

Zebra Finch CB1 Cannabinoid Receptor: Pharmacology and in Vivo and in Vitro Effects of Activation

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ABSTRACT

Zebra finches (*Taeniopygia guttata*) learn vocal behavior during sensitive developmental periods, similar to the way in which human language is acquired. As adults, they recite the learned song pattern in a stereotyped manner. Previously, we demonstrated that central nervous system-associated cannabinoid receptors (CB1) are expressed in brain regions known to control both juvenile song learning and adult recitation of song. Here we extend these findings by establishing the zebra finch as a behavioral model to study cannabinoid pharmacology, showing that the cannabinoid agonist WIN55212-2 inhibits both adult song production and locomotor activity, effects that are antagonist-reversed. Through radioligand binding assays we investigated the pharmacology of a number of cannabinoid ligands representing all structural classes and established an affinity profile that can be compared with that of other species.

To begin to characterize signal transduction mechanisms we isolated cDNA encoding the receptor protein. The zebra finch CB1 receptor (ZFCB1) is highly expressed in brain with amino acid sequence 92% identical to human CB1 receptor. Establishment of a Chinese hamster ovary cell line stably expressing ZFCB1 allowed demonstration that the cannabinoid agonist WIN55212-2 dose dependently and potently inhibits forskolin-stimulated adenylate cyclase activity ($IC_{50} = 9.0$ nM, maximum inhibition = 49% at 100 nM WIN55212-2, reversed by 1 mM SR141716A). Cyclase inhibition indicates that ZFCB1-mediated signal transduction is consistent with that of mammalian CB1 receptors. Overall, cannabinoid inhibition of adult song production and conserved pharmacology render the zebra finch a promising model to investigate cannabinoid effects on learning by juveniles.

Cannabinoids are the active constituents of marijuana, and have been used both medicinally and recreationally for centuries. Despite this long history, progress in understanding the molecular mechanisms responsible for cannabinoid action has been made only relatively recently (for review, see Pertwee, 1997). This progress began with identification of a specific CNS-associated cannabinoid receptor termed CB1 (Devane et al., 1988; Matsuda et al., 1990) and endogenous ligands capable of activating this receptor (Devane et al., 1992; Mechoulam et al., 1995; Priller et al., 1995). Later, a peripheral cannabinoid receptor termed CB2 was identified, which may be exclusively expressed in tissues of the immune system (Munro et al., 1993). Both CB1 and CB2 receptors are negatively coupled to adenylate cyclase consistent with activation of G proteins of the G_i/G_o subtype (Howlett et al., 1986), although CB1 coupling to G_s has also been demonstrated (Glass and Felder, 1997).

In spite of this progress, a clear physiological role for can-

nabinoid signaling has not been identified, although high densities of CB1 receptors within caudate-putamen, hippocampus, and neocortex suggest involvement in motor control, learning, and memory and cognitive function (Herkenham et al., 1991; Katona et al., 1999). The need for a better understanding of the physiological processes mediated by cannabinoid receptor activation is underscored by the fact that marijuana is the most widely abused illicit drug in the United States. Marijuana use by high school students increased dramatically over the last decade (Bachman et al., 1998). Recent evidence suggests that cannabinoid signaling plays a significant role in brain development (for review, see Fernandez-Ruiz et al., 2000). In humans, acute cannabinoid exposure is known to produce cognitive deficits that include difficulty focusing attention and filtering irrelevant information, effects that may persist for hours (for review, see Sullivan, 2000). Despite widespread abuse, especially by children, the effects of cannabinoid exposure during cognitive development remain essentially unevaluated, due to lack of appropriate model systems.

The zebra finch is a promising model for studying drug

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ABBREVIATIONS: CNS, central nervous system; CB1, CNS-associated receptor; CB2, immune system-associated cannabinoid receptor; ZFCB1, zebra finch CB1 receptor; CHO, Chinese hamster ovary; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; rRNA, ribosomal RNA; cDNA, complementary DNA; RA, robust nucleus of the archistriatum; HVC, higher vocal center; CI, confidence interval.

effects during development because much like human language learning (Doupe and Kuhl, 1999), zebra finch song learning involves multiple cognitive components. Juvenile males must attend to, hear, and memorize the song pattern of an adult (typically the father) and then practice reproducing the memorized song until they are able to produce a good copy (usually by early adulthood). As adults, zebra finches recite their vocal pattern in a highly stereotyped manner (Fig. 1), a behavior that is used to court females, although song is also produced spontaneously when birds are housed in isolation or with other males (for review, see Zann, 1996). Several brain regions involved in song learning and adult vocal production have been identified and many of their interconnections delineated (Fig. 1). Note that the song-control system is vested largely in the telencephalon, the same brain subdivision that houses much of the neural circuitry for human language learning (Doupe and Kuhl, 1999).

Previously, we reported the identification of a dense population of high-affinity cannabinoid binding sites in zebra finch CNS, isolation of a fragment of cDNA encoding the zebra finch CB1 receptor, and high-level expression of CB1 receptors in two telencephalic brain regions for song learning and production [higher vocal center (HVC) and robust nucleus of the archistriatum (RA), Fig. 1; Soderstrom and Johnson, 2000]. The present report builds on our earlier findings by demonstrating that the zebra finch provides a unique pharmacological model system to study cannabinoid effects on vocal, locomotor, and ingestive behaviors. Well tolerated

injections of the cannabinoid agonist WIN55212-2 produced concentration-dependent inhibition of adult vocal production and locomotor activity. At higher dosages food consumption was reduced. Coadministration of the antagonist SR141716A reversed effects on vocal production and locomotor activity. In addition to establishing a behavioral model, we have investigated the pharmacology of the zebra finch CB1, establishing a rank order of affinity profile that can be compared with that of other species. We also describe isolation of cDNA encoding the zebra finch CB1 receptor (ZFCB1) and its heterologous expression in Chinese hamster ovary (CHO) cells, which allowed demonstration that ZFCB1 receptors share signal transduction mechanisms with mammalian CB1 receptors. Our findings suggest that the behavioral and molecular aspects of CB1 receptor activation are well conserved across vertebrate species, and set the stage for further characterization and studies of the effects of cannabinoids on vocal learning by juvenile birds.

Experimental Procedures

Materials. Except where otherwise indicated all supplies and reagents were purchased from Sigma (St. Louis, MO), or Fisher (Pittsburgh, PA). Ro 20-1724, WIN55212-2, anandamide, and 2-arachidonyl glycerol were purchased from Research Biochemicals International (Natick, MA). HU-210 and methanandamide were purchased from Tocris Cookson (Ballwin, MO). SR141716A was generously provided first by the National Institute on Drug Abuse, and later by Sanofi (Montpellier, France). Levonantradol was a gift from

