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Research report

CB1 cannabinoid receptor expression in brain regions associated with zebra finch song control

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Abstract

Cannabinoids have been used for millennia through various preparations of *Cannabis sativa*. Despite this long history of use, the physiological significance of cannabinoid signaling in the vertebrate CNS is not well understood. High CB1 cannabinoid receptor densities in mammalian telencephalon and the results of behavioral studies suggest that cannabinoids play a role in cognitive function, learning, and memory. Since a network of discrete brain regions in zebra finch telencephalon controls song learning, we hypothesized that cannabinoid signaling may be relevant to songbird vocal development and behavior. Radioligand binding experiments using the cannabinoid agonist [^{3}H]CP-55940 allowed identification of a dense population of high-affinity cannabinoid binding sites in zebra finch neuronal membranes. Northern blotting and RT–PCR experiments demonstrated expression of a predominant zebra finch CB1 mRNA of ~5.5 kb. Expression of this CB1 mRNA appears to change over the course of vocal development within the caudal telencephalon. As zebra finch caudal telencephalon contains the higher vocal center (HVC) and the robust nucleus of the archistriatum (RA), regions involved in song learning and production, we further investigated CB1 expression in these areas using in situ hybridization. In situ hybridization revealed that CB1 mRNA is expressed at high levels within both HVC and RA. Overall, these data demonstrate the presence of CB1 signaling systems within songbird telencephalon, notably within regions known to be involved in song learning and production. High-level CB1 expression in song regions suggests a potential role for cannabinoid signaling in zebra finch vocal development.

Keywords: Cannabinoid; Songbird; Telencephalon; Cognitive; Development

1. Introduction

Despite a long history of use, an understanding of the mechanism of cannabinoid activity has only recently begun to emerge. Following the identification and cloning of a cannabinoid receptor (termed CB1 [7,18]), it was found to be densely expressed with a distinct pattern of distribution in mammalian CNS, particularly within telencephalon [17]. While high-level CB1 expression implies functional importance, the nature of this importance remains poorly understood. High CB1 expression within specific subregions of rat telencephalon (caudate-putamen, hippocampus, neocortex; [10,14]) and cannabinoid-induced deficits in delayed match to sample and spatial memory tasks [9,11,16] suggest possible roles in motor control, learning and memory, and cognitive function.

We are interested in the potential role played by cannabinoids during the development of vocal learning in the zebra finch (*Taeniopygia guttata*). In a manner similar to human language acquisition (see Ref. [8]), male zebra finches learn a song pattern during a sensitive period of juvenile development (for review see Ref. [32]). Song learning appears to involve multiple cognitive processes in that juvenile male zebra finches must first attend to, hear, and memorize the song pattern of an adult (termed auditory learning). Later in development birds practice in order to learn to reproduce the song memorized earlier in life, a process requiring auditory feedback and auditory–motor integration (termed sensory–motor learning). In addition to well-characterized behavioral development, much of the neural circuitry controlling zebra finch vocal behavior has been delineated. A set of interconnected brain regions, largely localized within the telencephalon, controls the learning and production of song (see Fig. 1).

The numerous cognitive aspects of learned vocal behavior and the telencephalic location of many song regions

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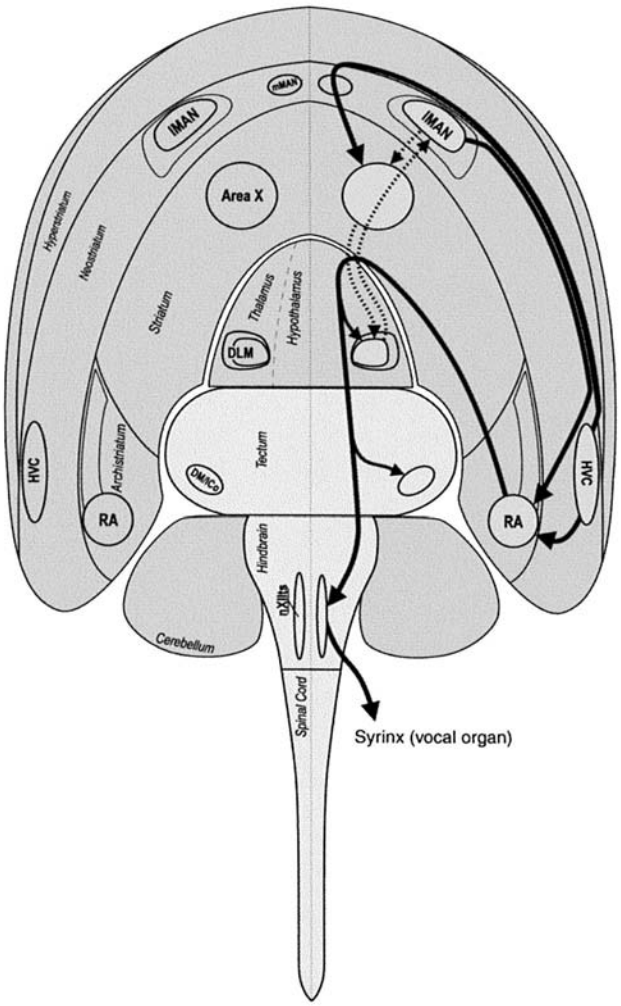

