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Cannabinoid mitigation of neuronal morphological change important to development and learning: insight from a zebra finch model of psychopharmacology

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Abstract

Normal CNS development proceeds through late-postnatal stages of adolescent development. The activity-dependence of this development underscores significance of CNS-active drug exposure prior to completion of brain maturation. Exogenous modulation of signaling important in regulating normal development is of particular concern. This mini-review presents a summary of accumulated behavioral, physiological and biochemical evidence supporting such a key regulatory role for endocannabinoid signaling during late-postnatal CNS development. Our focus is on data obtained using a unique zebra finch model of developmental psychopharmacology. This animal has allowed investigation of neuronal morphological effects essential to establishment and maintenance of neural circuitry, including processes related to synaptogenesis and dendritic spine dynamics. Altered neurophysiology that follows exogenous cannabinoid exposure during adolescent development has potential to persistently alter cognition, learning and memory.

Keywords

dendrite; spines; Arc; Caspase-3; Zenk; Egr-1; learning; cannabinoid; neuronal morphology; development

Introduction

It has become increasingly clear that cannabinoid exposure during peri-pubertal periods of late-postnatal development (loosely referred to as “adolescence”) can alter behavior in ways that persist through adulthood. These altered behaviors are not produced following the same treatments given to post-pubertal, adult animals (reviewed by Schneider, 2008). Similarly persistent changes in neuronal morphology have been found to accompany cannabinoid-altered behaviors, suggesting potential mechanistic relationships (e.g. Gilbert and Soderstrom, 2011, Bossong and Niesink, 2010).

Presented herein is evidence for such relationships in various species, with a focus on work done in a zebra finch model that allows pharmacological effects to be studied in the context

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of adolescent development-dependent vocal learning. The fact that drug abuse typically both begins in adolescence and involves *Cannabis*, makes distinct efficacy of cannabinoids during late-postnatal development of particular relevance to the problem of human substance abuse (Fontes et al., 2011).

Normal neuronal morphological change during adolescence

CNS gross anatomy is established early in postnatal development, in humans by age five (Giedd et al., 2009). Despite gross similarities between brains of juvenile and adult vertebrates, more subtle developmental changes associated with adolescence also occur. These changes are characterized by increased myelination, decreased grey matter, reduced synapse numbers and dendritic spine densities in some adult brain regions (Rakic et al., 1994).

It is becoming clear that activity-dependent, maturation-essential refinement of neuronal circuits occurs as a normal part of late-postnatal CNS development (Andersen, 2003). These maturational processes create distinct, periadolescent periods of psychopharmacological sensitivity that are of particular relevance to exogenous cannabinoid exposure (Bossong and Niesink, 2010).

Evidence for endocannabinoid involvement in controlling neuronal morphology and connectivity

Prior to discovery of a specific cannabinoid receptor (Devane et al., 1988), the mechanism of this drug class was thought to involve non-specific membrane effects (Tahir and Zimmerman, 1991). Supporting this was evidence that cannabinoids alter morphology of cultured cells (Kiosses et al., 1990, Tahir et al., 1992). Involvement of cannabinoid signaling in axonal and dendritic migration (discussed below) brings a new developmental perspective to this earlier line of research.

Receptor autoradiography studies during gestational and early post-natal development led to observation of cannabinoid binding sites within migrating neuronal fiber tracts (Romero et al., 1997). Lack of similar fiber tract localization of the CB₁ subtype of cannabinoid receptors in adult brain provided early evidence of a developmental role for cannabinoid signaling in normal CNS development (Berrendero et al., 1998).

The ability for CB₁ receptor activation to directly regulate neuronal connectivity was elegantly demonstrated through experiments using hippocampal cultures. These studies determined that CB₁ receptors are distinctly expressed within axons of developing GABAergic neurons, with enrichment in migrating growth cones. Upon maturation, CB₁ receptor populations are largely restricted to presynaptic terminals (Irving et al., 2000). Further experiments demonstrated that presynaptic CB₁ receptor activation effectively prevents cAMP-dependent production of new synapses (Kim and Thayer, 2001).

