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Novel Song-Stimulated Dendritic Spine Formation and Arc/Arg 3.1 Expression in Zebra Finch Auditory Telencephalon are Disrupted by Cannabinoid Agonism

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Abstract

Cannabinoids are well-established to alter processes of sensory perception; however neurophysiological mechanisms responsible remain unclear. Arc, an immediate-early gene (IEG) product involved in dendritic spine dynamics and necessary for plasticity changes such as long-term potentiation, is rapidly induced within zebra finch caudal medial nidopallium (NCM) following novel song exposure, a response that habituates after repeated stimuli. Arc appears unique in its rapid postsynaptic dendritic expression following excitatory input. Previously, we found that vocal development-altering cannabinoid treatments are associated with elevated dendritic spine densities in motor- (HVC) and learning-related (Area X) song regions of zebra finch telencephalon. Given Arc's dendritic morphological role, we hypothesized that cannabinoid-altered spine densities may involve Arc-related signaling. To test this, we examined the ability of the cannabinoid agonist WIN55212-2 (WIN) to: (1) acutely disrupt song-induced Arc expression; (2) interfere with habituation to auditory stimuli and; (3) alter dendritic spine densities in auditory regions. We found that WIN (3 mg/kg) acutely reduced Arc expression within both NCM and Field L2 in an antagonist-reversible manner. WIN did not alter Arc expression in thalamic auditory relay Nucleus Ovoidalis (Ov), suggesting cannabinoid signaling selectively alters responses to auditory stimulation. Novel song stimulation rapidly increased dendritic spine densities within auditory telencephalon, an effect blocked by WIN pretreatments. Taken together, cannabinoid inhibition of both Arc induction and its habituation to repeated stimuli, combined with prevention of rapid increases in dendritic spine densities, implicates cannabinoid signaling in modulation of physiological processes important to auditory responsiveness and memory.

Keywords

dendritic spines; cannabinoid; auditory; sensory; songbird; Arc/Arg 3.1; learning; memory

1. Introduction

Cannabinoids modulate neural activity important to learning and memory and a range of vertebrate behaviors (Cocchetto et al., 1981; Guindon and Hohmann, 2009; Soderstrom et

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al., 2004). The predominant receptor subtype expressed in CNS, thought to mediate most central cannabinoid effects, is CB₁. Activation of these predominantly presynaptically-located receptors is associated with reduced probability of neurotransmitter release (Elphick and Egertova, 2001). Distinct CB₁ expression within cerebellum, hippocampus, frontal cortex, and basal ganglia correlates with well-documented modulation of locomotor coordination, memory, anxiety, and reward (El Manira and Kyriakatos, 2010; Fernandez-Ruiz et al., 2010; Herkenham et al., 1991a; Herkenham et al., 1991b; Herkenham, 1992; Hill et al., 2011; Kinsey et al., 2011). Particularly intriguing are physiological effects cannabinoids produce on neuronal activity essential for processes of learning and memory (Hoffman et al., 2010; Wilson and Nicoll, 2002).

Using a songbird model of developmental psychopharmacology, we have previously found that once daily administration of the cannabinoid agonist WIN to zebra finches alters their vocal development (Soderstrom and Johnson, 2003; Soderstrom and Tian, 2004), and prevents normal developmental decreases in dendritic spine densities within telencephalic regions important for both sensorimotor learning (Area X) and production of stereotyped song (HVC, (Gilbert and Soderstrom, 2011). Effects on spine densities were only produced following developmental exposure; treatments given to adults that had completed vocal learning were not effective.

Others have shown that cannabinoid receptor activation disrupts both long-term depression (LTD) and long-term potentiation (LTP); persistent changes in neuronal activity associated with learning and memory (reviewed by (Chevalayre et al., 2006). Also critically-involved in learning-associated neural plasticity is the dendritically-located immediate early gene product *Arc/Arg3.1* (*Arc*, reviewed by (Bramham et al., 2008). *Arc* is involved both in dendritic spine dynamics and, in zebra finches, auditory processing essential for vocal learning (Velho et al., 2005). In the songbird, *Arc* mRNA is induced in auditory forebrain in response to novel song. This expression occurs within zebra finch caudomedial nidopallium (NCM), a brain region that receives prominent input from the primary auditory thalamorecipient region Field L2 (L2, see Fig. 1). This arrangement suggests that NCM may be analogous to mammalian auditory association cortex (reviewed by (Bolhuis and Gahr, 2006) and implicates the region as important to sensorimotor vocal learning requiring auditory feedback (London and Clayton, 2008). This pattern of song-stimulated *Arc* expression closely resembles that of another immediate early gene, *Zenk* (Mello et al., 1995; Mello et al., 2004; Velho et al., 2005; Whitney et al., 2003). Decreased *zenk* expression following habituation to novel song is notably associated with reduced electrophysiological responsiveness within NCM (Chew et al., 1995; Chew et al., 1996; Stripling et al., 1997).