Fig. 1. Flatmap of zebra finch CNS shows relative positions of major song regions and their axonal connections. Song regions located in the rostral forebrain play an important role in song learning but are not necessary for adult vocal behavior (lMAN, DLM, Area X), whereas two regions in the caudal telencephalon (HVC and RA) are important during song learning and during adult recitation of already learned song [4,19,20,22,24,27,30]. Abbreviations: 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, higher vocal center; lMAN, lateral magnocellular nucleus of the anterior neostriatum; mMAN, medial magnocellular nucleus of the anterior neostriatum; RA, robust nucleus of the archistriatum; nXIIts, hypoglossal nucleus, tracheosyringeal portion.

suggested to us that cannabinoid receptors might be highly expressed within the zebra finch song control system. Thus, utilizing a combination of radioligand binding, Northern blotting, RT–PCR, and in situ hybridization techniques, we determined that high-affinity CB1 receptors are present within zebra finch telencephalon and that cells expressing CB1 mRNA are present at high density within at least two brain regions for song (HVC and RA, see Fig. 1). These findings suggest that in birds, as in mammals, CB1 expression may play an important role in telencephalic processes that involve motor control, learning and memory, and cognition. Moreover, given the anatomical and behavioral specificity of song control brain regions, the zebra finch may provide a particularly useful model to examine the physiological role of cannabinoids in forms of learning and behavior that are developmentally regulated.

2. Materials and methods

2.1. Subjects

Subjects were male zebra finches raised in our breeding aviaries. Birds were 20 days of age (auditory learning stage birds), 55 days of age (sensory–motor learning stage birds) or >90 days of age (adult birds) at the time of sacrifice.

2.2. Chemicals and reagents

Except where otherwise indicated all supplies and reagents were purchased from Life Technologies, Sigma, or Fisher.

2.3. Radioligand binding

CP-55940 in tritiated form (180 Ci/mmol, DuPont/NEN) and well-washed zebra finch neuronal membranes (from the whole brain of an adult male zebra finch) were used for equilibrium saturation isotherm experiments. Binding reactions were conducted in a final volume of 200 μl containing: 0.5% BSA, 0.1% ethanol, and 50 μg membrane protein in 25 mM HEPES/10 mM $MgCl_2$, pH = 7.4. Nonspecific binding was defined as that occurring in the presence of 1 μM WIN55212-2(RBI). Equilibrium binding reactions were conducted over 120 min at 25°C and terminated by rapid filtration over GF/C glass fiber filters. Filters were washed immediately after termination of binding reactions with 3 ml ice-cold assay buffer. Filter-trapped radioactivity was quantified using a Beckman LS 5801 scintillation counter. Curves were fit to data and K_d and B_{max} values ± S.E.M. calculated using GraphPad Prism software (GraphPad, San Diego, CA). Fits of equations describing one- and two-site models were compared through F-tests. The one-site equation used was: $B = (B_{max} L)/(K_d + L)$, where B is specific binding, and L is the concentration of radioligand.