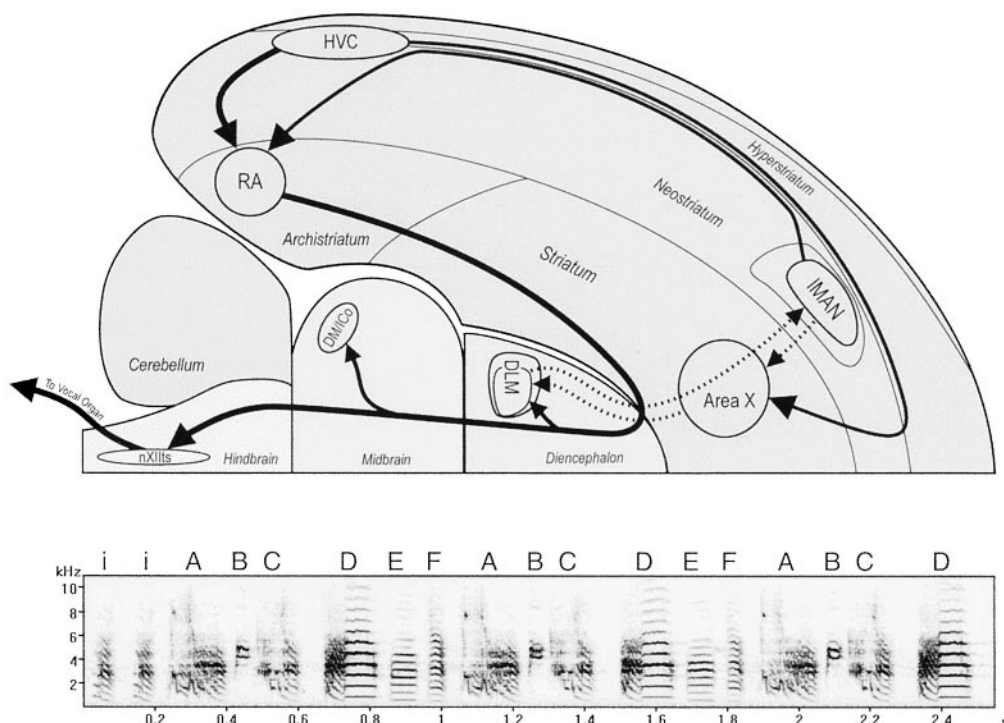


Fig. 1. Sagittal schematic of zebra finch CNS shows relative positions of major song regions and their axonal connections. Song regions located in the rostral forebrain play an important role in song learning but are not necessary for adult vocal behavior (IMAN, DLM, Area X), whereas two regions in the caudal telencephalon (HVC and RA) are important during song learning and during adult recitation of already learned song (Nottebohm et al., 1976; Bottjer et al., 1984; Nordeen and Nordeen, 1988; Simpson and Vicario, 1990; Sohrabji et al., 1990; Volman, 1993). Area X, Area X of striatum; DLM, medial portion of the dorsolateral nucleus of the thalamus; DM/ICo, dorsomedial nucleus of the intercollicular complex; HVC, lateral magnocellular nucleus of the anterior neostriatum; mMAN, medial magnocellular nucleus of the anterior neostriatum; RA; nXIIIts, hypoglossal nucleus, tracheosyringeal portion. Shown below is an example audiospectrogram of a song bout produced by an adult male zebra finch. Song bouts (defined as 2–5-s bursts of song elements produced in rapid succession) are produced spontaneously by male zebra finches housed in visual isolation (as in the present study). Song bouts typically begin with a series of introductory notes (i) followed by several iterations of the bird's song pattern (ABCDEF).

Pfizer (Groton, CT). [^3H]Adenine was purchased from Sigma. [^{14}C]cAMP, [^{32}P]dCTP, and [^3H]CP-55940 were purchased from NEN-DuPont (Boston, MA). Enzymes and reagents used for cDNA synthesis reactions were purchased as a kit (TimeSaver cDNA Synthesis kit) from Pharmacia (Piscataway, NJ). Lambda phage and packaging extracts used to construct cDNA libraries in gt11 were purchased from Stratagene (San Diego, CA). Deep Vent and Klenow-(exo⁻) DNA polymerases were purchased from New England Biolabs (Beverly, MA). LipofectAMINE reagent, oligonucleotide primers, cell culture media, and other cell culture reagents were purchased from Life Technologies (Grand Island, NY). Cosmic calf serum was purchased from Hyclone (Logan, UT). Nitrocellulose disks for plaque lift screening were purchased from Schleicher & Schuell (Keene, NH). Alkamuls EL-620 was a generous gift from Rhodia (Cranbury, NJ). Tri-Reagent was purchased from Molecular Research Center (Cincinnati, OH). The human 28S rRNA cDNA was provided by Dr. Cathy Levenson (Program in Neuroscience, Florida State University, Tallahassee, FL). pcDNA3.1(+) was purchased from Invitrogen (Carlsbad, CA). CHO cell cultures were provided by Dr. Joan Hare (Institute of Molecular Biophysics, Florida State University). Prism data analysis software was purchased from GraphPad (San Diego, CA).

Cannabinoid Effects on Incidence of Singing, Locomotor Activity, and Food Consumption. Subjects were adult (>90 days old) male zebra finches raised in our breeding aviaries. Animals were cared for in accordance to protocols approved by the Animal Care and Use Committee at Florida State University. Every opportunity was taken to minimize animal discomfort. Animals were maintained in visual but not auditory isolation on a 14:10-h light/dark cycle and given free access to food and water. Birds were allowed to habituate to recording cages and daily handling for 1 week before commencement of experiments. The cages in which birds were housed were equipped with perches that contained switches connected to a computer that recorded landing events. Vocal behavior was monitored using microphones placed in front of cages and connected to a computerized monitoring system (Avisoft SASLab Pro). This monitoring system uses software that compares recordings to programmed frequency criteria. Recordings matching the frequencies and duration of birdsong bouts are saved.

Cannabinoids were diluted from concentrated DMSO stocks (60 mM) and suspended in a vehicle consisting of saline:DMSO:Alkamuls EL-620, 18:1:1. Injections of 50 μl were made into the pectoralis muscle 30 min before the beginning of the light phase. Recordings of song bout incidence and perch landings began with lights-on and continued for 90 min. In an initial dose-response experiment four animals received the following WIN55212-2 dosages in order: 0, 0.03, 0.1, 0.3, and 1 mg/kg (average zebra finch weight of 13 g). Each dosage was given for 3 consecutive days and was followed by a treatment-free period of at least 72 h. To assess potential changes in the effectiveness of cannabinoid injections due to repeated exposure, a second experiment was done in reverse order from that described above. Naïve birds received the 1 mg/kg WIN55212-2 dosage first, followed by 0.3 and 0.1 mg/kg. No significant differences in the effectiveness of the dosages given in different order were observed. A third four-bird experiment was done in which naïve animals were given 1 mg/kg WIN55212-2 followed by 1 mg/kg WIN55212-2 + 3 mg/kg SR141716A. Again, each treatment was given for 3 consecutive days and was separated by 72 h. Combining the three experiments described above, the following number of birds was evaluated at each WIN55212-2 dosage level: 0 mg/kg ($n = 12$), 0.03 mg/kg ($n = 4$), 0.1 mg/kg ($n = 8$), 0.3 mg/kg ($n = 8$), and 1 mg/kg ($n = 12$).

A final behavioral experiment was performed to investigate the effects of WIN55212-2 on ingestive behavior. Injections of 0, 0.3, and 1 mg/kg were given as described above. After injections food cups were emptied and refilled with 15 g of finch seed. Seed was removed immediately after completion of the testing period and weighed. The mass of seed removed from food cups during the testing period was

recorded. No attempt was made to control for seed that may have been removed from the food cup and not eaten.