Given clear roles for both *Arc* and CB₁ in auditory processes important to sensorimotor vocal learning, we have investigated the possibility that these two proteins interact in a manner disruptive to learning-related dendritic spine plasticity. Here we have used behavioral, pharmacological, and immunohistological methods to examine the role of CB₁ activation to acutely modulate song-induced *Arc* expression, and to alter dendritic spine densities normally associated with vocal learning.

2. Results

2.1 Anti-Arc Antibody Selectivity

Western blotting was performed to assess selectivity of the anti-Arc antibody used. In an effort to capture *Arc* protein expression at its maximum levels, brains of animals exposed to novel song were used for these experiments. SDS-PAGE separation of 20 µg of brain protein revealed the presence of a single predominant band of approximately 55 kDa labeled by the anti-Arc antibody. The size of this labeled protein is consistent with that reported

from mammalian species, including mouse, rat and human (see Figure 2A, and (Bramham et al., 2008). Anti-Arc immunoreactivity was eliminated following preabsorption of antisera with the immunizing peptide (see Figure 2A). This peptide was also used immunohistochemically to demonstrate that preabsorption eliminated selective tissue staining. To determine the cellular localization of anti-Arc binding within neurons, images of double immunofluorescence labeling of dendritically-associated MAP2 (Figure 3B, red) with activity-stimulated arc protein within NCM (Figure 3B, green) were captured at 630 X and 1000 X, via confocal laser scanning microscopy. We observed considerable spatial overlap between the dendrite-associated protein MAP2 (Figure 3, red) and the Arc protein, which appears present both somatically and dendritically (see Figure 3, yellow merge). Note the robust expression of Arc protein both within dendrites (Figure 3, solid arrows), and cell bodies (Figure 3, dashed arrows). This pattern of both somatic and dendritic expression is consistent with that reported for *Arc* mRNA (Velho et al., 2005).

2.2 Cannabinoid Inhibition of Novel Song-Stimulated Arc Expression

The ability of novel song exposure to stimulate *Arc* mRNA expression within zebra finch auditory telencephalon has been previously demonstrated (Velho et al., 2005). In-terms of protein expression, we found that the different treatments employed significantly altered optical densities of anti-Arc staining within both NCM and L2. These treatment effects were confirmed by one-way ANOVA: NCM ($F(2,252) = 48.22$; $p < 0.001$) and Field L2 ($F(2,226) = 23.77$; $p < 0.005$). Results are summarized in Figure 4. Tukey's post hoc analyses further revealed that within both NCM and L2 that novel song significantly increased staining densities (indicated by asterisks in Figure 4J and K). Within NCM, this increase was from $OD = 0.049 \pm 0.003$ in VEH-Silence group to 0.091 ± 0.004 in the VEH-Novel Song group ($p < 0.05$). Within L2, this increase was from $OD = 0.051 \pm 0.003$ in the VEH-Silence group to 0.093 ± 0.005 in VEH-Novel Song group ($p < 0.05$). Post-hoc analyses also confirmed that WIN treatment (3 mg/kg given 30 min. prior to novel song exposure) significantly decreased staining intensities within both regions relative to that produced following novel song stimulation alone (indicated by daggers in Figure 4J and K). Within NCM, this decrease was from 0.091 ± 0.004 in the VEH-Novel Song group to 0.059 ± 0.002 in the WIN-treated group ($p < 0.05$). Within L2 this decrease was from 0.093 ± 0.005 to 0.068 ± 0.005 in the WIN-treated group ($p < 0.05$).

In order to assess whether cannabinoid effects on Arc expression were selective and not due to a general stimulation, we examined optical densities of Arc staining within the thalamic auditory relay region, Ov. Interestingly, optical densities of Arc labeled cells within this thalamic auditory relay region did not vary as a function of novel song exposure or cannabinoid treatment. This lack of a treatment effect was confirmed by one-way ANOVA: ($F(2,105) = 0.1118$; $p > 0.05$). Ov results are summarized in Figure 5.

2.3 WIN Prevents Habituation of Arc Expression Following Repeated Auditory Stimulation

Prior work demonstrates that Zenk expression is decreased following habituation to sequential presentations of the same conspecific song, supporting a relationship between Zenk expression and novel song recognition (Mello et al., 1992; Whitney et al., 2003). Similar patterns of Arc expression within auditory telencephalon led us to test whether Arc protein expression also habituates following repeated presentation of the same song, and whether this habituation is influenced by CB₁ receptor activation.