2.4. In situ hybridization

A free-floating, fixed tissue method for in situ hybridization was employed as described (see Ref. [13]). Male zebra finches in the sensory–motor stage of vocal development ($n = 4$) were overdosed with anesthetic and perfused with saline followed by 4% paraformaldehyde. Brains were removed and the caudal half of the telencephalon (containing the song regions HVC and RA) was frozen-sectioned in the coronal plane (30 μm thickness). Alternate sections were collected into 20-ml scintillation vials containing ice-cold 2× SSC buffer. RNAse A (100

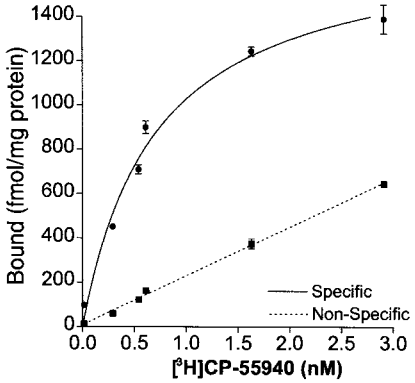

Fig. 2. Equilibrium saturation binding of [^{3}H]CP-55940 to zebra finch neuronal membranes (50 μg protein). Membranes were incubated with radioligand concentrations shown at 25°C for 120 min. Data points represent means of triplicate measures, bars represent S.E.M. Specific binding data were best fit with an equation describing interaction with a single binding site ($K_d = 0.55$ nM, $B_{max} = 1.76$ pmol/mg protein). Nonspecific binding was determined with 1 μM WIN55212-2.

μg, Qiagen) was added to one set of alternate sections as a control. [^{32}P]dCTP-labeled probes were synthesized with random hexamer primers and Klenow fragment using the zebra finch CB1 PCR fragment described below as a template. Following film exposure, slides were coated with and allowed to expose photoemulsion for 2 weeks. After developing, autoradiograms were lightly counterstained with thionin and coverslipped with Permount (Fisher). Percentages of cells labeled by in situ hybridization in various brain regions were determined through counts of silver grains over cells in randomly selected high-power fields. A 99% Poisson criterion was used to distinguish labeled from nonlabeled cells [2].

2.5. Isolation of total RNA

Tissue was isolated from male zebra finches at three stages of vocal development (auditory learning stage, sensory–motor learning stage, adult). Birds were anesthetized, decapitated and caudal telencephalon (containing the song regions HVC and RA) was isolated and immediately homogenized by Polytron in RNA isolation buffer (Tri Reagent, Molecular Research Center). RNA was isolated following Tri Reagent manufacturer's instructions.

2.6. RT–PCR

Five micrograms of total RNA was treated with DNAase I (1 U [unit] to digest contaminating genomic DNA), phenol/chloroform extracted, and used for Superscript II reverse transcriptase-mediated synthesis of oligo(dT)12–18-primed cDNA. Reactions were terminated by addition of RNAse H (2 U). cDNA (100 ng) was used for *Taq* polymerase-catalyzed PCR. Control reactions, stopped at three-cycle intervals, were used to determine the linear amplification range. These control reactions ensured that plateau conditions were avoided. A total of 25 cycles of the following temperature and duration steps were employed: 94°C × 30 s; 52°C × 45 s; 72°C × 60 s. Relative amplification of fragments of CB1 and the housekeeping gene GAPDH were assessed across stage of vocal development. GAPDH was selected as a control as its expression in rat brain has been reported to be stable over the course of development [25]. Degenerate PCR primers for CB1 and GAPDH were designed based on alignments of the amino acid sequences of various species. Each primer incorporates a 5′ three-base spacer followed by a restriction site. The sense CB1 primer was designed based on the amino acid sequence ''YHFIG'': ATGAAGCTTTACCACTTCATHGG. The antisense CB1 primer was designed based on the amino acid sequence ''FCSMLC'': CATTCTAGAGCACAGCATRCTRCARAA. The CB1 primers amplified a single 693 base pair (bp) fragment. The sense GAPDH primer was designed based on the amino acid sequence ''PMFVMG'': ATGCTGCAGCCNATGTTYGTNATGGG. The sequence of the antisense GAPDH primer was based on the amino acid sequence ''WYDNEY'': ATGCTGCAGCCNATGTTYGTNATGGG. GAPDH degenerate primers were used to amplify a 564 bp fragment. Identities of zebra finch CB1 and GAPDH fragments were confirmed by sequencing.