Radioligand Binding Assays. Well washed neuronal membranes were prepared from adult male zebra finch brains using the method described previously by Soderstrom et al. (2000). The synthetic cannabinoid agonist CP-55940 in tritiated form (180 Ci/mmol) was used in the binding studies described. This radioligand was selected because of its high specific activity and because it has been widely used in the characterization of cannabinoid receptors in other species (Pertwee, 1997). Binding reactions were conducted in a final volume of 200 μl containing 25 mM HEPES/10 mM MgCl_2 , 5 mg/ml bovine serum albumin, and 0.1% DMSO. In our system we have found that bovine serum albumin at 5 mg/ml significantly reduces nonspecific radioligand binding without significantly affecting specific binding. For competition assays using anandamide and 2-arachidonolyl glycerol, ethanol was used in place of DMSO and reactions were conducted in the presence of 50 μM PMSF. Fifty micrograms of membrane protein was used for equilibrium saturation isotherms, and 10 micrograms was used for equilibrium competition binding experiments. Nonspecific binding was defined as that occurring in the presence of 1 μM HU-210. The Millipore (Bedford, MA) Multiscreen equipment in 96-well format was used for binding assays. Reactions were conducted in GF/C glass fiber filter plates at 30°C for 90 min and terminated by rapid filtration. Filters were immediately washed with a total of 1.2 ml of ice-cold assay buffer. Filter-trapped radioactivity was quantified with a Beckman model LS 5801 scintillation counter. Tritium efficiency was approximately 50%. At radioligand concentrations approximating the K_d , specific binding exceeded 70% of total. Inhibitory binding constants (K_i) were calculated from IC_{50} values using the method of Cheng and Prusoff (1973).

Radioligand binding data were analyzed by computer-assisted nonlinear regression analysis using GraphPad (San Diego, CA) Prism software. This program fits appropriate equations to data using iterative least-squares curve-fitting algorithms. The relative appropriateness of one-binding-site models compared with more complex models was evaluated through F tests.

Production of a Zebra Finch Brain cDNA Library. To secure a permanent supply of zebra finch cDNA we constructed a zebra finch cDNA library using mRNA isolated from whole brain tissue. To increase the representativeness of the library, three zebra finches were used, including an adult female, adult male, and a juvenile male (55 days old). Total RNA was isolated using Tri-Reagent according to the manufacturer's instructions. Polyadenylated RNA was purified twice over oligo(dT)-cellulose columns. Zebra finch brain cDNA was synthesized from 5 μg of polyadenylated RNA using oligo(dT)₁₂₋₁₈ primers and a kit manufactured by Pharmacia (TimeSaver cDNA synthesis kit) and was cloned and amplified in lambda gt11 phage arms (Stratagene).

Northern Blotting. To assess potential peripheral expression of zebra finch CB1 receptors, a Northern blot of RNA prepared from various zebra finch tissues was probed with ^{32}P -labeled zebra finch CB1 cDNA. The methods previously described were used (Soderstrom and Johnson, 2000). After a suitable film image had been obtained using a zebra finch CB1 probe, the membrane was stripped and probed again using a cDNA encoding human 28S rRNA. This additional hybridization was done to demonstrate that similar amounts of RNA had been loaded from each tissue type.

cDNA Library Screening and Expression Vector Construction. Standard plaque lift screening methods were used. Of 12 putative clones isolated, 10 contained zebra finch CB1 cDNA, however, none of the clones contained the 5' initiation codon. To obtain the missing sequence information a modified version of the uneven PCR method was used (Chen and Wu, 1997). The modification consisted of substituting cDNA for the genomic DNA template called for in the original description. Once the complete zebra finch CB1 coding sequence was known, PCR primers were designed to amplify it without untranslated regions. The 5' sense primer incorporated a 5' three-

base spacer, *Hind*III restriction site, and Kozak consensus sequence: TTGAAGCTTGCCACCATGAAGTCAATTCTAGATGGCC. The 3' antisense primer incorporated an *Xho*I restriction site and 5' three-base spacer: ATGCTCGAGTTACAACGCTTCAGCTGTTG. These primers were used to amplify zebra finch CB1 cDNA using the zebra finch brain cDNA library as a template and Deep Vent thermostable DNA polymerase (New England Biolabs) for 20 rounds of PCR. Cycling conditions were 94°C for 30 s, 54°C for 45 s, and 72°C for 60 s, followed by a final 5 min 72°C soak. The product of this reaction was cloned into the *Hind*III/*Xho*I restriction sites of pcDNA3.1(+), a mammalian expression vector conferring G418 resistance. A large-scale plasmid DNA preparation was made and designated pcDNA3.1ZFCB1. Both strands of a positive clone were sequenced by Dr. Chris Baco of the Florida State University Core DNA sequencing facility. The sequence of zebra finch cDNA is available from GenBank (accession number AF255388).

CHO Cell Transfections. CHO cell cultures (grown in Ham's F-12 supplemented with 10% cosmic calf serum) were transfected with pcDNA3.1ZFCB1 using LipofectAMINE reagent (Life Technologies) according to the manufacturer's instructions. Isolated, G418-resistant colonies became visible after 10 to 14 days. Six colonies were picked, amplified, and screened in the adenylate cyclase assay described below. Three of the six clones were positive for CB1 expression, and one clone (showing about 50% inhibition of forskolin-stimulated cyclase activity when treated with 1 μ M WIN55212-2) was selected for further study. This clonal cell line was designated CHO-ZFCB1.

Adenylate Cyclase Assays. Effects of cannabinoid treatment on adenylate cyclase activity in CHO-ZFCB1 cultures were evaluated using a modified version of the method described by Salomon (1979), as detailed in Soderstrom et al. (1997). Nonlinear regression analysis of dose-response data from adenylate cyclase assays was analyzed by fitting a logistic equation to data points using GraphPad Prism software. The equation used was as follows:

$$[^3\text{H}]\text{adenine incorporated to cAMP} = \min + \frac{(\max - \min)}{1 + (10^{[L]}/10^{IC_{50}})}$$

where max represents the amount of [^3H]adenine incorporated to cAMP in the absence of cannabinoids, min represents the amount of [^3H]adenine incorporated in the presence of a fully effective concentration of WIN55212-2, [L] represents the concentration of WIN55212-2, and IC_{50} represents the [L] that will produce a 50% reduction in the amount of [^3H]adenine incorporated.

Statistics. The relationship between multiple WIN55212-2 dosages and behavioral measures of song bout incidence, locomotor activity, and food consumption were evaluated using analysis of variance. Where appropriate, post hoc analyses were done using the Student-Newman-Keuls test. Significance was defined as $p < 0.05$. Single dosage experiments were evaluated with a χ^2 analysis and pairwise two-tailed t tests where appropriate. Data are reported as mean $\pm$ S.E.M.