Growth cone CB₁ receptors direct axonal and dendritic migration (Berghuis et al., 2007, Vitalis et al., 2008, Watson et al., 2008). CB₁ receptor activation effects a repulsive response; neurites migrate away from sources of cannabinoid agonist. Importantly, this turning response is correlated with increased activation of RhoA, an actin-depolymerizing enzyme involved in neurite retraction and growth cone collapse (Gallo and Letourneau, 2004, Tagliaferro et al., 2006).

Mechanisms of cannabinoid regulation of axonal and dendritic migration established in vitro are now being studied in the context of developing brain. Interestingly, embryonic initiation of cortical pyramidal cell axonal outgrowth appears to be promoted, rather than inhibited by

CB₁ activation (Mulder et al., 2008). This finding suggests that cannabinoid signaling plays opposing roles at different developmental stages, or initially promotes neurite outgrowth and negatively modulates following extension and migration.

Using a combination of CB₁ deficient mice and conditional mutants capable of expressing green fluorescent protein in thalamocortical- and corticothalamic projection neurons, Wu et al. impressively demonstrated that CB₁ activation is critical for appropriate axonal pathfinding and target recognition (Wu et al., 2010). Pathfinding deficits in these developing mice were associated with aberrant migration through the striatocortical boundary. This region is enriched with diacylglycerol lipase, the enzyme responsible for production of 2-arachidonylglycerol, the principal endocannabinoid released in vertebrate brain (Bisogno et al., 2003) suggesting that appropriate endocannabinoid signaling is critically important for successful axon migration through this region.

Persistent behavioral and neurophysiological effects following adolescent cannabinoid exposure

Given evidence above for endocannabinoid signaling in processes essential for: (1) establishing neural circuits and; (2) the maturation of these circuits that occurs during adolescence, it perhaps should be expected that exogenous modification of this signaling can disrupt normal development in ways that persistently alter behavior.

Behavioral abnormalities following cannabinoid exposure during adolescence, but not in adults, have now been reported from several vertebrate species. Deficits in learning (Cha et al., 2007) memory (Moore et al., 2010, Rubino et al., 2009b), and cognitive function, including increases in anxiety and depressive phenotypes (Rubino et al., 2009a), with correlates to results of human studies, have been documented (Dekker et al., 2010, Hanson et al., 2010, Kristensen and Cadenhead, 2007, Solowij and Michie, 2007, Realini et al., 2011).

We have observed similarly-persistent behavioral effects that last through adulthood in our zebra finch model of developmental psychopharmacology. Because these animals are among a small group of vocal learners that notably includes humans, they provide advantages for studying drug effects on developmentally-relevant learned behavior (Doupe and Kuhl, 1999). Songbird telencephalon comprises a nuclear arrangement of functionally-related neurons, rather than the distributed, laminar organization characteristic of mammalian cortex (Csillag and Montagnese, 2005). This results in distinct, easily visualized and dense CB₁ receptor expression within regions of established functional importance to vocal perception, production, and learning (Soderstrom and Tian, 2006, and Fig. 1). Developmental cannabinoid treatment during zebra finch adolescent sensorimotor vocal learning (when memorized song patterns are practiced until perfected) reduces numbers of song notes, and causes reduction in song stereotypy – characterized by disordered and repetitive note production (see Soderstrom and Johnson, 2003, and Fig 2). Interestingly, developmental cannabinoid exposure produces greater effects on stereotypy when given early in adolescence, and greater effects on note number later (Soderstrom and Tian, 2004).

