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Singing Under the Influence: Examining the Effects of Nutrition and Addiction on a Learned Vocal Behavior

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Abstract The songbird model is widely established in a number of laboratories for the investigation of the neurobiology and development of vocal learning. While vocal learning is rare in the animal kingdom, it is a trait that songbirds share with humans. The neuroanatomical and physiological organization of the brain circuitry that controls learned vocalizations has been extensively characterized, particularly in zebra finches (*Taeniopygia guttata*). Recently, several powerful molecular and genomic tools have become available in this organism, making it an attractive choice for neurobiologists interested in the neural and genetic basis of a complex learned behavior. Here, we briefly review some of the main features of vocal learning and associated brain structures in zebra finches and comment on some examples that illustrate how themes related to nutrition and addiction can be explored using this model organism.

Keywords Songbird · Auditory · Gene expression · Nutrition · Drug addiction · Alcohol · Cannabinoids · Alcohol · Retinoic acid · Vitamin A · Reward

Introduction

Songbirds have become a premier model for investigating the neurobiological and genetic basis of vocal imitation—a complex learned behavior that serves as a prerequisite for speech and language in humans [1–3]. Furthermore, there is mounting evidence that different aspects of the production and perception of birdsong are modulated by nutritional state and/or reward systems that operate in the brain. We present here some examples that illustrate how studies of nutrition and addictive substances (ethanol and marijuana) in songbirds may inform us on how these drugs exert their effects on the brain. We would like to propose that songbirds could be a highly informative model for studying the effects of drug addictions and nutrition on cognitive function and communication behavior.

Vocal learning is a rare trait known to be shared only by marine mammals (dolphins, whales), bats, birds (parrots, songbirds, hummingbirds), and possibly elephants [4–8]. Other mammals, including non-human primates, appear to lack the trait, making songbirds the most tractable model for studying the neurobiology of learned vocal communication. Zebra finches (*Taeniopygia guttata*) have been the workhorse of birdsong research owing to the facts that they breed well in captivity, are continuous year-round singers, and lend themselves to longitudinal studies of vocal development due to their relatively short generation time (~3 months). Moreover, song represents a remarkably robust and quantifiable behavior that lends itself well to integrated studies of sensory, motor, and cognitive brain function [9, 10]. In many songbird species, the acquisition and production of song is critical for territorial defense, mate attraction, and/or the establishment and maintenance of dominance hierarchies. Decades of study have revealed a wealth of information about the connective, physiological, and neurochemical properties of neural circuits that are involved in the acquisition, production, and perceptual processing of birdsong (for general reviews, see [2, 3, 5]). More recently, armed with modern molecular tools such as

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the complete sequence of the zebra finch genome, extensive brain cDNA libraries and EST databases, microarrays and transgenic techniques [11–15], songbird researchers are beginning to make great strides towards identifying the molecular bases of this complex learned behavior.

The process of song learning in songbirds shares many striking parallels with speech acquisition in humans ([1, 16], reviewed in [2, 3]). In zebra finches, young males (~20 days of age) begin to memorize the song of an adult male tutor, usually that of their father. This process of auditory memorization is thought to result in the formation of a long-term memory or "template" of the tutor song. A little later (~28 days of age), the young birds begin the sensorimotor phase of song learning, during which they sing a rudimentary song, or subsong, that in many respects can be considered analogous to the "babbling" observed in human infants during the early stages of speech acquisition. As the juvenile vocalizations develop further and become plastic song, they start displaying many elements of the tutor song, but still lack the stereotypy and complexity of the latter. Finally, following a period of vocal practice lasting up to ~90 days of age, the young birds gradually refine their vocal output and the song "crystallizes" into a stereotyped song that closely matches that of the tutor.