As was the case for zenk, repeated presentation of the same conspecific song resulted in habituation of Arc expression within both NCM and Field L2 (compare Figure 6 A-C to Figure 6 G-I, and Figure 6P, Vehicle + Silence to Vehicle + Song). One-way ANOVA confirmed treatment differences in Arc immunoreactivity ($F(4,247) = 31.68$; $p < 0.0001$)

and ($F(4,143) = 13.14$; $p < 0.0001$) for NCM and Field L2, respectively. Tukey post-hoc analyses revealed that pretreatment with WIN prior to the first two song presentations prevented this habituation from occurring in response to the same song on the third day within NCM (compare Figure 6 G-I to Figure 6 J-L, and Figure 6 P Vehicle + Song to Day 1-2 WIN, Day 3 Vehicle, from 0.139 ± 0.033 to 0.199 ± 0.063 ($q = 11.56$, $p < 0.0001$). Habituation was also prevented by WIN treatment within Field L2 where mean optical densities were significantly elevated from 0.129 ± 0.027 to 0.165 ± 0.0239 ($q = 7.892$, $p < 0.0001$). Importantly, in both NCM and Field L2, agonist-induced prevention of habituation was reversed by pretreatment with the CB₁-selective antagonist/inverse agonist, SR141716A (SR), demonstrating CB₁ mediation of the effect (compare Figure 6 J-L to Figure 6 M-O, and Figure 6 P Day 1-2 WIN, Day 3 Vehicle to Day 1-2 WIN/SR, Day 3 Vehicle).

2.4 WIN Disrupts Auditory Stimulation-Induced Increased Dendritic Spine Densities within NCM

We previously demonstrated that developmental cannabinoid treatment is associated with elevated dendritic spine densities within telencephalic regions essential for song learning (Area X) and production (HVC, (Gilbert and Soderstrom, 2011). In addition, others have shown that activity-induced Arc expression is essential for maintenance of LTP, and is positively correlated with various forms of learning and memory, and is required for more durable forms of synaptic plasticity (Chowdhury et al., 2006; Guzowski et al., 1999; Guzowski et al., 2000; Plath et al., 2006). These observations, combined with the findings above for cannabinoid inhibition of Arc expression that is a correlated with auditory recognition, suggests a potential physiological mechanism by which exogenous cannabinoid exposure may disregulate synaptic plasticity that takes place during learning (Roberts et al., 2010). As our prior experiments investigated effects of chronic cannabinoid exposure, we have extended these results to assess potential acute influences of novel song on dendritic spine densities, and potential cannabinoid modulation of this effect.

Novel song stimulation of vehicle-treated animals resulted in a robust and rapid increase in dendritic spine densities within NCM (Figure 7). WIN administration 30 min prior to novel song stimuli reversed these song-stimulated spine increases to levels not significantly different from non-song stimulated, vehicle-treated controls (Compare Figures 7B and 7D, $F(2, 1053) = 53.67$; $p < 0.0001$).

3. Discussion

In addition to demonstrating for the first time that auditory-stimulated Arc expression (within Field L2 and NCM, respectively) is mitigated by CB₁ receptor activation, our results importantly demonstrate that these cannabinoid effects on Arc are accompanied by rapidly-altered dendritic spine densities, implying a role in processes important to learning and memory. Such a role is consistent with that observed within mammalian hippocampus where Arc is critical for memory-related neuronal plasticity, including LTP and LTD (reviewed by Bramham et al., 2008). Endocannabinoid signaling is directly involved in short-term synaptic plasticity changes (depolarization induced suppression of inhibition [DSI] and –excitation [DSE]) as well as more persistent LTD, with a modulatory role in LTP (reviewed by Chevaleyre et al., 2006). Together, clear involvement of both Arc expression and endogenous cannabinoid signaling in long-term forms of synaptic plasticity suggest that CB₁-altered Arc expression may be a general mechanism critical for regulating changes in synaptic strength. The current report is the first to demonstrate a relationship between memory-essential Arc and CB₁ activation.

Evidence demonstrating the importance of Arc signaling to neural plasticity has rapidly accumulated. For example, Arc has been found to co-precipitate and share nearly identical

intracellular distribution with F-actin in dendritic spines, and has been shown as a necessary component of actin-dependent morphological change (Bramham et al., 2008; Bramham et al., 2010; Guzowski et al., 2005; Lyford et al., 1995). Note that we have found, in vitro, that F-actin dynamics are altered following CB₁ activation (Soderstrom et al., 2012). Both *Arc* mRNA and protein sequences are highly conserved across vertebrates, and their expression during learning is so robust, distinct, and reproducible that it is used as an indicator of neural circuitry that underlies learning and memory (Guzowski et al., 2001; Guzowski et al., 2005). *Arc* has been identified as a key component of protein synthesis-dependent LTP, and is necessary for the consolidation of synaptic plasticity and formation of long term memories (Bramham et al., 2008; Pastuzyn et al., 2011; Plath et al., 2006; Ploski et al., 2008). Reductions in *Arc* mRNA and protein are associated with reduced performance in fear conditioning and spatial memory tasks (Guzowski et al., 2000; Zhang et al., 2011). Interestingly, inappropriate activation of CB₁ receptors is linked to impaired memory consolidation and performance in the very *same* tasks (Abush and Akirav, 2010; Mackowiak et al., 2009).

