Adolescent Cannabinoid Exposure Induces a Persistent Sub-Cortical Hyper-Dopaminergic State and Associated Molecular Adaptations in the Prefrontal Cortex

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Abstract

Considerable evidence suggests that adolescent exposure to delta-9-tetrahydrocannabinol (THC), the psychoactive component in marijuana, increases the risk of developing schizophrenia-related symptoms in early adulthood. In the present study, we used a combination of behavioral and molecular analyses with in vivo neuronal electrophysiology to compare the long-term effects of adolescent versus adulthood THC exposure in rats. We report that adolescent, but not adult, THC exposure induces long-term neuropsychiatric-like phenotypes similar to those observed in clinical populations. Thus, adolescent THC exposure induced behavioral abnormalities resembling positive and negative schizophrenia-related endophenotypes and a state of neuronal hyperactivity in the mesocorticolimbic dopamine (DA) pathway. Furthermore, we observed profound alterations in several prefrontal cortical molecular pathways consistent with sub-cortical DAergic dysregulation. Our findings demonstrate a profound dissociation in relative risk profiles for adolescent versus adulthood exposure to THC in terms of neuronal, behavioral, and molecular markers resembling neuropsychiatric pathology.

Key words: adolescence, cannabis, dopamine, prefrontal cortex, ventral tegmental area

Introduction

Marijuana (MJ) is the most commonly used illicit drug by adolescents. Indeed, chronic MJ use among teens is occurring at earlier ages and with increasing global prevalence (Substance Abuse and Mental Health Services Administration 2013). Importantly, adolescence represents a critical neurodevelopmental period characterized by increased neural plasticity and vulnerability to extrinsic insult, particularly in cortical and limbic regions (Spear 2000). This maturational period also involves extensive reorganization of various neurotransmitter systems such as the glutamatergic, GABAergic, dopaminergic, and endocannabinoid systems (Spear 2000). Although it is still a matter of debate, there is evidence suggesting that chronic adolescent MJ exposure may be associated with a higher risk for neuropsychiatric diseases, including schizophrenia (Andréasson et al. 1987; Arseneault et al. 2002, 2004; Stefanis et al. 2004; Renard et al. 2014). Δ-9-Tetrahydrocannabinol (THC), the psychoactive component of MJ, acts as a partial agonist of CB1 cannabinoid receptors (CB1Rs), one of the most abundantly expressed G protein-coupled receptors (GPCRs) in the central nervous system (CNS) (Herkenham et al. 1990). CB1Rs are key players in the control and maintenance of synaptic plasticity (Kano et al. 2009) and are thought to be central components of the neurodevelopmental changes occurring during adolescence (Ellgren et al. 2008). Increasing evidence points to the clinical risks associated with exposure to popular street strains of cannabis containing higher and higher relative THC content. For example, the popularity of cannabis...
strains such as sinsemilla, which contain high levels of THC, has been linked to an increased risk of developing first-episode psychotic symptomology (Di Forti et al. 2009). Nevertheless, the precise neuronal and molecular mechanisms by which adolescent THC exposure may lead to schizophrenia-related symptoms are not well understood.

Schizophrenia represents a complex disorder associated with various disturbances in motivational, social, emotional, and cognitive processing that is linked to profound disturbances in mesocorticolimbic dopamine (DA) transmission. By regulating excitatory and inhibitory inputs controlling mesocorticolimbic DA neurons, CB1Rs modulate DAergic activity (French et al. 1997; Melis et al. 2004) in neural regions such as the ventral tegmental area (VTA) and prefrontal cortex (PFC) (Laviolette 2007), both of which contain high levels of CB1Rs (Kano et al. 2009) and which are functionally connected via ascending and descending neural pathways (Laviolette 2007). Importantly, considerable evidence demonstrates that chronic cannabis exposure can alter mesocorticolimbic DAergic signaling (Bloom et al. 2011; Sutton and Rushlow 2012; Gururajan and Van Den Bussche et al. 2014). However, the precise mechanisms by which chronic adolescent THC exposure may disturb neuronal and molecular pathways within the mesocorticolimbic circuitry and thereby disrupt normal DAergic function are currently unknown.