Three main neural pathways are required for various aspects of song perception, production, and learning (Fig. 1): (A) *The Auditory Pathway* (Fig. 1a)—including nucleus ovoidalis in the thalamus, field L in the telencephalon (L1, L2, and L3 subdivisions), and the adjacent high-order regions, the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM). NCM and CMM can be considered analogous to the supragranular layers of the auditory cortex in mammals and are thought to play a role in the perceptual processing and memorization of birdsong [17–22]; (B) *The Song Motor Pathway* (SMP; Fig. 1b)—including HVC (proper name), the robust nucleus of the arcopallium (RA), the tracheosyringeal portion of the hypoglossal nucleus (not shown), and brainstem respiratory nuclei [23–27]. The SMP is considered the primary pathway for the production of learned song; lesions to nuclei in this pathway prevent singing [28]; (C) *The Anterior Forebrain Pathway* (AFP; Fig. 1b)—including HVC, the lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN), striatal Area X (X), and the medial part of the dorsolateral thalamic nucleus (DLM; [29–31]). The AFP plays central roles in the sensorimotor phase of song learning, and in the long-term maintenance of adult song [32–37]. Together, the SMP and AFP are commonly referred to as the song control system, with HVC serving as a central node that sends projections to both pathways. HVC is also exquisitely sensitive to sex steroids and undergoes extensive adult neurogenesis (reviewed in [2, 3]).

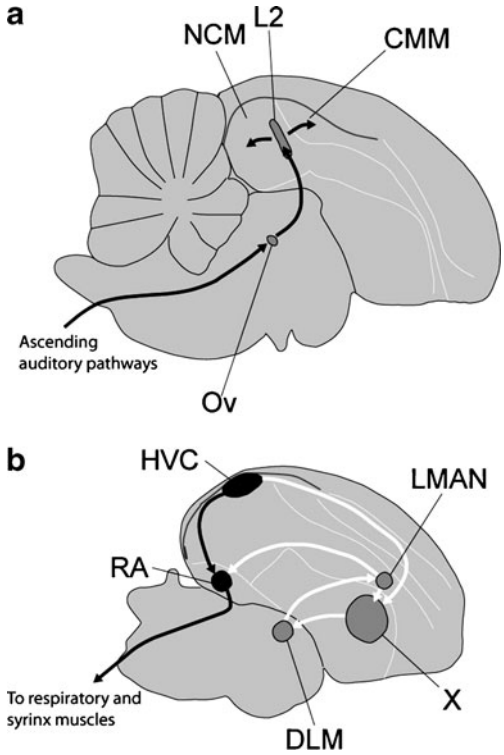

Fig. 1 Brain areas required for learning, production, and perception of song in zebra finches. **a**, **b** Schematic diagrams depicting the songbird brain in the parasagittal plane illustrate the approximate positions and pathways connecting various nuclei in the **a** auditory and **b** song control systems. **a** Nucleus ovoidalis (Ov), caudomedial nidopallium (NCM), caudomedial mesopallium (CMM), and field L (including subfields L1, L2, and L3) are thought to be important for the perceptual processing and memorization of birdsong. **b** The song system consists of the direct motor pathway (nuclei and projections in black), including connections from song nucleus HVC to the robust nucleus of the arcopallium (RA) and from RA to midbrain and brainstem nuclei (not shown) involved in vocal-motor and respiratory control. The anterior forebrain pathway (nuclei in *gray*; projections in *white*) includes a loop from Area X in the striatum to thalamic DLM, from DLM to LMAN, and from LMAN back to Area X (for abbreviations see text)

Some parts of the auditory system, particularly high-order areas like NCM and CMM (Fig. 1a), have received considerable attention for their proposed role in birdsong perceptual processing [38–41], as well as for the formation and storage of auditory memories of birdsong, particularly that of the tutor song ([42, 43], reviewed in [3, 22]). Of particular note, when zebra finches are presented with novel conspecific song, these areas respond with an increase in neuronal activation that rapidly habituates [44–46] and with the expression of immediately early genes (IEGs), including transcription factors (e.g., *ZENK*, *FOS*, and *JUN*; we use the human gene nomenclature convention [47], i.e., uppercase italics for gene and uppercase for protein) and direct effectors (*ARC*; [48–51]) that have been linked with synaptic plasticity and learning and memory in mammals ([52, 53]) as well as some late effectors (e.g., synapsins; [54]) that may

mediate some IEG actions. Overall, these studies indicate that NCM may play a critical role in tutor song memorization and suggest that components of the inducible gene expression response to song might play mechanistic roles in birdsong memory formation or storage. Regardless of such a link, however, song-inducible genes provide songbird researchers with an important tool for investigating birdsong representation in the auditory system [55]. As discussed later, this response is also useful for investigating the physiological effects of nutrition and psychotropic drugs on the perceptual processing of birdsong.