In other systems, *Arc* is associated with AMPA receptor expressing, calmodulin kinase-positive neurons (Vazdarjanova et al., 2006). As cannabinoid receptors are largely expressed presynaptically (Elphick and Egertova, 2001), this suggests that effects reported herein are likely attributable to receptor activation on glutamatergic terminals within both NCM and Field L2, that will effectively reduce excitatory glutamatergic signaling. If confirmed, this arrangement has similarities to the circuitry between CB₁-expressing, CA3-emanating Schaeffer collaterals that synapse on dendrites of pyramidal neurons within the CA1 region of mammalian hippocampus (Hoffman et al., 2010) associated with potent WIN inhibition of auditory sensory gating activity in rat CA3 (Dissanayake et al., 2008). Thus our results further implicate nidopallial regions in formation of novel, auditory-related memories (reviewed by (Moorman et al., 2011)). These findings also demonstrate interaction of cannabinoid signaling with the auditory memory-related function of songbird nidopallium that accumulating evidence suggests may retain the song “template” memorized during early, auditory stages of vocal development (Gobes et al., 2010; London and Clayton, 2008; Terpstra et al., 2004; Thompson and Gentner, 2010).

Cannabinoid effects to inhibit rapid spine density increases within auditory telencephalon (Fig. 7E) contribute to earlier evidence demonstrating CB₁ activation alters spine densities in both vocal motor HVC and a learning-essential region of basal ganglia, Area X (Gilbert and Soderstrom, 2011). This expands evidence for a cannabinoid signaling role in both auditory procedural-type memory and basal ganglia-related declarative memory processes (reviewed by (Izquierdo et al., 2006)), and demonstrates the value of the song bird model in studying these distinct memory-related processes.

The pattern of novel song-stimulated *Arc* protein expression that we observed differs from that of mRNA expression previously reported (Velho et al., 2005). Specifically, song-stimulated expression of *Arc* mRNA is distinctly higher within NCM than within Field L2. Note that although novel song exposure is associated with more robust *Arc* mRNA expression within NCM, distinct increases in *Arc* mRNA expression are also observed in L2 following novel song stimulation (see Figure 2 in (Velho et al., 2005)). In this context it is important to note evidence of differential control of transcriptional and translational processes following song-stimulated *Zenk* expression in zebra finch NCM (Whitney et al., 2000). In contrast to a higher level of *Arc* mRNA expression in NCM relative to L2, we have found similar levels of protein expression within these regions, although variance in song-stimulated *Arc* densities appears greater in NCM than L2 (compare Day 1-2 WIN Day 3 VEH groups, Fig. 6P and Q). This greater variance implies a more dynamic NCM responsiveness in-terms of protein expression relative to L2 that may be related to more

abundant transcript. The pattern of Arc protein expression also includes apparent somatic staining, that may be unexpected given the well-characterized dendritic function of Arc. Most Arc-related studies have focused on mRNA expression and translocation to dendrites for translation at the site of excitatory synapses (Bramham et al., 2008). Focus on this compelling dendritic function, that is clearly related to synaptic plasticity, may have eclipsed appreciation of a somatic function of Arc protein that appears also to be expressed in neuronal somata (see (Rodriguez et al., 2005; Shepherd et al., 2006). Although the Arc staining patterns observed within both NCM and L2 that we report here are consistent with presence of protein within somata, the methods we employed do not rule-out the possibility that the staining is of processes of cells residing outside of the region. Projections from L2 to NCM are established (Vates et al., 1996). The extent to which dendrites of NCM neurons are present within L2 is less clear, but the possibility for somatodendritic Arc staining remains.

Involvement of CB₁ in WIN inhibition of habituation to novel song is clearly demonstrated by reversal of the effect with the CB₁-selective antagonist/inverse agonist, SR. Agonist inhibition suggests that under normal conditions endocannabinoid tone within auditory telencephalon is likely low. As endocannabinoid signaling is increased under conditions of stress (reviewed by (Hill and Tasker, 2012) and in the zebra finch by brief periods of food deprivation (Soderstrom et al., 2004) these environmental factors may also prevent auditory habituation in a CB₁-antagonist-reversible manner. This is a testable hypothesis worthy of investigation. As treatments were administered systemically, a route most relevant to the problem of drug abuse, we do not know if effects measured are related to selective effects on receptor populations within auditory regions (that, outside of L2, express only modest densities of CB₁) or to a more general inhibition of neurotransmitter release throughout the brain. Because WIN-altered Arc expression was not observed in the thalamic auditory relay, Ov, we can be confident that effects in auditory telencephalon are unlikely to be attributable to modulation of thalamic input.

In summary, our results demonstrate that Arc is induced following novel auditory song stimulation in a manner inhibited by CB₁ receptor activation. Stimulation of Arc expression within auditory telencephalon is associated with rapid increases in dendritic spine densities, a process that is also acutely cannabinoid-reduced. Taken together, these results suggest an Arc-dependent mechanism involved in cannabinoid-modulated processes of auditory responsiveness, integration and memory. These findings may extend and provide insight to endocannabinoid signaling in other sensory systems, and CB₁-related roles in memory extinction, stress responses and deleterious effects following exogenous cannabinoid exposure, particularly during CNS development.