Results

The cannabinoid agonist WIN55212-2 produced dose-dependent reductions in song bout incidence, perch landings, and food consumption (Fig. 2). Analysis of variance revealed that the relationship between dosage and effect on each behavior was significant ($***p < 0.001$ in each case). Pairwise comparisons demonstrated that the magnitude of WIN55212-2 inhibition of all behaviors was significant at the 1-mg/kg dosage level ($***p < 0.001$ in all cases). In addition, the 0.1- and 0.3-mg/kg dosages significantly inhibited perch landings ($*p = 0.012$ and $**p = 0.005$, respectively). Song bout incidence was significantly inhibited at the 0.3-mg/kg dosage level ($**p = 0.003$). At 1 mg/kg the reductions of song

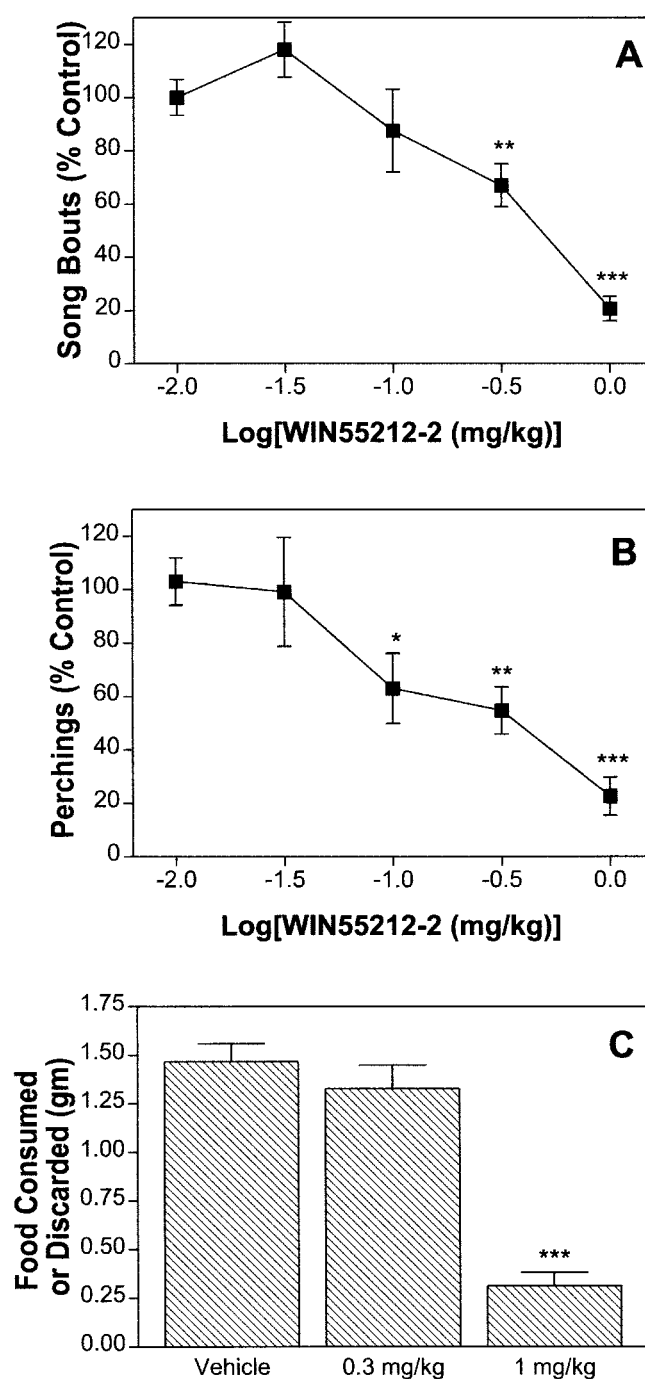


Fig. 2. WIN55212-2 dose dependently inhibits song bout incidence (A), perch landings (B), and food intake (C). Male zebra finches were injected with the dosages indicated 30 min before initiation of recording. Recording commenced with lights-on and continued 90 min. For song bout incidence and perch landings (A and B) each dosage was given for 3 consecutive days. Shown are pooled means of one (0.03 mg/kg), two (0.1 and 0.3 mg/kg), or three (0 and 1 mg/kg) experiments using four animals each ($n = 4, 8, \text{ and } 12$, respectively). For food intake (C) a single four-bird experiment was done. Analysis of variance revealed significant relationships between dosage and each behavior ($*p < 0.05$ in each case). Significant pairwise comparisons are indicated ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-tailed t tests). Cannabinoid effects on behavior were transient and resolved within 4 h postinjection.

bout incidence and perch landings were 79.4 ± 4.6 and $80.3 \pm 8.9\%$, respectively. Despite inhibition of song bout incidence at 0.3 and 1 mg/kg, differences in the acoustic features of

song (song bout length, rate of note production, note order, fundamental frequency of notes) were not observed in WIN55212-2-treated birds (data not shown). Food consumption was not altered by the 0.3-mg/kg dosage but was significantly reduced at 1 mg/kg from control values of 1.47 ± 0.09 to 0.31 ± 0.07 g consumed ($***p < 0.001$, Fig. 2C). In an independent experiment, 3 mg/kg of the cannabinoid antagonist SR141716A partially reversed the inhibition produced by 1 mg/kg WIN55212-2 on both song bout incidence and perch landings (Fig. 3). The magnitude of the antagonist reversal was significant in each case ($**p = 0.003$ for perch landings, $*p = 0.010$ for song bout incidence, each two-tailed). Antagonist effects on food intake were not evaluated.

Equilibrium saturation binding experiments were done with [3 H]CP-55940 to determine binding site density ($B_{\max} = 2100$ fmol/mg of protein; 95% CI, 1917–2283) and affinity ($K_d = 1.51 \pm 0.14$ nM, Fig. 4A). Determination of K_d was necessary for inhibitory binding constant (K_i) calculations. Affinities of various cannabinoids representative of each structural class were determined through equilibrium competition

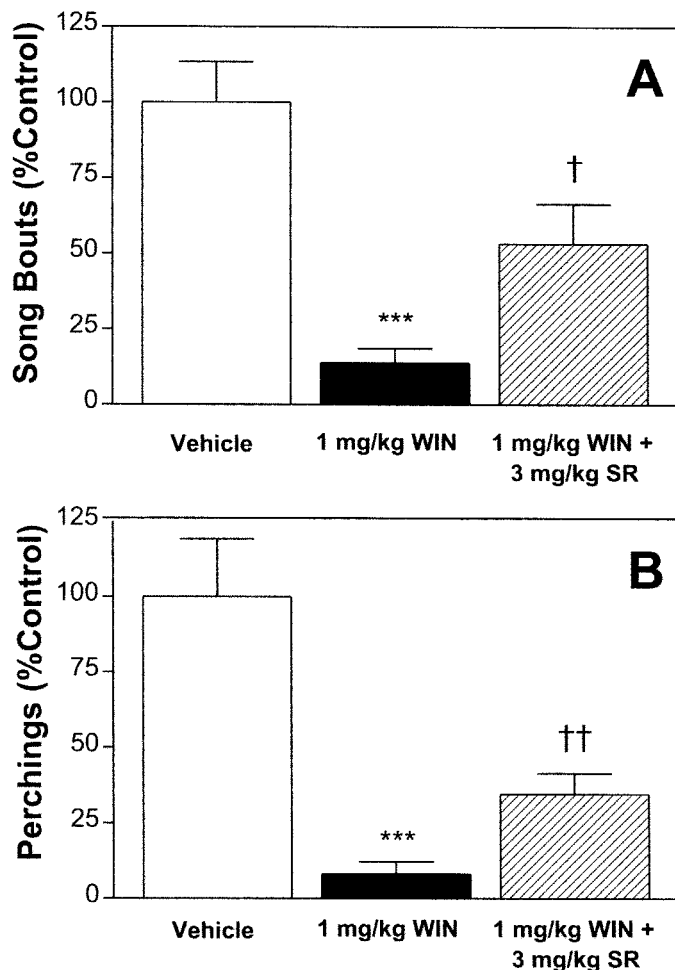


Fig. 3. Cannabinoid antagonist SR141716A significantly reverses agonist inhibition of song bout incidence (A) and perch landings (B). Male zebra finches were injected with the dosages indicated 30 min before initiation of recording. Recording commenced with lights-on and continued 90 min. Each dosage was given for 3 consecutive days. Shown are pooled means of a single experiment using four animals. Significant inhibition of both behaviors by WIN55212-2 (1 mg/kg, compared with vehicle controls $***p < 0.001$ in each case, two-tailed t test) was significantly reversed by SR141716A (3 mg/kg, compared with 1 mg/kg WIN55212-2 results $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$).