Because the behavioral prerequisites of avian vocal learning are well understood, and the neuroanatomy of the avian song system is discrete and well studied, the songbird model offers considerable advantages for investigating the effects of substance of abuse and nutrition on learned behaviors and associated brain mechanisms. Below, we highlight how singing behavior and auditory processing of song are affected by diet and nutrition and identify possible links between endogenous brain pathways and ethanol and marijuana from recent examples in the songbird literature. We also discuss how songbird studies may be informative for understanding the effects of some drugs of abuse on cognitive, perceptual, and learned behavior.

Retinoid Signaling and Ethanol in the Songbird Brain

A remarkable molecular specialization of the song control system is the highly specific expression of retinaldehyde dehydrogenase 2 (*ALDH1A2)* in song nucleus HVC ([56]; Fig. 2a). *ALDH1A2* (a.k.a. *zRalDH, RalDH2*) encodes an enzyme that is responsible for the last step in the synthesis of retinoic acid, the main active metabolite of diet-derived vitamin A, an essential nutrient [57]. The discovery of *ALDH1A2* expression in HVC suggested that retinoic acid might play an important role in the development and/or maintenance of the song control system. Indeed, when the synthesis of retinoic acid is blocked in the HVC of juvenile zebra finches through the local application of the aldehyde dehydrogenase inhibitor disulfiram, song remains abnormally plastic into adulthood [56]. Similarly, excess retinoic acid (supplemented orally in the diet) results in abnormally variable song with low complexity in adult finches [58].