At the molecular level, considerable evidence implicates the interconnected wingless (Wnt) signaling cascade (i.e., protein kinase B [Akt], glycogen synthase kinase-3 [GSK-3], β-Catenin) and the mammalian target of rapamycin (mTOR) (i.e., p70S6 Kinase [p70S6K]) signal transduction pathways as crucial molecular substrates underlying schizophrenia-related psychopathology and antipsychotic efficacy (Alimohamad et al. 2005; Valjent et al. 2011; Sutton and Rushlow 2012; Gururajan and Van Den Bussche et al. 2014). For example, haloperidol, clozapine, and risperidone alter GSK-3 and β-catenin protein expression levels in the PFC and striatum of rats (Alimohamad et al. 2005). In addition, haloperidol increases phosphorylation level of p70S6K in D2R-expressing striatal neurons (Valjent et al. 2011). Importantly, CB1R activation can modulate these downstream signaling pathways in various neural regions (Ozaita et al. 2007; Puighermanal et al. 2009), raising the possibility that THC-induced dysregulation in these pathways may underlie schizophrenia-related psychopathology.

In the present study, we used a combination of behavioral assays, molecular analyses, and in vivo neuronal electrophysiology to model endophenotypes similar to those observed in schizophrenia and other neuropsychiatric disorders. We report that THC exposure during adolescence leads to an array of persistent cognitive and behavioral disturbances and enduring molecular abnormalities in the PFC bearing remarkable similarity to adaptations observed in neuropsychiatric populations, including changes in the Akt, Wnt, and mTOR pathways. Finally, we report for the first time that adolescent THC exposure induces a persistent state of hyper-DAergic activity in the VTA and associated molecular adaptations in the PFC.

Materials and Methods

Animals

Adolescent and adult male Sprague-Dawley rats were obtained at postnatal day (PND) 30 and 60, respectively, from Charles River Laboratories (Quebec, Canada). At arrival, rats were pair-housed under controlled conditions (12 h light/dark cycle, constant temperature, and humidity) with free access to food and water. All procedures were performed in accordance with Governmental and Institutional guidelines for appropriate animal care and experimentation.

Drug Preparation and Administration

Rats treated with THC (Tocris, UK) received twice daily injections of THC (2.5 mg/kg; Days 1–3; 5 mg/kg; Days 4–7; 10 mg/kg, Days 8–11). Control groups received the same injection schedule (volume adjusted per body weight) with vehicle (VEH). Increasing doses of THC were administered to counter the development of drug tolerance (González et al. 2005). This THC dosing regimen was chosen based on previous studies (Rubino et al. 2009) and is known to produce long-term behavioral impairments in rats. THC was dissolved in ethanol, cremophor, and saline (1:1:18). Ethanol was then evaporated using nitrogen gas to remove it from the final THC solution. All injections were administered intraperitoneally (i.p) at a volume of 1 ml/kg. The adolescent and adult THC exposure experiments began at PND 35 and 65, respectively. Behavioral tests were initiated following a 30-day drug-free period (at PND 75 for the adolescent group and PND 105 for the adult group).

Social Motivation and Social Cognition Testing

Rats were tested using a social interaction procedure as described previously (Loureiro et al. 2014). Briefly, this task evaluates 2 distinguishable aspects of social behavior: 1) social affiliation/motivation and 2) social recognition memory. Rats were habituated to the test arena for 13 min, 24 h before testing. Testing consisted of 2 successive 8-min phases. During the first phase, we analyzed social motivation, that is, the propensity to spend time with an unfamiliar male rat (stranger rat) enclosed in a small wire cage compared with time spent with an identical but empty cage. Thus, the test rat had a choice between the stranger rat and an empty cage. During the second phase, occurring just after the first one, we analyzed social recognition, that is, the propensity to spend time with a novel unfamiliar rat (novel stranger) rather than with the familiar stranger rat (previously encountered). The novel stranger was placed in the chamber that had been empty during the first 8-min session. Therefore, the tested rat had a choice between the first, already investigated familiar stranger and the novel stranger. In this situation, control rats will spend significantly more time with the novel stranger, demonstrating a natural preference for social novelty. The locations of stranger versus novel rats in the left versus right side chambers were counterbalanced between trials. Times spent in exploration were videotaped with a video-tracking system (ANY-maze; Stoeling) and analyzed by an experimenter blind to the treatment conditions.