Zebra finch vocal learning is accompanied by normal decreases in telencephalic dendritic spine densities within regions essential for auditory learning and vocal motor production. Results of elegant two-photon imaging experiments show that song region spine densities increase rapidly following initial exposure to tutor song during the early auditory learning stage of zebra finch vocal development (Roberts et al., 2010). Similar to the case of mammalian species (discussed above), initial profusion of dendritic spines within song regions are diminished with experience and behavioral maturation. Spine densities normally

decrease within some telencephalic song regions from adolescence to adulthood, coincident with vocal practice and auditory feedback associated with sensorimotor learning (Wallhauser-Franke et al., 1995). Coincident spine density changes and normal vocal development led us to test whether cannabinoid-altered vocal development may be attributable to effects on dendritic spines. We found that vocal development-altering cannabinoid treatments persistently elevate dendritic spine densities, implying that adolescent exposure interferes with normal reductions (see Fig. 3 and Gilbert and Soderstrom, 2011).

Do cannabinoids alter CNS development through interfering with cytoskeleton-related protein expression?

As discussed above, one way in which cannabinoids influence CNS development is through alteration of axonal and dendritic migration. It is now becoming clear that CB₁ receptors modulate activity and regulation of structural proteins. Because these processes are of particular importance during the maturation of neural circuits, this suggests a potential distinct period of sensitivity. For example, coincident with persistent deficits in object recognition memory, adolescent THC exposure results in significant long-term reduction in hippocampal expression of several oxidative stress- and cytoskeleton-related proteins. These proteins include mortalin/GRP75, important to cellular differentiation and malignant transformation (Quinn et al., 2008, Wadhwa et al., 2002) and NP25, a CNS-specific, F-actin binding protein necessary for actin assembly (Mori et al., 2004).

Finding that cannabinoid-altered vocal development is associated with inappropriate maintenance of dendritic spine densities (Fig 3 and Gilbert and Soderstrom, 2011) led us to question whether CB₁ regulation of structurally-related proteins may play a role. Such structurally-relevant proteins with established involvement in the zebra finch vocal development model include Caspase-3 (Huesmann and Clayton, 2006) and Arc/Arg 3.1 (Moorman et al., 2011, Velho et al., 2005).

Caspase-3 is a protease activated by cleavage that is most well-known as the trigger for cellular apoptosis. Within neurons it has an apoptosis-independent role in regulation of dendritic spine dynamics. Dendritic expression of Caspase-3 is associated with synaptic plasticity, pruning, processes of learning and memory and habituation to sensory input (D'Amelio et al., 2010). Caspase-3 regulated habituation was discovered in the zebra finch model where perception of a novel song induces rapid expression of the classic immediate early gene, ZENK within auditory telencephalon (reviewed by Mello et al., 2004). Song perception-stimulated ZENK expression appears dependent upon caspase-3, as inhibition with the Caspase-3 antagonist, DEVD prevents the response (Huesmann and Clayton, 2006). In addition to Caspase-3 dependence, ZENK is subject to inhibition by CB₁ (see Fig. 4 and (Soderstrom et al., 2004, Whitney et al., 2003), implying that CB₁ inhibition of auditory perception-related ZENK expression may be attributable to upstream inhibition of Caspase-3 activation. We have found that in a manner similar to ZENK, novel song-stimulated Caspase-3 activation is subject to cannabinoid agonist inhibition (see Soderstrom and Gilbert, submitted and Fig. 5 and 6).

Similar to Caspase-3, Arc/Arg 3.1 is an effector-type immediate early gene induced by depolarization (Tzingounis and Nicoll, 2006). It has been well-studied in mammalian hippocampus and is necessary for long-term-potential, -depression and memory consolidation (Bramham et al., 2008). Arc regulation of synaptic plasticity appears related to promotion of AMPA receptor internalization (Waung et al., 2008). This internalization involves interaction with morphologically-relevant proteins including endofilin, dynamin and cofilin, that in turn modulate F-actin dynamics (Huang et al., 2007).

Like ZENK and activated Caspase-3, Arc is also induced in auditory telencephalon following zebra finch exposure to novel song (Velho, Pinaud, 2005). We have discovered that in a manner similar to ZENK, CB₁ activation effectively prevents novel song-induced Arc expression, and habituation to repeated song stimulation (see Figs 7,8 and Gilbert and Soderstrom, submitted).