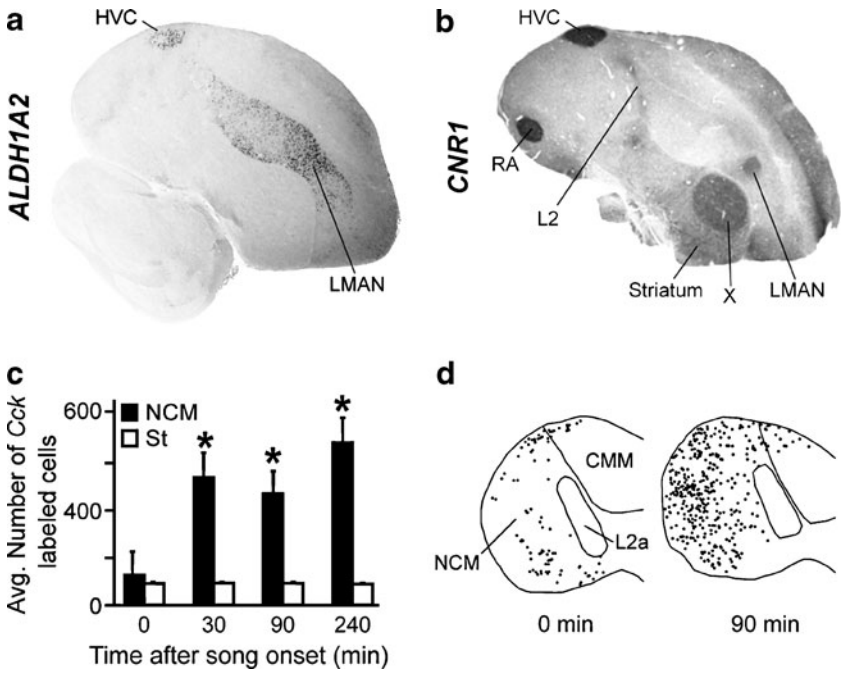

Fig. 2 Expression of genes related to retinoic acid synthesis (*ALDH1A2*), endocannabinoid signaling (CB_1), and cholecystokinin (*CCK*) in the brain of an adult male zebra finch. **a** Expression pattern of *ALDH1A2* in a parasagittal brain section (~1 mm from the midline) revealed by in situ hybridization (see [58] for details). **b** Immunohistochemical staining reveals selective expression of CB_1 in the song system (HVC, LMAN, RA, Area X) and field L2, which projects to NCM (see Fig. 1 for schematics). **c** Song stimulation induces *CCK* mRNA expression in NCM. Average counts of *CCK*-expressing cells within NCM (*black bars*) and the striatum (*white bars*) measured 0, 30, 90, and 240 min after song stimulation onset. *Asterisks* denote values that were significantly different from the unstimulated control group (time 0) according to an ANOVA and post hoc *t* tests. **d** Representative maps (from parasagittal sections at ~0.6 mm from the midline) showing *CCK*-expressing cells (*black circles*) detected in NCM by fluorescent in situ hybridization 0 and 90 min after the onset of song stimulation (see [109] for details). Panel **b** is modified, with permission from [91] © (2004) The Society for Neuroscience. Panel **c**–**d** are modified with permission from [109] © (2011) John Wiley & Sons, Inc. For abbreviations see text

These findings are in line with evidence from deprivation studies in rodents that vitamin A plays an important role in brain physiology, especially in areas like the hippocampus and in cognitive functions like learning and memory, likely mediated through retinoid-dependent signaling [59–62]. Indeed, retinoic acid receptors (RARs) α, β, and γ are widely expressed postnatally in the rodent brain [63, 64] and are required for neuronal plasticity and learning and memory [65, 66]. In songbirds, retinoic acid receptors are also broadly distributed throughout the brain, with some enrichment in auditory NCM and in Area X within the song system [67], suggesting that retinoic acid could have effects outside regions of *ALDH1A2* expression. Together, these studies provide intriguing evidence that retinoic acid, previously thought to be exclusively involved in embryonic development, can exert important post-embryonic actions in the vertebrate brain. They also indicate that tissue levels of retinoic acid in adulthood need to be kept within tight boundaries, since both deficiencies and excesses can have serious deleterious effects.

In addition to degradation enzymes and retinoid carrying proteins, tight control of retinoic acid levels is achieved by a battery of oxidative and reductive enzymes in the vitamin A (retinol) metabolic pathway. These enzymes are members of the same families of enzymes that metabolize ethanol and are responsible for its detoxification. In a first step within these pathways, enzymes of the alcohol dehydrogenase (ADH) and short-chain dehydrogenase/reductase (SDR) families facilitate conversion of retinol or ethanol to retinaldehyde and acetaldehyde, respectively (Fig. 3; [68]). Because acetaldehyde is highly toxic, causing cell and tissue damage, its prompt removal is essential. The second step, representing the oxidation of aldehydes, is thus particularly critical. Importantly, tissue activity of aldehyde dehydrogenases involved in acetaldehyde clearance is upregulated by ethanol [69]. Within the liver, an important consequence of this upregulation is an increased catabolism and eventual depletion of vitamin A, which is normally stored in the form of retinyl esters. Another potential consequence is that upregulated non-specific aldehyde dehydrogenases may end up taking over the retinoic acid-generating action of retinaldehyde-specific enzymes like *ALDH1A2*. In the brains of alcoholics, therefore, the upregulation of ethanol-metabolizing enzymes may result in the increased synthesis of endogenous retinoic acid. Hence, a potential serious effect of alcohol consumption is an increase in tissue levels of retinoic acid, which can have its own deleterious consequences, including suppression of naturally occurring adult neurogenesis [62]. Indeed, exposure of mice to ethanol was recently found to significantly increase retinoic acid levels in the hippocampus [70], a structure involved in various aspects of cognitive function, including memory formation and spatial navigation. Specifically, acute ethanol exposure increased retinoic acid in the hippocampus by 1.6-fold, while chronic maternal ethanol exposure increased retinoic acid in fetal hippocam-