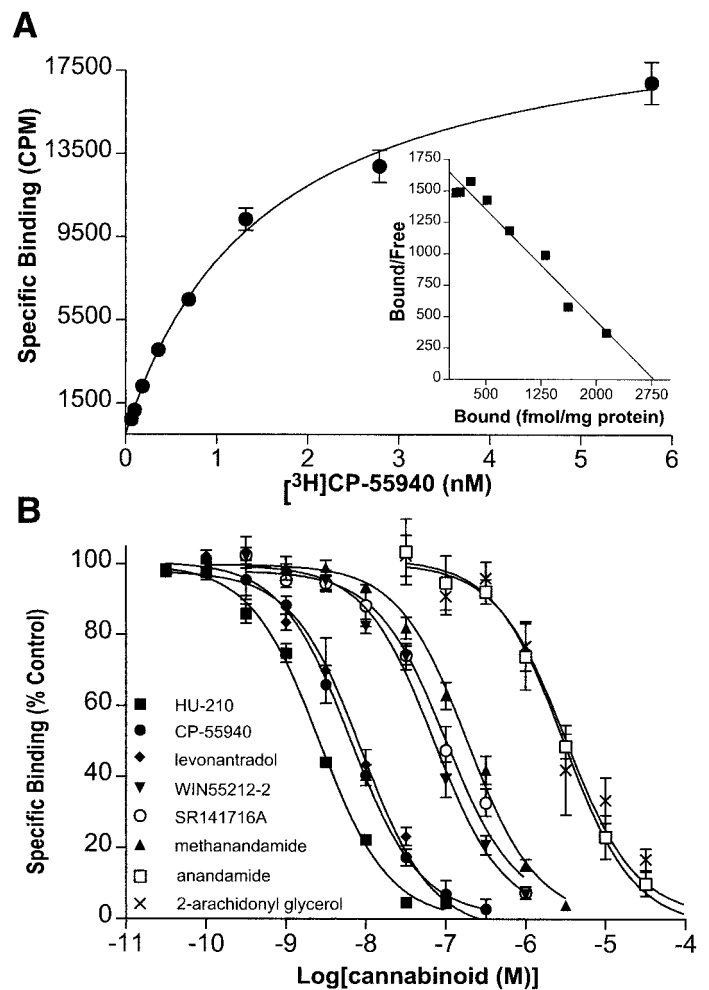


Fig. 4. Equilibrium binding of [3 H]CP-55940 to zebra finch brain membranes. A, saturation binding to 50 μ g of membrane protein at 30°C for 90 min. Data were best fit by single binding site with $K_d = 1.51 \pm 0.14$ nM, $B_{\max} = 2100$ fmol/mg of membrane protein (95% CI, 1917–2283). Inset, linear Scatchard replot supports a single binding site. B, specificity of displacement of [3 H]CP-55940 binding to zebra finch brain membranes by cannabinoids of various structural class. Ten micrograms of membrane protein was incubated with 0.10 to 0.18 nM radioligand and the concentration of competitor shown. Data points represent the means of two (anandamide and 2-arachidonyl glycerol) or three (all other compounds) pooled experiments each performed in triplicate.

TABLE 1

Displacement of [3 H]CP-55940 binding from zebra finch brain membranes

Cannabinoid	K_i nM	95% Confidence Interval
HU-210	2.32	1.96–2.75
CP-55940	5.92	4.38–8.00
Levonantradol	8.24	5.89–11.5
WIN55212-2	63.3	47.5–84.6
SR141716A	89.3	59.6–134.0
Methanandamide	166	125–221
Anandamide + PMSF	2795	1684–4638
2-Arachidonyl glycerol + PMSF	2941	1571–5503

binding assays (Fig. 4B; summarized in Table 1). Ligands used included the classic Δ^9 -tetrahydrocannabinol-related compound HU-210, the aminoalkylindole WIN55212-2, the bicyclic synthetic cannabinoids CP-55940 and levonantradol, the diarylpyrazole antagonist/inverse agonist SR141716A, the eicosanoid endogenous compounds anandamide and

2-arachidonyl glycerol, and the amidase-resistant modified form of anandamide methanandamide. The rank order of affinity of these compounds was (K_i , nM $\pm$ S.E.M.) as follows: HU-210 (2.32 ± 0.04) > CP-55910 (5.92 ± 0.06) $\sim$ levonantradol (8.24 ± 0.07) > WIN55212-2 (63.3 ± 0.06) $\sim$ SR141716A (89.3 ± 0.09) > methanandamide (166 ± 0.06) $\gg$ anandamide (2795 ± 0.11) $\sim$ 2-arachidonyl glycerol (2941 ± 0.14).

The cDNA sequence encoding zebra finch CB1 has been deposited in GenBank (accession number AF255388). Expression of the gene encoding zebra finch CB1 was assessed through Northern blotting (Fig. 5A). Of the tissues evaluated zebra finch CB1 mRNA is most highly expressed in brain, although detectable signal is also present in RNA isolated from testes, heart, and lung. For interpretation of cannabinoid effects on vocal behavior, the absence of CB1 mRNA in the vocal organ (syrinx) is notable. Reprobing of the blot with cDNA encoding human 28S rRNA demonstrated that similar amounts of RNA were loaded from each tissue (Fig. 5B).

The amino acid sequence deduced from the zebra finch CB1 cDNA shows a high degree of similarity to the CB1 receptors that have been cloned in other species, including 92% identity with human CB1. A summary of amino acid sequence identity shared by clones demonstrated through functional expression to encode bone fide cannabinoid receptors is presented in Table 2. An alignment of amino acid sequences encoded by expression-confirmed CB1 receptors is presented in Fig. 6. This figure also indicates the locations of the domains known or suspected to contribute to specific aspects of CB1 function. Every notable domain is present within CB1 from each species (Table 3).

Lipid-mediated transfection of CHO cells with pcDNA-3.1ZFCB1 allowed establishment of both nonclonal and clonal cell lines stably expressing the zebra finch CB1 cannabinoid receptor. Treatment of nonclonal cultures resulted in a modest, yet significant $25.6 \pm 3.7\%$ inhibition of cyclase activity (from two pooled experiments each done in triplicate, $***p < 0.001$, two-tailed t test; data not shown). Using the clonal cell line that was selected for further study (CHO-ZFCB1), WIN55212-2 produced a dose-dependent and potent inhibition of [3 H]adenine incorporation to intracellular cAMP, a measure of adenylate cyclase activity. A maximal inhibition of $49.0 \pm 1.9\%$ occurred with an $IC_{50} = 9.0 \pm 0.1$

nM (Fig. 7A). The cyclase inhibition produced by a fully effective concentration of WIN55212-2 (100 nM) was completely reversed by the antagonist SR141716A at 1 μ M (Fig. 7B). The combination of 100 nM WIN55212-2 and 1 μ M SR141716A actually produced a significant $10.0 \pm 2.3\%$ increase in the amount of [3 H]adenine incorporated to cAMP (Fig. 7B, $***p = 0.004$, two-tailed).