2.7. Northern blotting

Standard northern blotting protocols were used [5]. Briefly, total RNA (5 μg) was resolved on a formaldehyde-containing agarose gels, blotted to nylon mem-

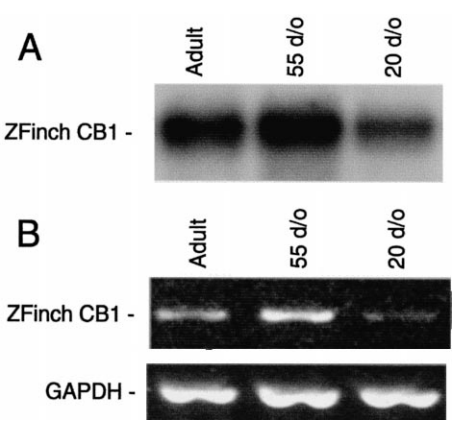

Fig. 3. Northern blotting and RT–PCR suggest that zebra finch CB1 expression changes over the course of vocal development. RNA was isolated from caudal (RA- and HVC-containing) telencephalon taken from adult, 55-day-old, and 20-day-old birds. Panel A. Total RNA (5 μg) was separated and blotted to a nylon membrane. The blot was probed with [^{32}P]-labeled zebra finch CB1 cDNA. Results shown are representative of three experiments. Panel B. RT–PCR analysis of CB1 expression confirms Northern blotting results (Panel A). After 25 PCR cycles amplifications had not plateaued. Similar intensities of fragments of the housekeeping gene GAPDH indicate that similar amounts of RNA from each group were used (bottom).

branes, and immobilized by UV cross-linking. Gels were stained with ethidium bromide and intensities of ribosomal RNA bands assessed to confirm that equal amounts of RNA had been loaded from each sample. Blots were prehybridized in a solution containing 50% formamide, 0.8 molar (M) NaCl, 20 mM PIPES (pH 6.5), 0.5% SDS, 100 μg/ml sonicated herring sperm DNA and hybridized in the same solution containing 1×10^6 counts per minute (cpm)/ml of [^{32}P]-labeled probe. Membranes exposed film between intensifying screens at −80°C for 1–2 weeks.

3. Results

3.1. High affinity cannabinoid binding sites are densely expressed within zebra finch neuronal membranes

Equilibrium saturation binding experiments demonstrated that the interaction of [^{3}H]CP-55940 with zebra finch neuronal membranes was specific, saturable, and of high affinity (Fig. 2). The fit of a single binding site model