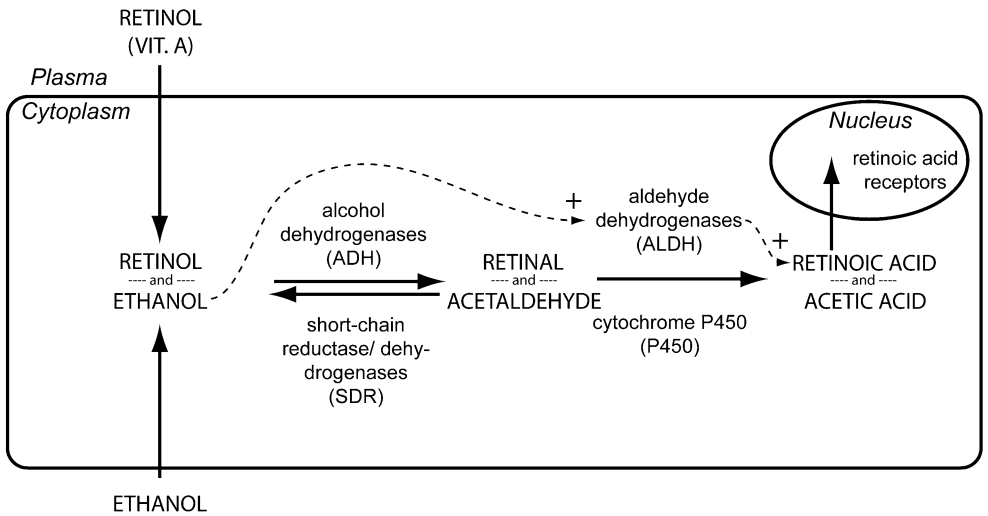

Fig. 3 Major substrates and common enzyme families in the metabolism of retinoids and ethanol. Retinol bound to carrying proteins (not shown) is delivered in plasma to target tissues where it is converted to retinoic acid for signaling in the nucleus. The first step that converts retinol to retinal is reversible and is catalyzed by alcohol dehydrogenases (ADH) and short-chain dehydrogenase/reductases (SDR). Several SDRs that differ in their enzyme kinetics may be present in the same cell, and thus, the direction and rate of this step is based on the balance of substrates and catalytic enzymes. In contrast, the oxidation of retinal to retinoic acid in the second step is irreversible and is catalyzed by aldehyde dehydrogenase (ALDH) and CYP450 enzymes. Various ALDHs differ in their affinity for their aldehyde substrate; retinal is normally oxidized by ALDH1A2. Ethanol metabolism relies on many of the same enzyme families and its presence in cells may increase ALDH activity (indicated by the *dotted line*) facilitating the elimination of toxic acetaldehyde intermediates. Thus, a consequence of the presence of ethanol in tissues may be elevated levels of retinoic acid and altered signaling in the nucleus

pus by 50-fold. Thus, brain centers that function in cognition and memory formation may become vulnerable to the deleterious effects of excess retinoic acid in consequence of ethanol consumption.

Excessive ethanol consumption in humans has been linked to cognitive impairments and memory deficits that can arise during different stages of brain development. For example, fetal alcohol syndrome, adolescent binge drinking, and chronic alcohol consumption in adulthood all impair cognitive function, though the exact mechanisms for each of these are still not well understood [71]. The recent findings linking ethanol and retinoic acid pathways suggest that some of the deleterious effects of ethanol on cognitive function might be mediated by disrupted retinoid signaling in the brain. The hypothesis above may be readily testable in songbirds. Song can be easily recorded and various sensitive tools for quantitative analysis of song acoustic properties, vocal imitation, and song ontogeny are available. Furthermore, *ALDH1A2* is selectively expressed in the large neurons that project to Area X in the songbird AFP, which is centrally involved in song learning [56]. Although X-projecting neurons in HVC are relatively stable after hatching [72], they are conceivably a source of neurotrophic support for the replaceable RA-projecting neurons in the SMP that control the timing of song motifs [73]. While effects of retinoic acid on neuronal precursor proliferation and neuronal replacement have been observed in rodents [62, 74], such a link has not yet been demonstrated in songbirds. If present, it would open the doors for examining the effects of ethanol consumption on a learned vocal behavior and its associated brain circuitry.

