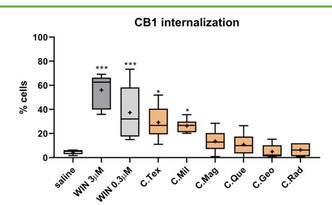


Immunostaining of HEK 293 cells expressing CB1 (labeled red) after the drug treatment. Receptors are located in the membrane and upon activation are translocated into cytoplasm. Strong activation of both CB1 and CB2 receptors was observed with synthetic mixed CB receptor agonist WIN55212-2 (positive control). Both CBD and BCP elicited minimal CB1 receptor internalization individual. The combination produced strong effects on CB1 internalization. CB1 internalization with combined CBD/BCP was blocked by CB1-selective antagonist AM251. Media and Vehicle treatment groups served as negative controls. CB2 internalization was also moderately enhanced with the CBD/BCP combination, and this was blocked by CB2-selective antagonist AM630. A dose response study was also conducted which yielded significant dose-related increases in internalization with higher drug doses, particularly for the combinations. *, **, ***, **** denote p < 0.05, 0.01, 0.001 and 0.0001 compared to the group indicated on the graph.

Screening of venom samples for identification of CB1 peptide



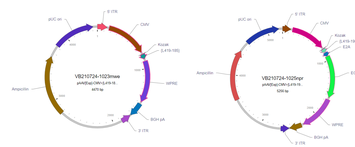
Examples of internalization of CB1 (red) in HEK293 cells, DAPI used as a nuclear staining. Top row: full stars indicate cells with internalized receptors, open stars indicate cells with receptor in the cytoplasmic membrane.



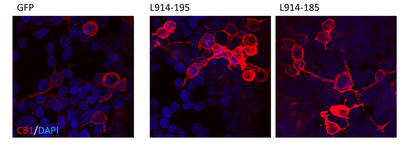
CB1 internalization of venom samples. WIN 55,212-2 as positive control. From these results, we selected C. Tex for further subfractionation and purification.
*,***P<0.05, 0.001 respectively vs saline.

Conopeptides

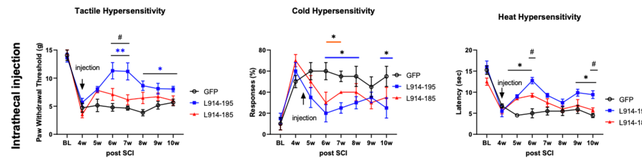
Screening of venom samples (CB1) and Identification



Two fractions derived from C. Tex were identified in the process of HPLC fractionation, immunoprecipitation and Mass Spectrometry. Sequences of fractions L914-185 and L914-195 were inserted into AAV plasmid (Vector Builder) and AAV particles were synthetized.



Analysis of the CB1 receptor internalization by CSF taken from treated animals treated with the two C. Tex subfractions L914-195 and L914-185 selected from initial screening. The internalization indicates the presence of CB1 active recombinant peptides in the samples.



Pain hypersensitivity in SCI animals treated by AAV2/8 encoding L914-195 and L914-185 with GFP as a control vector after intrathecal injections. Recombinant viral particles were injected at 4 weeks post SCI. *, **p<0.05, 0.01 vs GFP, #p<0.05 between treated groups. Results showed that both AAV-CB1 conopeptides reduced SCI pain, with the L914-195 peptide appearing most potent in all three pain tests.

Conclusions

- Analgesic effects of the CBD/BCP combination in reducing SCI pain appeared to be mediated by CB1, but not CB2 or opioid receptors.
- In vitro experiments showed that combined CBD/BCP could increase internalization of CB receptors, especially CB1, more than the individual treatments, supporting the in vivo findings using this combination.
- Together, our data suggests that the combination of CBD/BCP can reduce SCI pain via a novel interaction on CB receptors. This may be an improved approach to manage SCI pain with non-psychoactive cannabinoid combinations.
- In vitro screening of Conus venoms revealed CB1 receptor activity particularly in C. textile. Subfractions resulted in two promising conopeptides that may be developed for therapeutic application.
- cDNA sequences of promising CB1-active conopeptides inserted into AAV2/8 were showed promise as a gene therapy for reduction of SCI pain and are being further tested.
- Together, these results suggest that compounds targeting the CB1 receptor may prove beneficial in the management of SCI neuropathic pain.

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COI: The University of Miami and Drs Sagen and Jergova hold the rights to intellectual property used in the study and may financially benefit from the commercialization of the intellectual property.