4. Experimental Procedure

4.1 Materials

Except where noted, all materials and reagents were purchased from Sigma (St. Louis, MO), or Fisher Scientific (Pittsburgh, PA). Immunochemicals were purchased from Vector Laboratories (Burlingame, CA), and the primary anti-Arc/Arg3.1 antibody was purchased from Abcam (Cambridge MA, Catalog # AB85656). Equithesin was prepared from reagents (40 % propylene glycol, 10 % ETOH, 5 % chloral hydrate, 1 % pentobarbital). The synthetic cannabinoid agonist WIN and inverse agonist SR141716A were suspended in vehicle from concentrated DMSO stocks (10 mM). Vehicle consisted of a suspension of 1:1:18 DMSO:Alkamuls EL-620 (Rhodia, Cranberry, NJ):phosphate-buffered saline.

4.2 Animals

Animals were cared for and experiments conducted according to protocols approved by East Carolina University's Animal Care and Use Committee. Adult male zebra finches bred and raised in our aviary were used as subjects in these experiments. Prior to the start of experiments, birds were housed singly with free access to grit, water, mixed seeds (Sunseed VitaFinch), and cuttlebone, and provided multiple perches. Animals were maintained on a 14:10 light/dark cycle, and ambient temperature was maintained at 78° F.

4.3 Acute/Chronic Treatments

Twenty-four hours prior to the start of experiments, animals were housed singly in visual and auditory isolation, in recording chambers, based on a design developed by engineering personnel in the Florida State University Neuroscience Program (Whitney et al., 2003). The method developed to induce Arc protein expression in the zebra finch auditory telencephalon was adapted from protocols used for *Arc* and *Zenk* mRNA co-induction, and *Zenk* protein expression (Lonergan et al., 2010; Mello and Ribeiro, 1998; Velho et al., 2005). Birds were exposed to a tape recording of 15 s of novel conspecific song, followed by 45 s of silence, repeated 30 times. The novel song consisted of a collection of five second song bouts from three adult males from an outside aviary. Song playbacks were presented in a fifteen second medley, followed by forty five seconds of silence, for a total of a one minute. This procedure was repeated a total of thirty times. Unstimulated control groups were only exposed to silence. Following these 30 min stimulus periods, birds were left in silence for an additional 90 min to reach peak protein expression.

In experiments that involved drug treatments, injections were given thirty minutes prior to beginning of the song stimulus procedure. A well-established protocol was used in order to determine whether WIN treatment would disrupt habituation of Arc induction following repeated presentation of the same song stimulus (Whitney et al., 2003). Animals were exposed to the same thirty minute song playbacks for three days. On two consecutive days, birds received an intramuscular injections of WIN (3 mg/kg), WIN + SR141716A (SR, 6 mg/kg), or vehicle thirty minutes before the start of song playbacks. For groups administered both WIN and the CB₁-selective antagonist SR, SR was administered five minutes prior to the agonist, WIN. Silent controls not exposed to song were given either a single intramuscular injection of WIN (3 mg/kg) or vehicle for two days. On the third day of song exposure, all groups were given a single injection of vehicle. At the end of experiments on the third day, animals were given an overdose of Equithesin (100 µl), and brain tissue was processed for immunolabeling of Arc protein.

4.4 Western Blotting

The presence of Arc protein was detected in the zebra finch brain by using a rabbit polyclonal antibody targeting the internal sequence of amino acids 288-337 of Human Arg 3.1 (LDLPQKQGEP LDQFLWRKRD LYQTLYVDAD EEEIIQYVVG TLQPKLKRFL.) This epitope was compared to the zebra finch sequence, and found to differ by a single residue (LDLPQKEGEP LDQFLWRKRD LYQTLYVDAD EEEIIQYVVG TLQPKLKRFL, Genbank accession number XP_002186861). To assess the selectivity of the Arc antibody against zebra finch protein, western blotting experiments were done. Protein extraction protocols were adapted from methods described by (Schafer et al., 1998). Adult male zebra finches, subjected to song stimulation procedures described above, were killed by Equithesin overdose. Brains were removed, homogenized with a polytron in an ice-cold extraction buffer containing 1 M Tris-HCl, pH = 7.0, 0.6 M KCl, 0.5 mM ATP, 1 mM DTT, 0.5 mM MgCl₂, along with a cocktail of protease inhibitors (0.25 M sucrose/1 mM EDTA/4 mM phenylmethylsulfonyl fluoride/1 mM 4-amino benzamidine/0.1 % w/v bacitracin), and briefly micro-centrifuged at 1000 × g. Supernatants were stirred on ice for