Potential Mechanisms

How does CB₁ receptor activation result in inhibition of morphologically-relevant, auditory perception-related ZENK, Arc and Caspase-3 expression? A simple possibility, and one based-upon well-established cannabinoid effects, is through reducing probability of presynaptic excitatory transmitter release (Elphick and Egertova, 2001). Presynaptic inhibition following CB₁ activation has been described in several systems (Maier et al., 2011, Wilson and Nicoll, 2001, Yoshida et al., 2002) and implies that presynaptic CB₁ receptors are present on glutamatergic terminals within zebra finch auditory telencephalon.

As Arc expression appears limited to AMPA receptor-containing terminals (Waung, Pfeiffer, 2008), the signaling relationship in zebra finch auditory telencephalon appears similar to that between CB₁-expressing Schaffer collateral terminals that synapse on AMPA receptor-expressing dendrites of hippocampal CA1 pyramidal cells (Hoffman et al., 2010). Here, through presynaptic inhibition of glutamate release, cannabinoid exposure reduces probability of depolarization-dependent immediate early gene expression. Reduced excitation also reduces Arc-promoted AMPA receptor internalization, increasing sensitivity to later excitatory input (Vazdarjanova et al., 2006). Disregulated glutamatergic sensitivity will disrupt activity-dependent processes important to developmental selective stabilization of neural circuits. This hypothesis, adapted from Changeux and Dehaene, is summarized in Fig. 9A (Changeux and Dehaene, 1989).

Pharmacological Implications

Distinct cannabinoid sensitivity during periods of adolescent development raises two pharmacological possibilities: (1) that the potency of exogenous cannabinoids are enhanced during adolescence or; (2) that the maximal efficacy of the endocannabinoid system is augmented during this period. Affinity changes that would be consistent with potency modulation following chronic exposure seem unlikely given current evidence (Villares, 2007). Conversely, several studies including our own suggest that normal adolescent development is associated with initially high-level CB₁ receptor expression that decreases in adulthood, potentially leading to higher maximal efficacy during adolescence, followed by lower levels in adulthood (Soderstrom and Tian, 2006). Early high-density CB₁ expression may reflect receptors present on pathfinding neurites that are absent in adults (Berrendero, Garcia-Gil, 1998). A similar developmental sensitivity has been reported to occur within the visual system, where cannabinoid-mediated long term depression is necessary for normal development of neuronal activity within visual cortex (Jiang et al., 2010).

Open Questions

It is clear that CB₁ cannabinoid receptor activation is capable of altering normal processes of late-postnatal CNS development. The extent to which this occurs in humans, and the substrates altered as a result of *Cannabis* abuse, remain unclear. The songbird model provides important insight. Altered densities of dendritic spines in pre-motor (HVC) and regions of basal ganglia (Area X) imply altered processes of motor output and behavioral selection. These morphological changes may be attributable to altered expression patterns of structurally-relevant proteins, such as Arc and Caspase-3 discussed above, although causal relationships between CB₁ activity, protein expression and morphological change still await

testing. Such experiments will require direct manipulation of the proteins in question, either through pharmacological intervention using selective inhibitors (such as DEVD in the case of Caspase-3) or agents to upregulate or inhibit protein expression (perhaps through lentivector delivery of RNAi against Arc).

Establishing a causal link between cannabinoid inhibition of Caspase-3, Arc and other proteins, and inappropriately-maintained dendritic spine densities in these regions will immediately suggest treatments that may be effective in reversing behavioral effects of developmental *Cannabis* exposure. For example, it is possible that treatments designed to stimulate Caspase-3 and/or Arc will effectively mitigate deleterious effects. In zebra finches, this suggests intensive and repeated exposure to novel song that is known to increase expression of these proteins within auditory cortex. Such experience-induced expression is expected to promote pruning of dendritic spines inappropriately maintained by developmental cannabinoid exposure – a discovery that may translate to, and inform mechanisms responsible for, the efficacy of behavioral therapies to resolve human cognitive pathology.