Discussion

We have investigated the pharmacology and consequences of zebra finch CB1 cannabinoid receptor activation at behavioral and molecular levels. These data extend our previous identification of developmental changes in zebra finch CB1 mRNA levels and notable expression within brain regions associated with song control (Soderstrom and Johnson, 2000). They also establish the zebra finch as a suitable model system to study cannabinoid pharmacology and provide a solid foundation for further investigation of cannabinoid effects on song learning during juvenile development.

Interesting features of the dose-dependent behavioral effects that we measured include the similarities between the magnitude of cannabinoid-mediated inhibition of both locomotor activity and song bout incidence (Figs. 2 and 3). The specificity of cannabinoid effects on each of these behaviors is demonstrated by partial reversal with the antagonist SR141716A. The incomplete nature of this reversal may be attributable to a combination of low SR141716A affinity for the zebra finch receptor (Fig. 4B; Table 1) and a modest antagonist dosage administered relative to that of agonist. We expect that an increased antagonist dosage, perhaps an order of magnitude greater than that of the lowest fully effective agonist dosage (as in the *in vitro* experiments, Fig. 7B), would have produced complete reversal. Cannabinoid production of hypomotility is well documented in mammalian species and is one of the tetrad of indices comprising the "Martin Multiparameter Mouse Model" of cannabinoid action. It is possible that the reductions we observed in song bout incidence are at least partially attributable to a general decrease in motor output, although frank immobility was not observed even at the highest WIN55212-2 dosage level used (1 mg/kg). The cannabinoid-mediated decrease in the incidence of song bouts and locomotor activity is consistent with high-level CB1 expression in the song nucleus RA and surrounding archistriatum (Soderstrom and Johnson, 2000) where neurons contributing to the motor output of the telencephalon are located (Nottebohm et al., 1976; Dubbeldam, 2000). However, HVC (which drives adult vocal production by activating RA, Fig. 1) shows specific expression of CB1 (92% of HVC cells express CB1 compared with only 9% in the surrounding neostriatum, Soderstrom and Johnson, 2000), raising the possibility of specific cannabinoid effects on the initiation of vocal behavior. Dissecting the potential differential effects of cannabinoids in HVC versus RA may require direct infusion of cannabinoids into each region. The discrete, nuclear arrangement of telencephalic song regions makes these types of experiments possible.

Significant effects of WIN55212-2 on food intake were only observed at 1 mg/kg, a distinctly higher dosage than required to alter perch landings and song bout incidence (Fig. 2C). Cannabinoid effects on ingestive behavior remain unclear. Recent reports have shown that 1 to 2 mg/kg of orally admin-

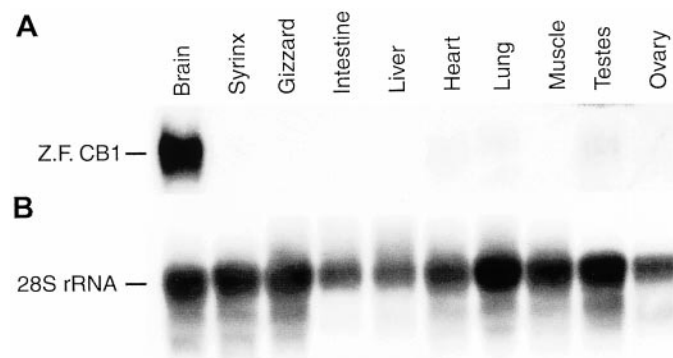


Fig. 5. Expression of the zebra finch CB1 cannabinoid receptor gene in various tissues. A, Northern blot probed with zebra finch CB1 receptor cDNA. Bands corresponding to zebra finch CB1 receptor transcripts are estimated to be 5.5 kb and are prominent in brain samples although visible in testes, lung, and heart. B, same blot reprobed with a human 28S rRNA probe. Similar intensities of 28S rRNA bands indicate that similar amounts of total RNA (5 μ g) were loaded across tissue types.

TABLE 2

Percentage of amino acid sequence identity between functionally expressed cannabinoid receptors

	CB1 Zebra Finch	CB1 Human	CB1 Rat	CB1 Mouse	CB1 Newt	CB2 Mouse	CB2 Rat	CB2 Human
CB1 zebra finch	100							
CB1 human	92	100						
CB1 rat	92	97	100					
CB1 mouse	92	97	97	100				
CB1 newt	86	84	84	84	100			
CB2 mouse	44	43	44	44	44	100		
CB2 rat	44	43	44	44	43	93	100	
CB2 human	40	40	41	41	40	82	81	100