After each rat testing, chambers and cages were cleaned with 50% ethanol to avoid olfactory cue bias. Two rats (1 adolescent VEH pre-treated rat and 1 adult THC pre-treated rat) were excluded from the experiment due to a lack of spontaneous exploratory behavior (defined as an exploration time <10 s during both phases of the test).

Light–Dark Box Test

This test is based upon a rat’s natural aversion to bright environments and attributes greater time spent in an illuminated environment as reflecting lower anxiety levels. The test apparatus consisted of two 50 × 25 × 37 cm compartments. Between the compartments was a 10 × 10 cm door allowing the rat to enter either side. One compartment was black and covered with a black lid (the dark box), while the other compartment was...
white, left uncovered, and brightly illuminated by a lamp located 120 cm above the apparatus floor, providing 1500 lux at floor level (the light box). At the start of the experiment, a rat was placed in the center of the lighted box with its head facing the wall opposite the door and was allowed to freely explore both compartments for a period of 8 min. A zone entry was considered to have begun when the animal placed all 4 paws in that zone. Experiments were videotaped with a video-tracking system (ANY-maze; Stoelting) and analyzed by an experimenter blind to treatment conditions. Behaviors analyzed included: 1) latency time to first entry in the dark box; 2) latency time for re-entering the light box, and 3) total number of transitions between compartments. The latency to leave the dark box and enter the light box is thought to be the most reliable indicator of anxiety-like behavior and is sensitive to both anxiogenic and anxiolytic treatments (Ardayfio and Kim 2006).

Prepulse Inhibition of Startle Reflex

Rats were acclimatized to the startle chambers (Med Associates, USA) for 5 min over 3 days. On the last day of acclimatization, rats were tested in an input/output (I/O) function consisting of 12 increasing startle pulses (from 65 to 120 dB, 5 dB increments) to determine the appropriate gain setting for each individual rat. The testing paradigm consisted of the following phases: the acclimatization phase, a habituation phase (Block 1), and PPI measurement (Block 2). During acclimatization, rats were exposed to the chambers and white background noise (68 dB) for 5 min. During Block 1, 10 pulse alone trials (110 dB white noise, 20 ms duration) were delivered at 15–20 s intervals. Block 2 consisted of the presentation of 9 different trials presented 10 times, each in a pseudorandomized order, and at 30 s intervals: 10 pulse-alone trials and 10 of each of 3 different prepulse–pulse trial types (72, 76, 80) with interstimulus intervals (ISI) of 100 ms. “Pulse-alone” trials consisted of a startle stimulus-only presentation, while “prepulse-pulse” trials consisted of the presentation of a weaker nonstartling prepulse (white noise, 20 ms duration, volume as indicated) before the startling stimulus. PPI was calculated for each animal and each trial condition as PPI (%) = (1 – average startle amplitude to pulse with prepulse/average startle amplitude to pulse only) × 100.

Open Field Test

Rats were placed in an automated open field activity chamber (Med Associates, San Diego, CA, USA). Total distance travelled and total stereotypy and vertical counts were recorded for 60 min.

