Altered patterns of filopodia production in CHO cells heterologously expressing zebra finch CB₁ cannabinoid receptors

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Abbreviations: CHO cells, Chinese hamster ovary cells; DMSO, dimethyl sulfoxide; M/C, membranecyttoplasm; PBS, phosphate-buffered saline; SR, the CB₁ receptor-selective antagonist/inverse agonist SR141716A; WIN, the cannabinoid agonist WIN55212-2; zfCB1, zebra finch CB₁ cannabinoid receptor

Recent findings indicate that cannabinoid-altered vocal development involves elevated densities of dendritic spines in a subset of brain regions involved in zebra finch song learning and production suggesting that cannabinoid receptor activation may regulate cell structure. Here we report that activation of zebra finch CB₁ receptors (zfCB₁, delivered by a lentivector to CHO cells) produces dose-dependent biphasic effects on the mean length of filopodia expressed: Low agonist concentrations (3 nM WIN55212-2) increase lengths while higher concentrations reduce them. In contrast, treatment of zfCB₁-expressing cells with the antagonist/inverse agonist SR141716A causes increases in both mean filopodia length and number at 30 and 100 nM. These results demonstrate that CB₁ receptor activation can differentially influence filopodia elongation depending on dose, and demonstrate that manipulation of cannabinoid receptor activity is capable of modulating cell morphology.

Introduction

We and others have found that cannabinoid exposure during late-postnatal development leads to persistently-altered behavior in adulthood. For example, in rats, pubertal cannabinoid treatment results in persistent deficits in sensorimotor gating,1 recognition memory,2 and increased tendency to consume other drugs of abuse.3,4 In a vocal learning songbird we have found that early cannabinoid exposure persistently alters the course of vocal development5-7 and expression of endogenous cannabinoid signaling elements.8 Persistent alterations in behavior that last through adulthood must be attributable to induced changes in the physiology of the CNS substrates responsible.

We are currently working to identify these changes. We have found that development-altering cannabinoid exposure leads to an inappropriate elevation in dendritic spine densities in a subset of brain regions responsible for vocal learning.9 This implies a cannabinoid effect to alter neuronal morphology. To begin to study the mechanisms responsible for cannabinoid-altered cell morphology we have developed a lentivector to functionally express the zfCB₁ in mammalian cells. The lentivector system will be useful for studying effects of in vivo delivery of the receptor. Using this vector to transfect CHO cells we have found that agonist activation and antagonist inhibition of zfCB₁ produce distinct effects on both the length and number of filopodia expressed.

Filopodia are fine, microfilament-based cellular protrusions involved in migration, adhesion and environmental sensing.10 These structures are dynamic, and depend upon F-actin polymerization and depolymerization for elongation and contraction.11 Given evidence for a role for cannabinoid signaling in regulating dendritic spine densities and neuronal growth cone migration, another F-actin-dependent process, we hypothesized that cannabinoid signaling may interact with the cytoarchitecture of all cell types.9,12 This hypothesis was tested through the experiments described below.

Results

CB₁ expression, translocation and agonist-induced internalization. In order to assess transfection and functional expression of zfCB₁ delivered by the lentivirus, CHO cells were grown to 40–50% confluence in 6-well plates, each well with a diameter of 35 mm, and infected with an optimized concentration of the lentivector. Approximately 48 h following infections, cells were fixed, permeabilized and treated with the anti-zebra finch CB₁ receptor antibody that we previously developed.13

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Immunocytochemistry revealed that zfCB\textsubscript{1} successfully traffics to plasma membranes and filopodia of the subpopulation of filopodia-expressing CHO cells. Filopodial expression appeared punctuate, with clustered aggregations of receptor protein (see Fig. 1B). These aggregations appear to be a distinct feature of filopodial expression, as they are not characteristic of plasma

**Figure 1.** The zfCB\textsubscript{1} receptor successfully translocates to CHO cell plasma membranes and filopodia. (A) TRITC-phalloidin stains F-actin; and (B) the same cells stained with a specific antibody against zfCB\textsubscript{1}. The arrows in (B) indicate zfCB\textsubscript{1} targets the membrane and travels to filopodia in CHO cells. Cells with particularly distinct intensity of filopodial staining are presented to most clearly illustrate punctate aggregation of receptor protein. (C–E) Agonist treatment of CHO cells stably expressing zfCB\textsubscript{1} receptors are internalized. Cultures were treated for 30 min with: (C) Vehicle; (D) the agonist WIN55212-2 at 100 nM and (E) the CB\textsubscript{1}-selective antagonist SR141716A at 100 nM. (F) Optical density analysis of relative intensities of membranecytoplasm immunofluorescence (M/C) reveals a significant reduction from control levels after agonist exposure (n = 20 cells, bars = 15 microns).
membrane expression that we and others have observed in non-filopodia-expressing cells14 (e.g., Fig. 1B).