Conclusions

Taken together, our findings in the context of those reported by others, have led us to propose the following general hypothesis: Distinctly-dense CB₁ cannabinoid receptor expression associated with CNS development during adolescence, renders normally-occurring activity-dependent processes of synaptic refinement sensitive to dysregulation by exogenous cannabinoids. This maturational dysregulation may involve decreased levels of excitatory activity within neural circuits important for memory and sensorimotor learning, such as mammalian hippocampus and learning- and vocal motor-essential regions in our songbird model. Decreased excitatory activity is associated with lack of morphological change in the neurons involved, including inappropriately-elevated dendritic spine densities. Decreased morphological change is associated with inhibition of the activity of cytoskeletal proteins. A thorough understanding of the morphologically-relevant signaling systems involved remains incomplete, and represents an important area for further study.

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Figure 1.

Images ($\times 12.5$) of immunohistochemical staining of zebra finch brain with anti-zebra finch CB1 receptor antibody. Sections shown were reacted together. A–D: Parasagittal sections represent planes about 2.5 mm lateral from the midline. Rostral is to the left and dorsal is top. Bar in Panel E = 1 mm. E: Diagram based on C illustrates regions of distinct staining (shown in black), including song regions important to auditory learning (IMAN, Area X) perception (L2) and vocal motor production (HVC and RA). Adapted from Soderstrom, et al 2004, permission requested.

Figure 2.

Effects of cannabinoid exposure during sensory-motor learning on song patterns produced at adulthood. Individuals from sibling pairs raised by the same adult tutor were randomly assigned to treatment (1 mg/kg WIN55212-2) or vehicle control groups ($n=7$ pairs). Treatments were given from 50 to 100 days of age. Songs were recorded at adulthood (110 days of age) and 10 bouts from each animal were randomly selected for analysis by two independent observers blind to treatment. Shown are means $\pm$ S.E.M. (A) Cannabinoid-treated birds learned significantly fewer note types (mean = 5.6 ± 0.5 vs. 7.8 ± 0.3 , $*P=0.025$, paired t-test, two-tailed). (B) Songs of cannabinoid-treated birds were less well-stereotyped (mean scores = 0.56 ± 0.06 vs. 0.81 ± 0.02 , $**P=0.004$, paired t-test, two-tailed). (C) Mean note duration did not vary as a function of treatment (mean duration [ms] = 177 ± 21 vs. 176 ± 13 , $p=0.97$, paired t-test, two-tailed). Representative adult song patterns produced by a sibling pair. (E) Vehicle-treated animal B379 and (F) WIN55212-2-treated animal B379. Shown are

sonograms produced at 110 days of age. Introductory notes are indicated by 'i'. Song notes are indicated alphabetically according to order of production by the control animal. A unique note type (with similarities to notes 'h' and 'f') produced by the WIN55212-2-treated animal is indicated by '*'. Evident are fewer note types and decreased stereotypy decreased stereotypy in the song pattern produced by the WIN55212-2-treated sibling. Adapted from Soderstrom and Johnson, 2003 with permission.

Figure 3.

Panels A – C, Representative image of Golgi-Cox impregnated spiny dendrites used for analysis within HVC of birds developmentally treated from 50 to 75 days of age with 1 mg/kg WIN (A), or vehicle (B). Animals were allowed to mature to adulthood (> 110 days), and brains were dissected and stained with Golgi-Cox solution (see Experimental procedures). Bar = 10 μm . (C) Representative high power image of a typical HVC neuron used for analysis. Measurements made from 10 randomly selected neurons for each of the 4 representative song regions of both WIN and vehicle-treated animals. Bar = 30 μm . Panels D – G, Effect of developmental treatments from 50 to 75 days on song region spine densities at adulthood (n = 8). Developmental WIN treatments resulted in significantly elevated dendritic spine densities within Area X and HVC but not IMAN or RA. (p < 0.05). Panels H – K, Effect of treatments given to adults for 25 days on song region spine densities (n = 8). WIN (1 mg/kg/day) did not result in a significant change in dendritic spine densities in the four song regions of adult males that had already learned song. Adapted from Gilbert and Soderstrom, 2011, with permission.

Figure 4.