30 min, collected and centrifuged at $24,000 \times g$. Following this, protein preps were ultra-centrifuged at $100,000 \times g$ for 1 hour. Pellets were discarded and supernatants were filtered through cheesecloth to remove traces of excess lipid, and dialyzed against 10 L of ice-cold distilled water for 4 hours at 4°C . The fractions were then dialyzed overnight against two changes of ice-cold extraction buffer containing 10 mM Tris-HCl, pH 8.0, 0.2 mM CaCl_2 , 0.5 mM ATP, 0.1 mM DTT, 100 mM KCl, and 0.1 mM phenylmethylsulfonyl fluoride. Samples were spun a final time at $24,000 \times g$ at 4°C to remove trace precipitate. Supernatants were collected and protein concentrations determined using the Bradford method. 30 μg of soluble protein were loaded into a 10 % polyacrylamide gel, separated by SDS-PAGE, and blotted onto a PVDF membrane. Blots were blocked with a non-mammalian blocking reagent (Odyssey Blocking Buffer, LI-COR Biosciences, Lincoln, NE) and incubated overnight at 4°C in a 1:1000 dilution of the Arc antibody in Odyssey Blocking Buffer. The next day, the blot was washed three times with 1X PBS-T (Phosphate-Buffered Saline [pH = 7.4] with 0.05 % Tween-20), and incubated in a secondary antibody solution containing an infrared anti-rabbit secondary antibody (LI-COR Biosciences # 926-68021, 1:25,000) and 0.02 % SDS for 2 hours at room temperature in the dark. The blot was washed 4 times with 1X PBS (Phosphate-Buffered Saline [pH = 7.4]) for 10 minutes each, and visualized using the LI-COR Odyssey Infrared Imaging System. Masses of infrared bands were determined standard curves fit to distances migrated by molecular weight standards using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Separate experiments where primary antibody was preabsorbed with 20 μM of the immunizing peptide were performed as a negative control (Abcam, Cambridge, MA).

Additional western blotting experiments were performed with synaptosomal membrane preparation isolated from caudal telencephalon (containing NCM and Field L2) following drug treatments and song exposure procedures as described above. After behavioral experiments, animals were quickly anesthetized, decapitated, and brains rapidly dissected on ice. Brain sections containing regions NCM and Field L2 were placed into a glass and Teflon grinder containing 25 volumes of ice-cold 0.32 M sucrose in 5 mM Tris, pH = 7.4, and ground to homogeneity. Following this, homogenates were centrifuged at $1000 \times g$ at 4°C for 15 minutes. Pellets were discarded; supernatant was removed and placed into a fresh centrifuge tube on ice, and centrifuged at $30,000 \times g$ at 4°C for 60 minutes. Pellets were then snap-frozen at -80°C overnight, and resuspended in 150 volumes of 50 mM Tris, 10 mM EDTA, pH = 7.4. Membrane fractions were incubated at 4°C for 2 hours in an effort to chelate endogenous cations and dissociate endogenous ligands from receptors. These fractions were again centrifuged at $30,000 \times g$ at 4°C for 60 minutes, after which pellet fractions were collected. Pellets were washed by homogenizing with a glass and Teflon grinder in 150 volumes of 50 mM Tris, 5 mM MgCl_2 , pH = 7.4. Homogenates were centrifuged at $30,000 \times g$ at 4°C for 60 minutes and resuspended in 50 mM Tris, 5 mM MgCl_2 , pH = 7.4 a final time, and protein concentrations determined by the Bradford method. In addition to anti-Arc detection, these blots were also probed with an antibody directed against β -actin as an internal control (Sigma # A3853). Relative intensities of Arc and actin bands were determined and compared across treatment conditions. Potential treatment effects were assessed using t-tests.

4.5 Immunohistochemistry

Immediately following the end of song exposure procedures described above, animals were killed by Equithesin overdose (100 μl) and transcardially perfused with PBS, followed by phosphate-buffered 4% paraformaldehyde [pH = 7.0]. Brains were immersed in 4% paraformaldehyde overnight, and then transferred to an ice-cold 20% sucrose solution the next morning. Once tissues sank in the sucrose solution, brains were blocked down the midline and sectioned parasagittally, using a Leica VT 1000 vibrating microtome. For

experiments that required capture of NCM and Field L2, consecutive free-floating sections were collected from the midline (0.0mm) to 2.0 mm lateral of the midline. All tissue was processed using a standard immunohistochemical procedure adapted from (Whitney et al., 2000). Tissue was briefly rinsed in 1X PBS for 10 minutes followed by incubation in 1% H₂O₂ for 30 minutes, to quench endogenous peroxidase. The sections were again rinsed 3 times with 1X PBS, and then blocked with 5% normal goat serum for 35 minutes. Following blocking, sections were incubated overnight at room temperature in a solution containing anti-Arc antibody (1:3000 dilution), 1% normal goat serum, 0.01% sodium azide, and 0.3% Triton-X 100 in PBS. The following day, tissue was rinsed three times in PBS and incubated in a biotinylated anti-rabbit antiserum followed by an avidin-biotin complex from a kit following the manufacturer's instructions (VECTASTAIN ABC Elite kit, Vector Laboratories Inc., Burlingame, CA). Antibody labeling was developed using a 3,3'-diaminobenzidine (DAB) solution in the presence of hydrogen peroxide followed by three washes in 1X PBS. Tissue sections were mounted on glass slides coated with 0.3 % gelatin. After sections on slides were allowed to dry overnight, sections were dehydrated with graded concentrations of ethanol (70 %, 95 %, and 100 % respectively), cleared with xylenes, and coverslipped with Permount (Fisher).