In Vivo VTA Electrophysiological Recordings

Single-unit extracellular VTA recordings were performed on rats anesthetized with urethane (1.4 g/kg, ip) and placed in a stereotaxic frame with body temperature maintained at 37°C. A scalp incision was made, and a hole was drilled in the skull overlaying the VTA. For VTA recordings, glass microelectrodes (with an average impedance of 6–8 MΩ) filled with a 2% pontamine sky blue solution were lowered using a hydraulic micro-positioner (Kopf640) at the following coordinates: AP: −5.2 mm from bregma, L: ±0.8–1 mm, DV: −6.5 to −9 mm from the dural surface. Extracellular signals were amplified using a MultiClamp700B amplifier (Molecular Devices) and recorded through a Digidata1440A acquisition system (Molecular Devices) using pClamp10 software. Recordings were filtered at 1 kHz and sampled at 5 kHz. Presumptive VTA DA neurons were identified according to the following well-established electrophysiological features (Ungless and Grace 2012): 1) action potential (AP) with biphasic or triphasic waveform with duration >1.1 ms from the start of the action potential to the negative trough; 2) a slow spontaneous firing rate (~2–5 Hz), and 3) a single irregular or bursting firing pattern. Electrophysiological analyses were performed offline using the Clampfit10 (Molecular Devices) software package. The electrophysiological properties of spontaneously active DA neurons were sampled in the VTA by making 6 vertical passes of the electrode through the DA cell body region. These tracks were made in a predefined pattern, with each track separated by 200 μm. After an individual DA neuron was isolated, its spontaneous activity was recorded for 5 min. Two parameters of activity were sampled, the basal firing rate and the proportion of spikes fired by the DA neurons that occurred in bursts. The onset of a burst was defined as the occurrence of 2 spikes with an interspike interval of 80 ms (Grace and Bunney 1983). The percentage of spikes in bursts was calculated by dividing the number of spikes occurring in bursts by the total number of spikes occurring in the same period of time. The basal firing rates of all DA neurons and the % of spikes in bursts were calculated as an average value for each rat and analyzed using 2-tailed t-tests.

For histological analysis, recording electrode locations were marked with iontophoretic deposit of pontamine sky blue (~20 μA for 15 min). Brains were removed and rapidly frozen. Brains were cut using a brain block to obtain blocks of tissue containing the VTA or PFC. The VTA block was cut using a cryostat to obtain sections (60 μm) that were stained with neutral red to confirm the neuronal recording site. Cells recorded outside the anatomical boundaries of the VTA were excluded from data analysis.

Protein Extraction and Western Blotting

The block containing the PFC was cut using a cryostat to obtain coronal sections (60 μm) from which bilateral micropunches of the PFC were obtained for protein isolation. The western blotting procedure was performed as described previously (Lyons et al. 2013). Primary antibody dilutions were as follows: α-tubulin (1:120 000; Sigma-Aldrich), phosphorylated GSK-3α/β ser21/9 (p-GSK-3α/β; 1:1000; Cell Signaling Technology), total GSK-3α/β ser21/9 (t-GSK-3α/β; 1:1000; Cell Signaling Technology), phosphorylated Akt Ser473 (p-Akt-Ser473; 1:1000; Cell Signaling Technology), phosphorylated Akt thr308 (p-Akt-thr308; 1:1000; Cell Signaling Technology), total Akt (t-Akt; 1:1000; Cell Signaling Technology), β-catenin (1:10 000; Sigma-Aldrich), phosphorylated mTOR ser2448 (p-mTOR;1:2000; Cell Signaling Technology), total mTOR (t-mTOR; 1:2000; Cell Signaling Technology), phosphorylated p70S6K thr389 (p-p70S6K; 1:1000; Cell Signaling Technology), total p70S6K (t-p70S6K; 1:1000; Cell Signaling Technology). Species specific horseradish peroxidase (HRP)-conjugated secondary antibodies (Thermo Scientific) were all used at a dilution of 1:20 000.

Statistical Analyses

Data were analyzed with 2-tailed t-tests or 2-way repeated-measures ANOVA where appropriate. Post hoc analyses were performed with Fisher’s LSD. Values and SEMs for each behavioral test are presented in Supplementary Table 1. Densitometry values for western blots were obtained with Kodak digital analysis software and analyzed with t-tests.

Results

THC Exposure During Adolescence Versus Adulthood Differentially Modulates Social Interaction and Cognition

Disturbances in social interaction and cognition are commonly observed in schizophrenia (Marwick and Hall 2008). Accordingly,
we evaluated the long-term effects of adolescent versus adulthood THC exposure on social motivation/cognition (Fig. 1A). In adolescent THC rats, analysis of interaction times with a novel stranger revealed that THC rats spent significantly less time than VEH rats exploring the stranger rat ($t_{153} = -3.022; P < 0.01$; Fig. 1B). During the social recognition test, 2-way repeated-measures ANOVA comparing times spent with novel versus familiar rats showed a significant effect of novelty ($F_{1,33} = 9.734; P < 0.01$). Post hoc analysis revealed that while VEH-treated rats displayed a clear preference for novel versus familiar rats ($P < 0.05$; Fig. 1C), THC rats showed no preference ($P > 0.05$; Fig. 1C).