Rat	1	MKSILDLGLADTTFTTITDLYLVGSNDIQVEDIKGDMSKLGYPFQKFPPLTSFRGSPFOE
Mouse	1	MKSILDLGLADTTFTTITDLYLVGSNDIQVEDIKGDMSKLGYPFQKFPPLTSFRGSPFOE
Human	1	MKSILDLGLADTTFTTITDLYLVGSNDIQVEDIKGDMSKLGYPFQKFPPLTSFRGSPFOE
ZFINCH	1	MKSILDLGLADTTFTTITDLYLVGSNDIQVEDIKGDMSKLGYPFQKFPPLTSFRGSPFOE
Newt	1	MKSILDLGLADTTFTTITDLYLVGSNDIQVEDIKGDMSKLGYPFQKFPPLTSFRGSPFOE
		I
Rat	61	KMTAGDNPST--VPAQTNTTEFYKSLSSPKENEENIQCCENFMDMECFMILNPSQOL
Mouse	61	KMTAGDNPST--VPAQTNTTEFYKSLSSPKENEENIQCCENFMDMECFMILNPSQOL
Human	61	KMTAGDNPST--VPAQTNTTEFYKSLSSPKENEENIQCCENFMDMECFMILNPSQOL
ZFINCH	61	KMTAGDNPST--VPAQTNTTEFYKSLSSPKENEENIQCCENFMDMECFMILNPSQOL
Newt	61	KMTAGDNPST--VPAQTNTTEFYKSLSSPKENEENIQCCENFMDMECFMILNPSQOL
		II
Rat	119	AAVLSLTGLTFTVLENLVLCVILHSRLRCRPSYHFGSLAVADLLGSVIFVYSPVDF
Mouse	119	AAVLSLTGLTFTVLENLVLCVILHSRLRCRPSYHFGSLAVADLLGSVIFVYSPVDF
Human	118	AAVLSLTGLTFTVLENLVLCVILHSRLRCRPSYHFGSLAVADLLGSVIFVYSPVDF
ZFINCH	120	AAVLSLTGLTFTVLENLVLCVILHSRLRCRPSYHFGSLAVADLLGSVIFVYSPVDF
Newt	120	AAVLSLTGLTFTVLENLVLCVILHSRLRCRPSYHFGSLAVADLLGSVIFVYSPVDF
		III
Rat	179	HVFHRKDSNVVFLFKLGGVTSFASVSGSLFLTAIDRYISIHRLPAYKRIVTRPKAVVAF
Mouse	179	HVFHRKDSNVVFLFKLGGVTSFASVSGSLFLTAIDRYISIHRLPAYKRIVTRPKAVVAF
Human	178	HVFHRKDSNVVFLFKLGGVTSFASVSGSLFLTAIDRYISIHRLPAYKRIVTRPKAVVAF
ZFINCH	180	HVFHRKDSNVVFLFKLGGVTSFASVSGSLFLTAIDRYISIHRLPAYKRIVTRPKAVVAF
Newt	180	HVFHRKDSNVVFLFKLGGVTSFASVSGSLFLTAIDRYISIHRLPAYKRIVTRPKAVVAF
		IV
Rat	239	CLHWITATVIAVLPGLGWCKKLSVCSDFPPLIDETVLMFHWIGVTSVLLFVIVAYMYI
Mouse	239	CLHWITATVIAVLPGLGWCKKLSVCSDFPPLIDETVLMFHWIGVTSVLLFVIVAYMYI
Human	238	CLHWITATVIAVLPGLGWCKKLSVCSDFPPLIDETVLMFHWIGVTSVLLFVIVAYMYI
ZFINCH	240	CLHWITATVIAVLPGLGWCKKLSVCSDFPPLIDETVLMFHWIGVTSVLLFVIVAYMYI
Newt	240	CLHWITATVIAVLPGLGWCKKLSVCSDFPPLIDETVLMFHWIGVTSVLLFVIVAYMYI
		V
Rat	299	LWKAHSHAVRMIOGCTQKSTIIHTSEDDGKVQVTRPDQARMDIRLAKTLVLVLVLIICWG
Mouse	299	LWKAHSHAVRMIOGCTQKSTIIHTSEDDGKVQVTRPDQARMDIRLAKTLVLVLVLIICWG
Human	298	LWKAHSHAVRMIOGCTQKSTIIHTSEDDGKVQVTRPDQARMDIRLAKTLVLVLVLIICWG
ZFINCH	300	LWKAHSHAVRMIOGCTQKSTIIHTSEDDGKVQVTRPDQARMDIRLAKTLVLVLVLIICWG
Newt	300	LWKAHSHAVRMIOGCTQKSTIIHTSEDDGKVQVTRPDQARMDIRLAKTLVLVLVLIICWG
		VI
Rat	359	PLLAIMYVDVFGKNNKLIKTVPFACSMCLLNSTVNPPIVALRSKDLRHAFRSKFPSCCG
Mouse	359	PLLAIMYVDVFGKNNKLIKTVPFACSMCLLNSTVNPPIVALRSKDLRHAFRSKFPSCCG
Human	358	PLLAIMYVDVFGKNNKLIKTVPFACSMCLLNSTVNPPIVALRSKDLRHAFRSKFPSCCG
ZFINCH	360	PLLAIMYVDVFGKNNKLIKTVPFACSMCLLNSTVNPPIVALRSKDLRHAFRSKFPSCCG
Newt	360	PLLAIMYVDVFGKNNKLIKTVPFACSMCLLNSTVNPPIVALRSKDLRHAFRSKFPSCCG
		VII
Rat	419	TAQPLDNSMGDSDDLCHHANNAGSHRAAESCIKSTVKIAKVTVMSVSTDTSAEAL
Mouse	419	TAQPLDNSMGDSDDLCHHANNAGSHRAAESCIKSTVKIAKVTVMSVSTDTSAEAL
Human	418	TAQPLDNSMGDSDDLCHHANNAGSHRAAESCIKSTVKIAKVTVMSVSTDTSAEAL
ZFINCH	420	TAQPLDNSMGDSDDLCHHANNAGSHRAAESCIKSTVKIAKVTVMSVSTDTSAEAL
Newt	420	TAQPLDNSMGDSDDLCHHANNAGSHRAAESCIKSTVKIAKVTVMSVSTDTSAEAL

Fig. 6. Amino acid sequence alignment of CB1 cannabinoid receptors that have been functionally expressed. Putative transmembrane domains are numbered and locations indicated by lines. Conserved amino acids are indicated by white letters against a black background, regions with similar amino acids are indicated in black letters against a shaded background, and regions of dissimilarity are in black letters against a white background. Notable domains and regions known to be involved in specific aspects of CB1 receptor function are summarized in Table 2. All notable domains identified to date are present in each receptor.

istered Δ^9 -tetrahydrocannabinol will increase food intake in satiated rats (Williams et al., 1998). Other studies indicate that synthetic cannabinoid agonists can reduce food intake and body weight in rats (Giuliani et al., 2000). The reduction of food intake that we measured may be related to the significant inhibition of locomotor activity produced by 1 mg/kg WIN55212-2, which may have resulted in a reduced number of food cup visits.

The affinity of [3 H]CP-55940 interaction with zebra finch neuronal membranes at 30°C ($K_d = 1.51 \pm 0.14$ nM) was similar to that observed previously at 25°C ($K_d = 0.55 \pm 0.13$ nM, Soderstrom and Johnson, 2000). Because of the high density of cannabinoid binding sites present in zebra finch neuronal membranes (525 pM was used in our equilibrium saturation binding experiments), inflation of K_d estimates related to ligand depletion was a concern. The magnitude of this distortion was small and corrected according to the

method described by Chang et al. (1975). A corrected affinity estimate of 1.24 nM was used with the Cheng and Prusoff (1973) equation to calculate inhibitory binding constants (K_i) from competition binding experiments.

Equilibrium competition binding experiments allowed investigation of the specificity of binding of a series of cannabinoids to zebra finch brain membranes, resulting in a pharmacological profile comparable to that obtained in other species. Cannabinoids representative of each structural class displaced [3 H]CP-55940 from an apparently single binding site. Despite ternary model prediction of at least two affinity states for agonist interaction with G protein-coupled receptors, [3 H]CP-55940 displacement from an apparently single binding site has consistently been reported (Pertwee, 1999). The rank order of affinity of the classical, synthetic bicyclic, and aminoalkylindole compounds was similar to that observed in other species. The most distinct feature of the zebra finch cannabinoid binding profile is the low-affinity interaction of the antagonist/inverse agonist compound SR141716A ($K_i = 89.3$ nM). In preparations of rat neuronal membranes this compound exhibits sub- to low-nanomolar affinities ranging from 0.89 to 2.35 nM, although lower affinities to 12.3 nM have been reported using membranes from human CB1-transfected CHO cells (for review, see Pertwee, 1997; Table 2). Distinctly lower affinity of SR141716A for zebra finch neuronal membranes may involve structural differences in the receptor, although amino acid sequences in transmembrane spanning domain regions implicated in ligand binding are well conserved (Fig. 5). Differential effector coupling is another potential mechanism for distinct affinities, a speculation supported by evidence for promiscuous coupling of CB1 receptors to various G protein subtypes (Abadji et al., 1999), including G_s . Distinct amino acid sequences within zebra finch CB1 regions implicated in G protein coupling include Gln³²²-Ser³²³-Thr³²⁴ within the third cytoplasmic loop region (Fig. 6). These amino acids appear avian-specific because the amphibian and mammalian sequences are conserved. A second notable feature of the zebra finch binding profile is the low, supramicromolar affinity of the endogenous eicosanoid compounds anandamide and 2-arachidonyl glycerol. Similar distinctly lower-than-rat affinities were also observed for anandamide binding to amphibian neuronal membranes (Soderstrom et al., 2000). Recent demonstration that chicken fatty acid amidohydrolase is less effectively inhibited than the rat homolog by PMSF (the amidase inhibitor that we used in binding assays), raises the possibility that amidase activity contributed to the low endocannabinoid affinities observed (Fowler et al., 2000).