4.6 Golgi-Cox Impregnation

A mixture containing 5 volumes of 5 % potassium dichromate solution, 5 volumes of 5 % mercuric chloride, and 4 volumes of 5 % potassium chromate solution was prepared and allowed to ripen in the dark five days before use. After the end of song exposure procedures described above, birds were killed by Equithesin overdose and transcardially perfused with PBS, brains removed and rinsed briefly with ice-cold distilled water, and immersed in a filtered (0.45 micron) aliquot of Golgi-Cox supernatant for 14 days in the dark at room temperature. The brains were then transferred to an ice-cold 30 % sucrose solution for five days. Following sucrose treatment, brains were blocked down the midline, and left hemispheres were sectioned parasagittally on a vibrating microtome. 125-micron sections of zebra finch brain were then gold-toned in order to preserve staining as follows. The sections were exposed to chilled solutions of 80, 60, 40, and 20 % glycerol, rinsed with water, then transferred to a 0.05 % ice-cold gold chloride solution for 80 minutes. After being briefly transferred to an ice-cold 0.2 % oxalic acid solution for two minutes, sections were immersed in 1 % sodium thiosulfate for a total of 60 minutes. The sections were developed in a 7 % ammonium hydroxide solution at room temperature, in the dark for thirty minutes, followed by further processing in Kodak Fixer. Sections were then mounted and dehydrated on glass slides, and neurons of zebra finch auditory nuclei assessed for dendritic spine densities and as a function of treatment. Tissue was stained and processed in multiple batches; however, in order to eliminate possible variance associated with reaction conditions, each of the treatment groups were represented in each batch.

4.7 Optical Density Measurements

Immunohistochemical staining was examined in various brain regions at 40, 100, and 1000 X using an Olympus BX51 microscope with Nomarski DIC optics. Images were captured using a Spot Insight QE digital camera and Image-Pro Plus 5.0 software (MediaCybernetics, Silver Spring, MD) under identical, calibrated exposure conditions. All images were background-corrected, and converted to 8-bit grey scale; borders that outlined brain regions of interest were traced manually. Two dimensional optical density measurements of both labeled nuclei and neuropil from areas enclosed within traced areas were performed on images without knowledge of treatment condition for each brain region of interest. For measurements pertaining to NCM and Field L2, All intact sections containing NCM brain regions from the midline (0.0 mm) to 2.0 mm from the midline per animal were included for analysis, and analyzed using Image-Pro Plus 5.0 software. Field L2 regions between the

midline and 0.5 mm from the midline were not included for analysis. Counting all NCM-containing sections was done to make results generally applicable to this large region. We made no attempt to distinguish potential differences in efficacy within subregions of NCM that have been identified (Pinaud et al., 2006). Mean optical densities (within brain region counts of stained nuclei and neuropil/area of the region) were compared across treatment groups using one-way ANOVA. Upon confirmation of treatment effects, post-hoc analyses were done with Tukey's test.

4.8 Measurement of Dendritic Spine Densities

Dendritic spines were visualized at 100 X and 1000 X under oil immersion using a Zeiss AxioImager M2 microscope with QIClick digital CCD optics, and analyzed using Neurolucida 3D reconstruction imaging software (MBF Bioscience, Williston VT). Dendritic spine densities, expressed as the mean number of dendritic spines per length (μm) of each dendrite, were analyzed based on criteria adapted from Norrholm, et al. (Norrholm and Ouimet, 2001). Neurons that were selected for measurement were fully impregnated, intact, located in areas of tissue free of debris and other imperfections, and possessed dendritic processes greater than 20 microns in length. In order to ensure random sampling, all neurons that fit the criteria above were assigned a number, and 5 neurons were randomly selected from each of 5 separate tissue sections containing NCM for each treatment. 3-D serial reconstructions were created for each neuron directly from slides, and quantitative morphometric analyses were conducted for each neuron (cell body, dendrites, and dendritic spines), and data pertaining to each outline was exported into Microsoft Excel Spreadsheet. All observable spines along the entire length of dendrites of 25 randomly selected neurons per each NCM brain region of animals in each representative treatment group (vehicle + novel song exposure, WIN + novel song exposure, and un-stimulated silence control groups, $n = 4$ for each treatment group) were analyzed.