The evidence linking song maturation to retinoic acid signaling suggests that dietary vitamin A may be a critical determinant of adult song quality. While most researchers would agree that birdsong plays important roles in territoriality and courtship behaviors, a widely debated issue is whether birdsong also conveys specific information about the fitness and/or developmental history of singing adults, which could have important implications for reproductive success. Much attention has been devoted to the developmental stress hypothesis [75], which in songbirds postulates that a complex trait like birdsong and its underlying brain circuitry may be sensitive to potential stressors like food deprivation [76]. Indeed, experimental studies have provided some support for this notion [76–79] and suggest that birdsong may be a sensitive read-out of the nutritional history of birds. To date, however, there is little evidence for the role of specific nutrients. In this context, further efforts in songbirds may provide unique opportunities for establishing conclusive links between a complex learned behavior and nutritional history during development.

Cannabinoids and Birdsong

When subjected to food restriction [80, 81] or administered drugs that favor the metabolism of stored lipids over dietary carbohydrates [82], adult male zebra finches sing less than control birds. These observations indicate that the production of birdsong can be modulated by brief periods of limited food availability. Intriguingly, Δ^9-tetrahydrocannabinol (or the synthetic agonist WIN55212-2), a known ligand of the endocannabinoid type 1 brain receptor (CB_1; a.k.a CNR1) and the main psychoactive component of *Cannabis sativa* has also been reported to depress singing behavior ([83, 84]; Fig. 2b) and to reduce song-specific neuronal activity in central auditory areas (as measured by ZENK induction; [85]). Consistent with these effects, CB_1 receptors (mRNA and protein) appear to be abundantly expressed throughout the song system [83, 86], and auditory field L2, though relatively few labeled cells are present in song-responsive auditory areas that express ZENK [50, 87]. In mammals, CB_1 receptor activation has been linked to appetite stimulation, food intake, and overeating behaviors, among others (for reviews see [88–90]), raising the intriguing possibility that some of the effects of nutrition on song behavior could be linked to the modulation of cannabinoid signaling pathways in the brain. To test this idea, Soderstrom et al. [91] injected food restricted birds with a specific CB_1 antagonist (SR141716A) and measured both singing behavior and the induction of ZENK by song in several auditory areas. They found that CB_1 receptor blockade partially reversed the suppressive effect of food deprivation on singing and also partially re-established the song responsiveness of NCM [85, 91]. Furthermore, food restriction could be correlated with a significant rise in the levels of the endogenous CB_1 receptor ligand, 2-arachidonoylglycerol (2-AG), but not anandamide, thus implicating enhanced 2-AG endocannabinoid signaling in modulating both song perceptual processing and production. Taken together, these findings suggest that food deprivation somehow triggers a rise in 2-AG through an as yet unspecified pathway, which in turn suppresses song-related behaviors through the activation of an endogenous cannabinoid signaling pathway.

In light of the widespread effects of cannabinoids on song perception and singing behavior, it should not come as a surprise that CB_1 signaling also influences the process of vocal learning in songbirds. For example, administration of CB_1 agonists during the sensorimotor period of song learning results in adult song that exhibits decreased stereotypy and possesses fewer notes than normal song [84, 92]. Recent studies suggest that these effects on vocal learning may be partly attributable to a cannabinoid-mediated increase in the expression of *FOXP2* in the striatum [93], including song nucleus Area X. In humans, mutations in the gene encoding the *FOXP2* transcription

factor are associated with developmental verbal dyspraxia—a speech-related disorder that results from imprecise movements of tongue, lips, palate, and jaw [94]. In zebra finches, knock down of *FOXP2* expression in Area X during the sensorimotor learning period results in only partial reproduction of the tutor song [95], suggesting a role for *FOXP2* in vocal plasticity. Of interest, these same neurons in Area X not only receive glutamatergic input from HVC, but also dopaminergic input from the substantia nigra [96, 97], suggesting that the activation of an endogenous reward pathway is implicated in the fine-tuning of vocal-motor control and/or in sensorimotor learning. Taken together, these findings imply an intriguing functional link between vocal learning, *FOXP2*, and the activation of endocannabinoid and dopaminergic-dependent signaling cascades, raising the value of songbirds as a research model for investigating the effects of drugs of addiction on speech and language development.