In filopodia-expressing neurons, CB1 receptors have been shown to be rapidly internalized following agonist stimulation.15 In order to assess effects of activation of the transfected CB1 receptor in our system, infected CHO cells were challenged with 100 nM of the cannabinoid agonist, WIN55212–2 (WIN). This agonist exposure resulted in a rapid internalization of both membrane and filopodial populations of receptor, as reflected by the ratio of membrane to cytoplasmic immunocytochemical OD measures (M/C, from 1.99 ± 0.57 to 0.62 ± 0.15, *p < 0.05, ANOVA followed by SNK post-test, Fig. 1C–E). This internalization was maximal by 10 min (data not shown). Pretreatment with 100 nM the CB1-selective inverse agonist/antagonist SR reversed internalization effects of WIN (Fig. 1E and F, 1.99 ± 0.57 vs. 1.85 ± 0.49). Reversal of agonist-induced internalization by SR is consistent with antagonist activity of this compound. Note that prior experiments have determined that both WIN and SR have high affinity for zfCB1 (Kd = 63 and 89 nM, respectively).16 SR has been shown to exert efficacy through the cannabinoid-like receptor GPR55 at concentrations above 1 μM.17 As the concentration we have used was 10-fold lower than that required for threshold effects in this other culture system, it is unlikely that GPR55 activation would have been produced in our studies.

**Dose-response effects on filopodia length.** After establishing functional zfCB1 expression in CHO cells, we began experiments to assess potential effects of activating the receptor on cellular morphology. Dose-response experiments employing various concentrations of the agonist WIN resulted in a clear and interesting biphasic effect on filopodia length in zfCB1-expressing cells. The relatively low concentration of 3 nM WIN significantly increased filopodia lengths over vehicle controls (from 34.3 ± 0.99 to 45.6 ± 1.65 μm, p < 0.05, Kruskal-Wallis ANOVA followed by Rank-Sum tests) while higher 30 and 100 nM concentrations produced significant reductions (from 34.3 ± 0.99 to 26.4 ± 0.92, and to 26.9 ± 0.93 μm respectively, p < 0.05, Kruskal-Wallis ANOVA followed by Rank-Sum tests, Fig. 2A). Similar to effects produced by agonist at low concentration, but in contrast to inhibitory effects of high agonist concentrations, the antagonist/inverse agonist SR increased mean filopodia lengths (from 45.2 ± 1.2 to 56.2 ± 1.3 at 30 nM, and to 57.9 ± 1.4 μm at 100 nM, Kruskal-Wallis ANOVA followed by Rank-Sum tests, Fig. 1B). This effect is consistent with an inverse-agonist type mechanism to reduce the probability of constitutive receptor activation.

Analysis of quantal frequency distributions of filopodia length following WIN treatment shows that effects of low concentrations (3 nM) to increase mean filopodia lengths is associated with increased variance around the mean. This variance is attributable to increased frequencies of longer filopodia, possibly indicating that processes related to regulation of filopodia length are more sensitive to cannabinoid inhibition than those involved in extension (see Fig. 3C). In contrast, effects produced by 30 and 100 nM WIN concentrations were associated with reduced variance; filopodia of more consistent and relatively short sizes were produced (see Fig. 3E and F), suggesting that these concentrations effectively reduce filopodial extension. Consistent with effects of 3 nM WIN, both 30 nM and 100 nM concentrations of SR increased the variance of filopodia length distributions (see Fig. 4E and F).

**Dose-response effects on filopodia number.** In order to determine if zfCB1 receptor activation may alter de novo production of filopodia, numbers of processes per cell were determined. Results of these studies show that numbers of filopodia expressed were not affected by the concentrations of WIN employed (3, 10, 30 and 100 nM, see Fig. 2C). In contrast, the antagonist/inverse agonist SR did significantly increase mean numbers of filopodia/cell from 33.1 ± 2.8 to 47.6 ± 3.9 at 3 nM, 46.1 ± 3.7 at 30 nM and 46.4 ± 4.6 at 100 nM, p < 0.05 Kruskal-Wallis ANOVA followed by Rank-Sum tests (Fig. 2D). Ten nanomolar SR was not associated with a significant change. Combined with the filopodia length results, these results suggest that low-level zfCB1 activity, either through constitutive activation or stimulation by endogenous agonist (possibly released as free fatty acid following phospholipase cleavage from lipid membrane precursors) reduces filopodia production, and at high concentrations also inhibits filopodia extension.