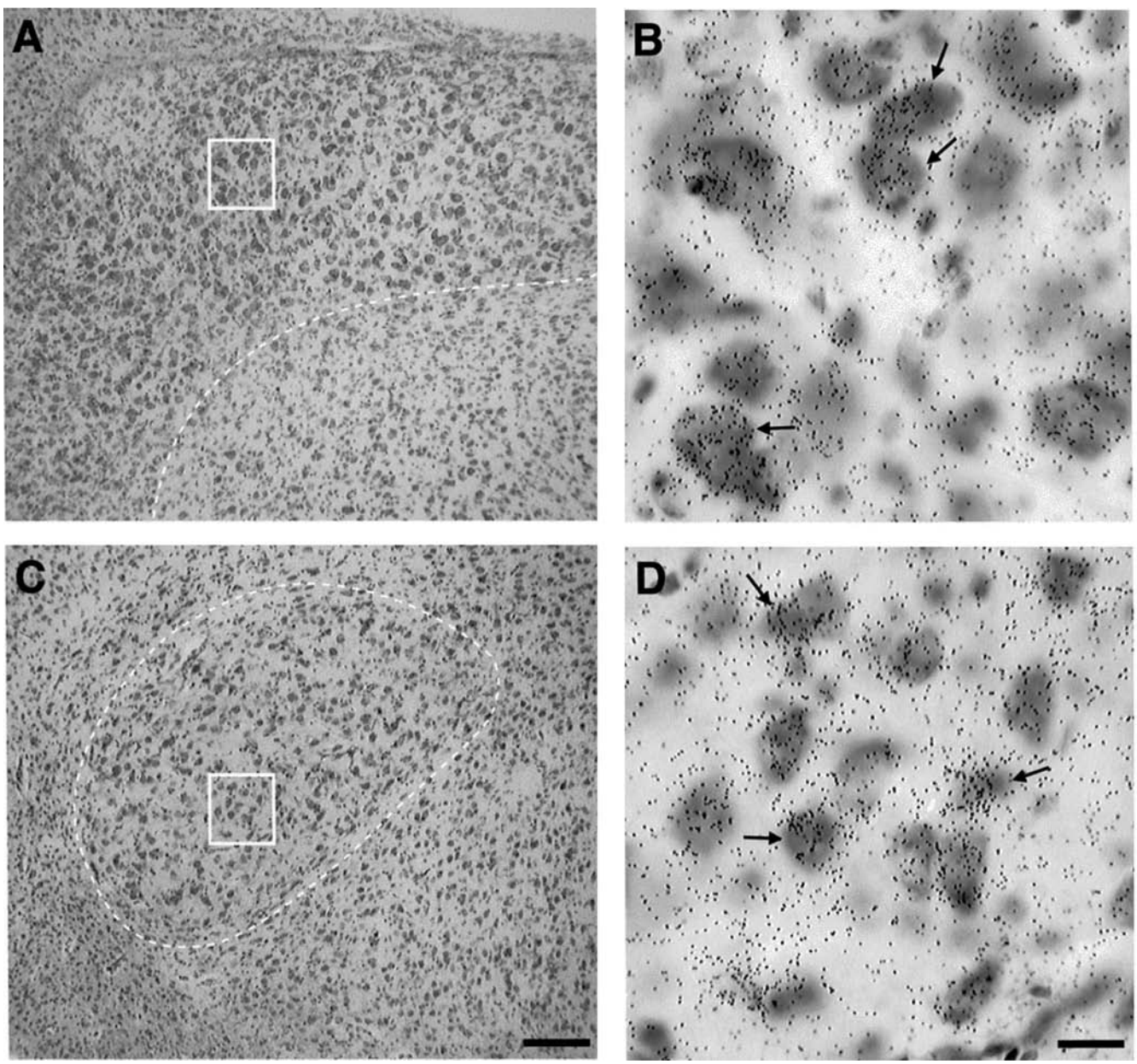

Fig. 4. Expression of CB1 mRNA in two zebra finch song regions determined through in situ hybridization. (A) Low-power photomicrograph shows the higher vocal center (HVC). The dashed line indicates the ventral border of HVC. (B) High-power photomicrograph (boxed area in A) shows silver grains concentrated over HVC cell bodies; arrows indicate examples of labeled cells. The density of labeled cells was much lower in the surrounding neostriatum (see Fig. 5). (C) Low-power photomicrograph shows the borders (dashed line) of the robust nucleus of the archistriatum (RA). (D) High-power photomicrograph (boxed area in C) shows silver grains concentrated over RA cell bodies; arrows indicate examples of heavily labeled cells. Specific cell labeling was not observed in RNAse-treated sections. In all photomicrographs medial is left. Low-power scale bar = 120 μm; high-power scale bar = 10 μm.

to data points was not improved by adding a second component ($F < 1$). Therefore, using 0.01–3.0 nM [^{3}H]CP-55940, a single population of high-affinity binding sites were labeled at 25°C with $K_d = 0.55 \pm 0.13$ nM and $B_{max} = 1.76 \pm 0.12$ pmol/mg protein. Note that the subnanomolar K_d observed in zebra finches is consistent with the high affinity of CP-55940 for mammalian neuronal membranes and membranes prepared from cells heterologously expressing mammalian CB1 receptors [21]. Moreover, the high density of [^{3}H]CP-55940 binding sites is similar to that reported using rat whole brain membranes (1.47 pmol/mg protein [3]). Thus, the presence of a population of specific cannabinoid binding sites, similar in number and affinity to those found in other species, is consistent with a CB1-mediated signaling system within zebra finch CNS.

3.2. Northern blotting and RT–PCR results suggest that zebra finch CB1 is differentially expressed over the course of song learning

Northern blots of 5 μg total RNA revealed the presence of a prominent zebra finch CB1 message of approximately 5.5 kb (Fig. 3A). The size of the mRNA labeled with the zebra finch CB1 probe is similar to that reported for rat ~ 6 kb [18].

Levels of zebra finch CB1 mRNA showed distinct differences over the course of song development (see Fig. 3A). The lowest CB1 mRNA levels were observed in RNA isolated from caudal telencephalon taken during the auditory learning stage of vocal learning. Interestingly, RNA isolated from telencephalon taken during the sensory–motor learning stage of vocal development showed increased CB1 mRNA levels. RNA isolated from adult telencephalon showed higher CB1 mRNA levels than that isolated during the auditory learning stage, but lower than the sensory–motor stage. These CB1 expression differences observed by northern blotting were subsequently confirmed by RT–PCR (Fig. 3B). Both northern blot and RT–PCR results are consistent with reports of a progressive increase in the number of cannabinoid binding sites in rat brain from birth to postnatal day 60 [3]. However, increased CB1 expression in the vicinity of HVC and RA during sensory–motor learning may indicate a specific role for cannabinoid signaling in vocal development.