Production of a cDNA library combined with use of a PCR-based method to clone cDNA ends (uneven PCR, Chen and

TABLE 3
Notable CB1 domains

Description	Amino Acid Position in ZFCB1	Amino Acids/Comments/Reference
Dual glycosylation sites in ECT	79–82, 85–88	NITE and NKSL in all except newt = NVTE and NRSV
PKC phosphorylation site in IL1	148–150	SLR in all except newt = TLR
Aspartate in TMDII	165	Mutation disrupts G protein coupling (Tao and Abood, 1998)
Lysine in TMDIII	194	Bicyclic ligand interaction (Song and Bonner, 1996)
PKC phosphorylation site in IL3	315–317	TQK in all species
Leucine-alanine pair in IL3	342–343	Implicated in G_s interaction (Abadji et al., 1999)
Dual serines in ICT	427, 430	Implicated in desensitization (Jin et al., 1999)
PKC phosphorylation site in ICT	454–456	TVK in all species
Thr-Met-Ser-Val in all species	462–465	Implicated in internalization (Hsieh et al., 1999)

ECT, extracellular tail; PKC, protein kinase C; IL1, first intracellular loop; TMD, transmembrane spanning domain; ICT, intracellular tail.

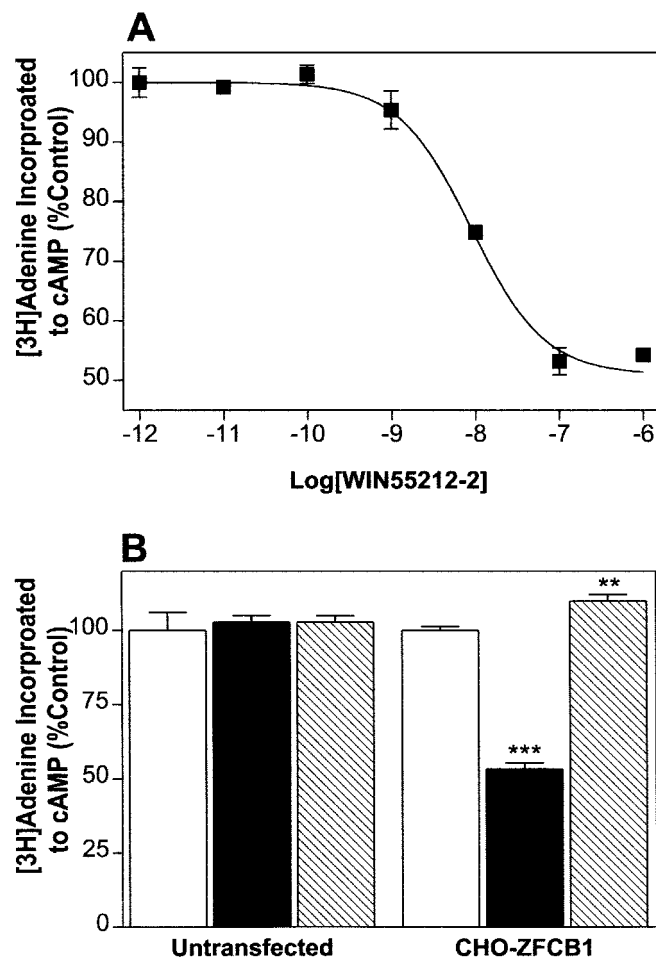


Fig. 7. Inhibition of forskolin-stimulated [3 H]adenine incorporation to cyclic AMP. CHO cells stably expressing the zebra finch CB1 cannabinoid receptor were treated for 30 min with 50 μ M forskolin (100% of control). A, WIN55212-2 inhibition of adenylate cyclase activity is dose-dependent and potent. Maximal inhibition was $49.0 \pm 1.9\%$ and occurred with an $IC_{50} = 9.0 \pm 0.1$ nM. Data points represent the pooled means $\pm$ S.E.M. of two experiments each performed in triplicate. B, WIN55212-2 (WIN, 100 nM) inhibition of adenylate cyclase activity is reversible by the cannabinoid antagonist SR141716A (SR, 1 μ M). The reversal was to levels significantly exceeding those of the vehicle-treated, forskolin-stimulated controls ($10.0 \pm 2.3\%$ stimulation, $**p = 0.004$, two-tailed). This suggests endogenous agonist release by the CHO-ZFCB1 cultures or inverse agonism by SR141716A. No effects on adenylate cyclase in untransfected cells were produced by either cannabinoid ligand. Vehicle-treated controls are indicated (VEH).

Wu, 1997) allowed us to obtain a complete zebra finch CB1 coding sequence. Availability of this zebra finch brain cDNA library has been useful in our other projects, and will be

important to our future investigation of gene expression related to zebra finch vocal development. The zebra finch CB1, along with the newt CB1 (Soderstrom et al., 2000) are the only nonmammalian cannabinoid receptors that have been cloned and functionally expressed to date. The cDNAs encoding nonmammalian CB1 receptors are remarkable in their high degree of homology with those of higher vertebrates (Tables 2 and 3), a fact that implies involvement in important physiological processes. We remain hopeful that comparative studies of the structure and function of cannabinoid receptors will be useful in illuminating the physiological roles they play, although this may require investigation of cannabinoid signaling in invertebrates such as those initiated with the leech (Stefano et al., 1997), mussel (Stefano et al., 1998), and hydra (De Petrocellis et al., 1999).

Expression of the zebra finch CB1 cDNA in CHO cells confirmed that it encodes a functional cannabinoid receptor. These experiments also allowed us to clearly determine that zebra finch CB1 is capable of negatively coupling to adenylate cyclase activity, an effect consistent with activation of G proteins of the G_o/G_i subtype. Inhibition of cyclase by WIN55212-2 was dose-dependent and potent, occurring at a concentration consistent with that reported for mammalian species (Tao and Abood, 1998). The potent $IC_{50} = 9.0$ nM indicates a higher affinity interaction than the estimate obtained in binding assays with neuronal membranes may predict ($K_i = 63.3$ nM). It is possible that differences in CHO cell membrane composition or differential G protein coupling contribute to apparent differences between affinity and potency.

Negative cyclase coupling is similar to that observed with CB1 of other species, demonstrated in cultures of neuroblastoma cells and transfected cell lines. Use of CHO-ZFCB1 cultures allowed us to show that agonist inhibition of cyclase was reversible with the cannabinoid antagonist SR141716A. However, this reversal was to cyclase activity levels significantly elevated from forskolin-treated controls (Fig. 7B). Stimulatory activity of the antagonist implies endogenous cannabinoid release by CHO-ZFCB1 cultures, or that SR141716A in this system has inverse agonist properties. Inverse agonism by SR141716A has been reported in other systems (Bouaboula et al., 1997) and may be attributable to preferential binding to CB1 receptors that are uncoupled from the G protein, driving equilibrium away from pre-coupled receptors, resulting in an overall reduction in effector activation. An alternate explanation for SR141716A activation of adenylate cyclase activity is related to the ability of CB1 receptors to promiscuously couple to multiple G protein subtypes, including G_s (Glass and Felder, 1997; Abadji et al.,

1999), raising the possibility that SR141716A is involved in agonism of G_s-coupled receptors.

Overall, the results presented here represent significant progress toward establishing a zebra finch model of cannabinoid pharmacology. This model will allow investigation of the effects of cannabinoid exposure during critical periods of juvenile learning, and a better understanding of the implications of marijuana abuse on cognitive development.

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