**Discussion**

Others have studied filopodia production in CHO cells in the context of exploring effects of altered cytoskeletal and related signaling proteins.19-21 CB1-mediated signaling is known to be involved in the control of axonal migration and growth cone structure in the developing retina22 and cortex.23-26 We have recently discovered that developmental, but not adult cannabinoid exposure increases dendritic spine densities in song control regions of zebra finch telencephalon.27 Our goal here was to develop a system relevant to understanding how these receptors biochemically contribute to altering cellular morphology. Our results provide insight to distinct, dose-dependent effects of cannabinoids to both promote, and inhibit production and length of filopodia. What is learned about cannabinoid effects on cell structure in these undifferentiated cells will provide a comparative foundation for understanding mechanisms responsible for potentially more complex neuronal processes. Development of the lentiviral delivery system will allow in vivo modulation of cannabinoid signaling systems in freely-behaving animals.

The lentivector developed delivers zfCB1 cDNA in a manner resulting in receptor protein that is trafficked and internalized in a manner consistent with mammalian isoforms delivered by lipid reagents.14 Development of this vector may become an important tool allowing delivery of the receptor in vivo via microinjection, and direct manipulation of zfCB1 expression during development.

The distinct, dense pattern of zfCB1 expression in the subset of CHO cells expressing filopodia is particularly notable, and suggests that endogenous cannabinoid signaling may play a role in modulating the sensing and motility functions of filopodia in non-neuronal cell types. Such a role is consistent with evidence demonstrating that cannabinoid signaling is important to axon guidance (mentioned above), and the migration of newborn neurons from ventricular zones.12,15,27
Modulation of zfCB₁ activity produced significant effects on filopodia lengths. In neurons, endocannabinoids are known to result in RhoA activation. RhoA activation induces cytoskeletal reorganization resulting in growth cone breakdown and inhibition of axon growth. Thus, it is possible that RhoA activation may be responsible for effects of high agonist concentrations to reduce filopodia length in our CHO cell system.

The biphasic effect of the agonist, WIN, where the low-dose of 3 nM increased, and higher dosages (30 and 100 nM) reduced filopodia length was surprising, but is not an isolated report. These biphasic effects are consistent with a conflicted literature, where CB₁ activation is linked to promotion and migration of cellular processes and to inhibition or stabilization of their growth. Such biphasic responses following G-protein-coupled receptor activation may involve differential coupling with cellular effectors, resulting in different affinity states of receptor-effector complexes for WIN. Higher affinity complexes are liable to activation at relatively low agonist concentrations, resulting in differential signaling, and potentially distinct effects. A possibility is that zfCB₁ receptors in high affinity states (and therefore bound at lower agonist concentrations) are less efficacious in inhibiting filopodia extension than lower affinity populations that require higher agonist concentrations for activation, either due to distinct localization and/or effector coupling. For example, non-filipodial zfCB₁ receptors in cell bodies may be coupled to a distinct population of signaling...
proteins that result in higher-affinity conformations, but are not relevant to control of filopodia extension.

The stimulatory effect of the SR antagonist/inverse agonist to increase filopodia lengths implies significant, inhibitory, constitutive receptor activation in the expression system we employed. Constitutive, agonist-independent activity of G-protein-coupled receptors is a well-established phenomenon and is widely viewed as the mechanistic foundation of inverse agonism that is consistent with effects of SR that we report here. According to this mechanism, inverse agonists like SR have distinctly high affinity for receptors in the unliganded, but constitutively activated state. Once bound, the inverse agonist effects an

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**Figure 3.** Frequency distributions of filopodia length show that higher cannabinoid agonist concentrations tend to decrease variance around arithmetic means. Significant reductions in mean filopodia length following treatment with 30 nM and 100 nM WIN are attributable to decreased frequencies of filopodia with lengths exceeding 50 µm.
inhibitory conformational change in the receptor that prevents further signal transduction. Thus, by driving equilibrium away from constitutive activation, the inverse agonist produces physiological effects that oppose those of agonists.