In contrast, in adult treated rats, analysis of times spent with the stranger rat during the social motivation test, showed that both VEH and THC rats spent the same length of time exploring the stranger rat ($t_{16} = -0.054; P > 0.05$; Fig. 1D). During the social recognition test, 2-way repeated-measures ANOVA comparing times spent with novel versus familiar rats showed a significant effect of novelty ($F_{1,35} = 6.83; P < 0.05$). Post hoc analysis revealed that while VEH rats displayed a significant preference for novel versus familiar rats ($P < 0.05$; Fig. 1E), THC rats spent the same length of time exploring novel versus familiar rats ($P > 0.05$; Fig. 1E). Thus, THC exposure during adolescence but not adulthood induced long-term deficits in social motivation. However, both adolescent and adulthood THC exposure induced deficits in social cognition/memory.

Adolescent THC Exposures Induces Long-Term Deficits in Exploratory Behaviors

We next evaluated the long-term effects of adolescent versus adult THC exposure on exploratory behaviors in a novel

**Figure 1.** Long-term effects of chronic THC exposure during either adolescence or adulthood on social motivation and social recognition. (A) Apparatus and rats’ placements for the social motivation and social recognition test, respectively. (B) During the social motivation test, adolescent THC pretreated rats spent significant less time than VEH rats exploring the stranger rat. (C) During the social recognition test, adolescent VEH pretreated rats displayed a clear preference for the novel rat in comparison with the familiar one, whereas adolescent THC pretreated rats spent the same length of time exploring both the novel and the familiar rats. (D) During the social motivation test, both adult VEH and THC pretreated rats spent the same amount of time exploring the stranger rat. (E) During the social recognition test, adult VEH pretreated rats displayed a clear preference for the novel rat in comparison with the familiar one, whereas adult THC pretreated rats spent the same length of time exploring both the novel and the familiar rats. Adolescent pretreated rats: $n = 8$ VEH and $n = 9$ THC; Adult pretreated rats: $n = 10$ VEH and $n = 8$ THC. Two-tailed t-tests or two-way repeated-measures ANOVA; ** indicated $P < 0.01$; * indicated $P < 0.05$. Error bars represent the standard error of the means (SEMs).
environment (i.e., the open field test). Statistical analysis revealed that adolescent THC rats travelled less distance relative to VEH controls \((t_{19} = -2.393; P < 0.05; \text{Fig. 2A})\). Examples of typical activity plots for adolescent VEH or THC-treated rats are presented in Figure 2B. In addition, adolescent THC rats displayed less rearing relative to VEH controls \((t_{19} = -2.861; P < 0.05; \text{Fig. 2C})\). In contrast, in adult pretreated rats, analysis of locomotor activity and rearing counts revealed no significant differences between VEH- versus THC-treated rats \((t_{19} = -1.812; P > 0.05 \text{ and } t_{19} = 0.092; P > 0.05; \text{Fig. 2D,E, respectively})\).

THC Exposure During Adolescence Induces Long-Term Increases in Anxiety Measures

We next compared the effects of adolescent versus adult THC exposure on anxiety levels using the light-dark box test (Fig. 3A). In adolescent THC rats, statistical analysis comparing latency to first entry in the dark box revealed that both adolescent THC and VEH rats displayed similar entry latency times \((t_{18} = -1.461; P > 0.05; \text{Fig. 3B})\). However, THC rats exhibited a significantly longer latency to emerge from the dark to the light box relative to VEH controls \((t_{18} = -2.374; P < 0.05; \text{Fig. 3C})\). In addition, THC rats performed less total transitions between compartments compared with VEH control \((t_{18} = 3.432; P < 0.01; \text{Fig. 3D})\). In adult treated rats, statistical analysis revealed that both VEH and THC rats spent similar amounts of time 1) entering the dark box \((t_{19} = 0.432; P > 0.05; \text{Fig. 3E})\) and 2) re-entering the light box \((t_{19} = 0.219; P > 0.05; \text{Fig. 3F})\). VEH and THC rats also displayed similar numbers of transitions between compartments \((t_{19} = 0.124; P > 0.05; \text{Fig. 3G})\). Thus, adolescent, but not adulthood THC exposure, induces long-lasting effects on anxiety levels.