Inhibition of song stimulus-induced zenk immunoreactivity in NCM is reversed with the CB1 receptor antagonist SR141716A. (A) The 61% reduction in zenk immunoreactivity in NCM of birds treated with 1 mg/kg of WIN55212-2 (** $p = 0.001$, SNK) was completely reversed with 3 mg/kg of SR141716A, demonstrating that the inhibitory effects of WIN55212-2 treatment of ZENK immunoreactivity are due to specific activation of the CB1 cannabinoid receptor. (B) Song stimulus-induced zenk immunoreactivity in Field L1 and L3 of birds treated with both SR141716A and WIN55212-2, was comparable to zenk immunoreactivity in Field L of birds treated with WIN55212-2 alone as well as vehicle controls. Histogram bars are means $\pm$ S.E.M. Adapted from Whitney et al, 2003, with permission.

Figure 5.

Cannabinoid agonist inhibition of novel song-stimulated Caspase-3 activation. All sections shown were reacted together and photographed at 40 X under identical, calibrated exposure conditions. Panel A shows basal levels of activated Caspase-3 immunoreactivity. Panel B shows increased staining following novel song exposure throughout avian hippocampus (Hp), and auditory telencephalon, including Field L2 and NCM (Song = 45 min of novel song followed by 10 min of silence as described by Whitney et al, 2003). Panel C shows that administration of 3 mg/kg of WIN55212-2 has little effect on staining intensity. Panel D shows that 3 mg/kg WIN prevents effects of novel song exposure. E is a diagram of the section shown in Panel B with the relative positions of Field L2 and NCM indicated. Rostral is approximately left, dorsal is up. The bar = 1 mm. Panel F, western blotting results indicate the antibody used (Cell Signaling #9661) selectively stains a single band of approximately 17 kDa, consistent with the cleaved, activated form of Caspase-3.

Figure 6.

WIN inhibition of novel song-stimulated Caspase-3 release. Shown are mean cell counts measures of results pooled from two experiments. Y-axis labels to the left of slashes indicate treatments administered prior to auditory exposure: Veh = vehicle control; WIN = 3 mg/kg of the cannabinoid agonist WIN55212-2. Y-axis labels to the right of hyphens indicate auditory exposure: Silence = 10 min of silence; Song = 10 min of novel song exposure. One-way ANOVA indicated significant effects of treatment. Asterisk indicates significant differences from Veh-Silence group (SNK post-test, $p < 0.05$).

Figure 7.

Inhibition of novel song-induced Arc/Arg 3.1 immunoreactivity in NCM and Field L2 regions of zebra finch auditory telencephalon by the cannabinoid agonist WIN. (A–C) Low-levels of Arc/Arg3.1 immunoreactivity are found in the absence of novel song exposure. (D–F) Novel song exposure increases Arc/Arg3.1 expression in both Field L2 and NCM regions of auditory telencephalon. (G–I) Pretreatment with the cannabinoid agonist WIN (3 mg/kg) reduces novel song-induced Arc/Arg3.1 immunoreactivity in both Field L2 and NCM. (J,K) Novel Song exposure significantly increases Arc/Arg3.1 expression within Field L2 and NCM. This increased expression is prevented by pretreatment with 3 mg/kg WIN.

Figure 8.

Habituation to novel song-stimulated increases Arc/Arg3.1 immunoreactivity occurs after 45 min exposure on two consecutive days. This habituation is prevented by pretreatment with 3 mg/kg of the cannabinoid agonist WIN. WIN reversal of habituation is prevented by pretreatment with the CB₁-selective antagonist SR. Exposure conditions were the same as those described in Figure 7.

Figure 9.

Diagrammatic models illustrating potential mechanisms responsible for cannabinoid mitigation of neuronal morphological change. Panel A is adapted from the model proposed by Changeux and Dehaene, 1989 for late-postnatal refinement of neural circuitry. Panel B illustrates the potential for CB₁-mediated presynaptic inhibition of glutamate release to prevent activation of structurally-relevant proteins important to zebra finch sensorimotor integration of auditory stimuli.