It may also be possible that SR has antagonized an inhibitory endocannabinoid tone. Potential sources of endocannabinoids include the serum component of the culture media used and synthesis/release from the cultured cells themselves, as endocannabinoids are fatty acids and known fractional constituents of cell membranes subject to phospholipase liberation. It is also possible that SR has intrinsic agonist efficacy, and is capable of producing effects in the absence of constitutive activity or endogenous

Figure 4. In contrast to agonist effects, the zfCB₁ antagonism by SR at 30 and 100 nM increased the frequency of longer filopodia. This is consistent with the well-established inverse agonist properties of SR, and suggests possible constitutative receptor activation and/or presence of inhibitory endogenous cannabinoid tone in the culture system employed.
agonist tone, potentially through activation of G_{i} G-protein subtypes, for which there is some evidence in the literature.59-41

A more complete appreciation of WIN and SR effects on filopodia length is aided by considering distributional changes (Figs. 3 and 4). Increases in mean filopodia lengths by 3 nM WIN and 30 and 100 nM SR were associated with increased variance around the mean, with skew toward longer filopodia (see Figs. 3C, 4E and F). In contrast, the effects of 30 and 100 nM WIN to decrease filopodia length is associated with reduced variance, and production of filopodia with more consistent and relatively shorter lengths. Assuming that distinct mechanisms control filopodia extension and regulation of length, the biphasic effect of low concentrations resulting in populations of longer filopodia may indicate that the process responsible for limiting filopodia length is more sensitive to cannabinoid inhibition than is the process of extension. This may be attributable to different cell signaling constituents and/or receptor conformational states within different compartments of filopodial processes. Distinct regulation within different regions of filopodia is suggested by distinct distal receptor aggregations (e.g., Fig. 1B).

The potential for constitutive receptor activity in reducing filopodia production is supported by the lack of effect of exogenous agonist on filopodia numbers (Fig. 2C). This may suggest that production of filopodia is sensitive to even low, constitutive levels of receptor activation, and that constitutive activity itself is maximally-effective in inhibiting filopodia production in this system. Significant effects of 30 and 100 nM of the SR antagonist to increase filopodia numbers further supports an inhibitory role of zfCB1 activation in filopodia proliferation (Fig. 2D). In contrast, extension of the relatively fewer filopodia that are produced under conditions of receptor activation is only inhibited by higher agonist concentrations (Fig. 2A), suggesting involvement of a less-sensitive, cannabinoid-inhibited system in regulating filopodia elongation.

In summary, we have discovered that cannabinoid agonism and antagonism differentially modulate the morphology of filopodia produced by zfCB1-expressing CHO cells. These results further demonstrate the potential of cannabinoid signaling to influence production and maintenance of structurally-relevant cellular projections. These experiments provide a foundation for beginning to study the molecular mechanisms responsible for differential cannabinoid effects on cell structure, and for investigating the role of endocannabinoid signaling in regulating the structure of more complex cell types that comprise neuronal systems.

**Materials and Methods**

**Cell culture and transfection.** CHO-K1 (ATCC CCL-61) and 293TN cells (System Bioscience, cat. #LV900A-1) were maintained in Dulbecco’s modified Eagle’s medium (D-MEM, Invitrogen, cat. #11995073) supplemented with 10% fetal bovine serum (Invitrogen, cat. #16000036), 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen) in an atmosphere of 5% CO_{2}. One day before transfection, 293TN cells were seeded in T-75 culture flasks in 12 ml complete medium and grown to 50–70% confluence. For each transfection, 30 µl of Lipofectamine 2000 (Invitrogen, cat. #1832411) and 2 µg of plasmid DNA were diluted into D-MEM, mixed gently and incubated at room temperature for 20 min to allow DNA-lipofectamine complexes to form. After complex formation, the cells were rinsed with D-MEM once and DNA-lipofectamine complexes overlaid onto cells with 10 ml of complete medium. Following incubation with complete medium for 48 h, the supernatants were collected.