Cholecystokinin: A Second Link Between Endocannabinoid Signaling and Song Auditory Processing?

Similar to endocannabinoids, the neuropeptide cholecystokinin (CCK) has been implicated in the regulation of a variety of cognitive processes, including feeding behavior and satiety, as well as learning and memory. Although first identified as a gut peptide, CCK is now recognized as one of the most abundant and widely distributed neuropeptides in the brain [98–101]. Intriguingly, unlike the CB_1 receptor (which is high in the song system and absent in secondary auditory areas), *CCK* mRNA is low in the song system, but highly expressed in auditory areas, including NCM [109]. Moreover, novel song presentation dramatically increases *CCK* (both the number of labeled cells and mRNA levels [109]; Fig. 2c) in a manner reminiscent of the induction of IEGs [48, 50]. Whether there is a role for *CCK* in regulating song auditory processing or singing remains to be tested, but that a peptide implicated in the regulation of feeding behavior and satiety [102, 103] is also regulated by song, raises interesting parallels with the effects of endocannabinoid signaling on songbird behavior.

Intriguingly, in mammals, the expression of the CB_1 and CCK receptors co-localizes in neocortex, hippocampus, and basal ganglia [104–107]. In some areas, CB_1 receptor activation can modulate the release of *CCK* through retrograde messenger signaling [108]. While a functional link between CB_1 receptors and CCK has not been tested in songbirds, available data indicate a possible overlap of their distributions in several basal ganglia structures, including dopaminergic cells in the substantia nigra [86, 109]. Thus, while speculative, it appears possible that CCK and endocannabinoid signaling pathways may functionally overlap in some regions of the songbird brain, including areas that have been implicated in reward and addictive behavior. A possible involvement of dopaminergic cells is particularly intriguing, given the extensive dopaminergic innervation of the song system [96, 97, 110–112] and mounting evidence, suggesting a role for dopaminergic modulation in various aspects of singing behavior and vocal learning [111, 113–117]. This possibility is also in line with the notion that endogenous brain signaling and/or metabolic pathways commonly associated with feeding behavior can be targets of specific drugs of abuse [118–122]. Clearly, more studies will be needed to understand how these various systems interact, but songbirds seem like an ideal model organism to explore these questions further.

Synopsis/Conclusions

The relative ease of breeding birds like zebra finches and of characterizing their vocal output, coupled with the extensive knowledge on vocal control pathways and the novel molecular tools that are increasingly available for birds, promise to make birdsong a highly tractable system to unravel the genetic basis of learned vocal behaviors. The studies discussed here also indicate that songbirds can be highly informative in the investigation of how factors related to nutrition and addiction modulate vocal production, perception and learning, and their underlying brain pathways. Although still in its infancy, an emerging theme in this field appears to be the considerable degree of overlap between pathways related to dietary or nutritional state and some molecular targets for drugs of addiction. Brain areas and neuronal populations related to reward mechanisms (e.g., the dopaminergic system) appear to be of particular interest, as these may be common modulators of addiction and satiety. While we illustrated these points with a few examples focusing on ethanol and marijuana, we believe that the songbird model might be applied more broadly to understand how other substances (e.g., methamphetamine, opioids, cocaine) affect cognitive and reward mechanisms in the brain as well. In turn, establishing mechanistic links between nutrition and addictive drugs may open many new avenues for understanding the phylogenetic, developmental, and pathophysiological implications of food uptake, energy metabolism, and drug addiction behavior.

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