3.3. In situ hybridization demonstrates that zebra finch CB1 is expressed in HVC and RA

In situ hybridization experiments were performed to further assess the possible relationship between cannabinoid receptor expression in song regions and vocal development. Sections through the caudal telencephalon (containing RA and HVC) were hybridized with a [^{32}P]-labeled zebra finch CB1 cannabinoid receptor probe. Results demonstrated that the song regions HVC and RA both contain a high density of cells expressing CB1 mRNA (see Fig. 4A, B).

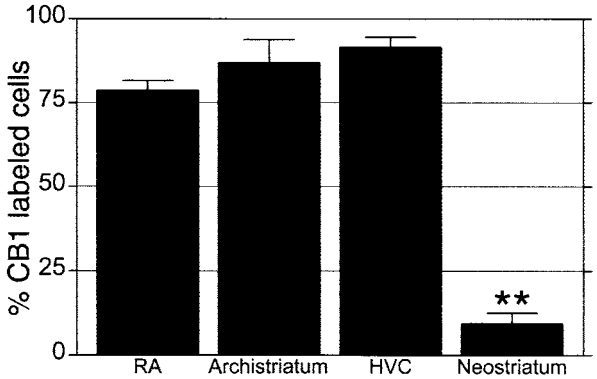

Fig. 5. Percentages of cells labeled with a CB1 probe in four zebra finch brain regions (see Fig. 1). Within RA, a high percentage of cells expressing CB1 mRNA were present both within the song region and in surrounding archistriatum. By comparison, the distribution of CB1 expressing cells in HVC was largely restricted to the song nucleus itself; a significantly lower percentage of labeled cells were present in the surrounding neostriatum (** $p < 0.01$). Shown are mean percentages ± S.E.M. from counts of four sections each from four birds.

While high percentages of cells expressing CB1 mRNA were present both within RA (mean ± S.E.M., 78.7 ± 2.9%) and the surrounding archistriatum (87.0 ± 6.8%), the distribution of CB1 expressing cells in HVC was largely restricted to the song nucleus itself (91.6 ± 3.0%); labeled cells were present but infrequent in the surrounding neostriatum (9.4 ± 3.1%, Fig. 5). A one-way repeated measures analysis of variance demonstrated a significant relationship between brain region and the percentage of CB1 labeled cells ($p < 0.001$). Pairwise comparisons (Student–Newman–Keuls method) revealed that the percentage of labeled cells in neostriatum is lower than in each of the other regions ($p < 0.01$ in each case). High-level CB1 expression in RA and HVC is notable as these regions are involved song development by juveniles and song production in adults [19,20,24,30].

4. Discussion

The data presented in this report are the most extensive characterization of the CB1 receptor in an avian species to date [12]. CB1 cannabinoid receptor expression has been confirmed by cDNA cloning in several mammalian species (human, rat, and mouse [6,18,23]) and in an amphibian (newt [26]) and teleost (pufferfish [31]). Broad taxonomic representation of CB1 expression suggests that the cannabinoid neurochemical system plays an important role in vertebrate CNS functioning. Moreover, taxonomic similarities in the affinity and density of CNS CB1 receptors imply that the regulation of this receptor system has been well preserved over the course of vertebrate evolution. The significance of CB1 signaling in vertebrate CNS is under-

scored by receptor densities that approach those of the amino acid transmitters [10].

The physiological processes under control of cannabinoid signaling in the vertebrate brain remain poorly understood. Experiments employing a recently developed CB1-deficient mouse should help provide answers [15], but clues to the role of cannabinoid systems may also be found in the distribution of expression of CB1 receptors in mammalian CNS. For example, high receptor densities in telencephalon (hippocampus and cortex) suggest roles in cognitive processes, learning, and memory [29]. A high level of telencephalic CB1 expression is consistent with the acute cognitive deficits produced by cannabinoid exposure in adult humans, such as loss of short-term memory and persistent changes in the ability to focus attention and filter irrelevant information [28]. In light of these observations, it is particularly interesting that the zebra finch shows high levels of CB1 expression in telencephalic brain regions for song learning and song production (HVC and RA).

CB1 expression appears to change over the course of zebra finch vocal learning. Since vocal learning involves multiple aspects of cognition (e.g., attention, memory, sensory–motor integration), changes in cannabinoid receptor expression during this period may have functional consequences. However, the effects of cannabinoid exposure on cognitive development remain unevaluated, due in part to the lack of appropriate model systems [1]. Since zebra finches learn vocal behavior during a restricted period of juvenile life, they may provide an ideal model for studying cannabinoid effects on cognitive development.

Acknowledgements

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