**Lentivirus production.** cDNA encoding zfCB1 was amplified by RT-PCR from a zebrafish brain cDNA library to introduce BamH1 and NotI restriction sites, and PCR products were cloned into the lentivector pCDH-CMV-MCS (System Bioscience, cat. #CD500B-1). The sequence of recombinant zfCB1-expressing lentivectors were confirmed by DNA sequencing. The lentivector expression system used contained a packaging plasmid mixture of pPACKH1-GAG, pPACKH1-REV and pVSV-G plasmids (System Bioscience, cat. #LV500A-1). Pseudoviral particles were produced following cotransfection of 293TN cells with the four vectors using lipofectamine 2000 (Invitrogen, cat. #18324-111) according to the manufacturer’s procedure as described above. The pseudoviral particle-containing supernatants were collected at 48 h post-transfection. Viral titers were determined by transfection of CHO cells, fluorescent staining and subsequent fluorescent microscopic analysis. Transfection was conducted according to the manufacturer’s recommendations. The day before infection, CHO cells were passed onto six-well plates to reach a density of 40–50%. For infection, the culture medium was removed, and 2 ml of new medium containing freshly produced virus suspension and 5 µg/ml of Polybrene (Sigma-Aldrich) was added to each well of the culture plates. After 12 h incubation at 37°C, 5% CO_{2}, the medium was changed with fresh complete culture medium. After further culture for another 32 h, cells were fixed and subjected to the immunostaining described as following.

**Immunofluorescent staining, confocal imaging, and analysis of filopodia morphology.** Immunofluorescent labeling was performed as following. Cells were fixed with 3.7% formaldehyde (Thermo, cat. #28908) and treated with phosphate-buffered saline containing 3% Bovine Serum Albumen and 0.05% Triton X-100 for permeabilization. The cells were then incubated with the anti-zfCB1 antibody in PBS-BSA for 30 min. They were then incubated with an FITC-conjugated goat anti-rabbit secondary antibody. Actin was labeled with phallolidin-TRITC.

Confocal images were acquired on an LSM 510 META with a 63X/1.4 Plan-Apochromat. Images were processed using LSM 510 software. Filopodia lengths and numbers were quantified using ImageJ analysis software. The length of individual filopodia was measured from the tip of the filopodia to the interface with the cell membrane. Filopodia less than 5 µm were not counted and only well-separated cells were selected for analysis. Cellular distributions of zfCB1 receptor expression were assessed by measuring mean optical densities of plasma membrane immunofluorescence to cytoplasmic immunofluorescence: resulting in M/C ratios that could be compared across treatment groups. The anti-zfCB1 antibody used is directed at the C-terminal tail region of the receptor, and has been previously characterized.13
Each data point analyzed represents pooled means ± SEM from three experiments. For each experiment and treatment condition at least 70 filopodia were measured for length, and at filopodia numbers were counted for at least 15 well-separated cells. Relationships between treatment groups were assessed with ANOVA and where appropriate followed by post-tests as described in the statistics section below.

**Drug treatments.** CHO cell cultures were treated with various concentrations of the cannabinoid agonist WIN55212-2 (WIN) and the antagonist/inverse agonist SR141716A (SR). These compounds were obtained from the National Institute on Drug Abuse drug provision program and diluted from 10 mM stocks dissolved in DMSO. DMSO was used as a vehicle control.

**Statistics.** As described above, treatment effects on M/C ratios were compared using ANOVA followed by Student-Neuman-Keuls post-tests (Fig. 1F). Several statistical analyses were performed to determine the effect of SR and WIN treatments on filopodia length and number of filopodia per cell. To assess the relationship between treatments of varying concentrations of WIN and SR, Kruskal-Wallis ANOVAs were employed. When warranted, Wilcoxon Rank-Sum post-tests were used to identify treatment conditions that produced results significantly different from vehicle control groups. This non-parametric test was chosen because the resulting data were positively skewed and not normally distributed (e.g., Figs. 3 and 4).

Frequency distributions displaying filopodia length after treatments of various concentrations of WIN and SR are shown in Figures 3 and 4. To create these distributions the arithmetic means and arithmetic standard deviations were obtained. To do so, a logarithmic transformation was performed on data from all groups. Using the logarithmic means and standard deviations of filopodia lengths, a back transformation on these descriptive statistics was executed. Meaning, 10 was raised to the power of the obtained logarithmic means, resulting in the arithmetic means. To obtain the upper tail of the arithmetic standard deviation, 10 was raised to the power of the obtained logarithmic mean plus the logarithmic standard deviation. The lower tail of the arithmetic standard deviation is equal to 10 raised to the power of the logarithmic mean minus the logarithmic standard deviation. These transformations were used to display the data in a more descriptive manner. By doing a logarithmic transformation, the skewed data were normalized and the back transformation provided arithmetic means which better represents the distribution of the data.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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