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Abbreviations

Arc	activity-regulated cytoskeleton-associated protein (also known as Arg3.1)
WIN	WIN55212-2
SR	SR141716A
L2	Auditory Field L2
NCM	Caudal Medial Nidopallium
Ov	Nucleus Ovoidalis
MAP2	microtubule-associated protein 2

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Highlights

- Arc is induced by novel song stimulation in a manner inhibited by CB₁ activation.
- Two-day WIN pretreatment prevents NCM habituation from occurring on day three.
- CB₁ effects on Arc expression were observed in higher-order telencephalic regions and not within the thalamic auditory relay, Ov.
- WIN-induced habituation prevention is reversed by a CB₁-selective inverse agonist.
- Spine density increases produced following novel song stimulation are also inhibited by cannabinoid treatments.

Figure 1.

Schematic representation of the avian auditory brain regions studied. The thalamic region nucleus ovoidalis (Ov) relays primary auditory sensory information to thalamorecipient Field L2 (L2, shown in light grey), which projects to caudal medial neostriatum (NCM, shown in darker grey).

Figure 2.

(A) Representative Western blot of the soluble fraction of protein isolated from male NCM brain homogenates. Similar to other species, the anti-Arc antibody labels a single predominant band of about 55 kDa (left lane). Staining is eliminated by preabsorption with 20 μ M of the immunizing peptide (right lane). (B) Novel song stimulation increases Arc protein levels in zebra finch caudal telencephalon that contains auditory regions NCM and Field L2 (compare Silence to Song). This stimulation was prevented by pretreatment with the cannabinoid agonist WIN (WIN + Song). Blots were re-probed with an antibody directed against β -actin as an internal control. (C) Summary of the relative intensities of Arc: β -actin bands. Shown are means $\pm$ SEM of five pooled experiments. Novel song stimulation (Song) increased Arc expression over that of silence-exposed controls (Silence). WIN treatment prior to novel song exposure (Song + WIN) reduced Arc expression. Asterisk indicates significant difference from Silence group, dagger indicates difference from Song group ($p < 0.05$, 1-tailed t-test).

Figure 3. Double immunofluorescence labeling of Arc protein with dendritically-associated MAP2 (630,1000X), visualized via confocal laser scanning microscopy. In an effort to capture Arc protein expression at its maximum level, brains of animals exposed to novel song were used for these experiments. Images demonstrating Arc distribution alone (green), and colocalized with MAP2 (yellow, image overlays) show both cellular and dendritic distribution of protein. Bar = 20 μ m.

Figure 4.

Immunohistochemical staining of NCM (solid boxes in A,D,G) and Field L2 (dashed boxes in A,D,G) with Arc antibody. Animals were exposed to either vehicle (D-F) or WIN pretreatment (G-I) 30 minutes before novel song exposure. Unstimulated control animals (A-C) received only silence. At 1000X magnification, novel song stimulation increases cellular and neuropil Arc expression within both NCM (E) and Field L2 (F). J-K: Optical densities of NCM (J) and Field L2 (K) Arc expression after novel song exposure, and after WIN pretreatment. Novel song stimulation increases Arc expression by about 90%, but only by about 20% after WIN exposure. Bar in G = 300 μ m. Bar in H-I = 30 μ m.

Figure 5. Optical densities (G) and immunohistochemical staining (A-F) of Arc expression within Ov in unstimulated controls, after acute novel song exposure, and after pretreatment with WIN. The absence of cannabinoid-mediated effects on Arc expression within this thalamic region contrast with those observed in telencephalon, supporting brain region selectivity of responsiveness. Scale Bar in E = 300 μ m. Scale Bar in F = 30 μ m.

Figure 6.

Birds were exposed to the same 30 min song for 3 days. On days 1 and 2, birds received WIN (3 mg/kg), the CB₁-selective antagonist SR (6 mg/kg) and WIN, or vehicle injections 30 min before song stimulation. On day 3, all groups received vehicle injections prior to song exposure, were euthanized 90 min afterward and brains processed for anti-Arc immunolabeling (A-O). (G-I): Habituation to novel song-induced Arc expression occurred in the vehicle treated group as evidenced by a return to baseline levels. WIN exposure disrupts habituation (J-L) however, and this disruption is reversed with the antagonist SR (M-O).

Figure 7.

(A) Representative high power image of an NCM spiny neuron used for analysis. Bar = 30 μ m. (B-D) Representative Golgi-Cox impregnated dendrites included in analysis of dendritic spine densities within (B) unstimulated control, (C) 30 min of novel song exposure and (D) cannabinoidpretreated groups (3 mg/kg WIN 30 min prior to song exposure). (E) Novel song stimulation results in significantly increased dendritic spine densities within NCM. Note that this effect was produced rapidly after acute auditory exposure (animals were killed 90 min following song exposure). Cannabinoid pretreatment (30 min prior to song exposure) with 3 mg/kg WIN prevents rapid increases in spine densities.