Adolescent THC Exposure Induces Long-Term Deficits in Sensorimotor Gating

Deficits in sensorimotor gating are a well-established endophenotype of schizophrenia (Braff and Geyer 1990). Accordingly, we next compared the effects of adolescent versus adult THC exposure on sensorimotor gating using the PPI procedure (see methods; Fig. 4). In adolescent treated rats, 2-way repeated-measures ANOVA on PPI responses revealed a nearly significant effect of treatment \((F_{1,50} = 4.23; P = 0.057)\) and a significant effect of prepulse intensity factor \((F_{2,50} = 7.25; P < 0.01)\). Post hoc comparisons revealed that THC rats displayed PPI deficits at prepulse intensity levels of 76 dB \((P < 0.05)\) and 80 dB \((P < 0.01)\) compared with VEH controls (Fig. 4A). The startle amplitude responses were unchanged between groups \((t_{19} = 0.423; P > 0.05; \text{Fig. 4B})\). In adult treated rats, 2-way repeated-measures ANOVA on PPI responses showed a significant effect of prepulse intensity factor \((F_{2,62} = 5.02; P < 0.05)\). Post hoc comparisons revealed that there were no significant differences between THC and VEH groups at the different prepulse intensities tested \((ps > 0.05; \text{Fig. 4C})\). Startle amplitude responses were unchanged between groups \((t_{19} = -0.122; P > 0.05; \text{Fig. 4D})\). These data demonstrate that THC exposure during adolescence, but not adulthood, induces lasting deficits in sensorimotor gating. Importantly the long-term PPI deficits observed in adolescent pre-treated rats are not confounded with reduced startle amplitudes, since no changes were observed between THC and VEH rats on this variable.

Adolescent THC Exposure Induces a Sub-Cortical Hyper-DAergic State

We next evaluated the long-term effects of adolescent versus adulthood THC exposure on spontaneous VTA DA neuronal

![Figure 2. Long-term effects of chronic THC exposure during either adolescence or adulthood on exploratory/motivation behavior. (A) Adolescent THC pretreated rats travelled less distance relative to VEH controls. (B) Examples of typical activity plots for adolescent VEH (top) or THC (bottom) treated rats. (C) Adolescent THC pretreated rats did less rearing counts relative to VEH controls. (D) Adult VEH and THC pretreated rats travelled the same distance and (E) did the same number of rearing. Adolescent pretreated rats: n = 9 VEH and n = 9 THC, Adult pretreated rats: n = 11 VEH and n = 10 THC. Two-tailed t-tests; ** indicated \(P < 0.01\), * indicated \(P < 0.05\). Error bars represent the standard error of the means (SEMs).](image-url)
activity parameters. A microphotograph of a representative VTA neuronal recording placement is shown in Figure 5A. In adolescent treated rats, statistical analysis revealed that THC-exposed rats displayed significantly increased VTA DA neuronal firing frequencies ($t_{(12)} = -3.733; P < 0.01; \text{Fig. 5B}$) and a significantly increased relative proportion of spikes firing in burst mode ($t_{(12)} = -2.702; P < 0.05; \text{Fig. 5C}$) relative to VEH controls. Representative rastergrams showing spontaneous activity of typical VTA DA neurons in THC versus VEH pretreated rats are presented in Figure 5D. Sample VTA DA neuronal recording traces from THC versus VEH pretreated rats are presented in Figure 5E.

In contrast, in adult THC-treated rats, analysis of VTA DA neuronal firing frequency and spikes firing in burst mode revealed no significant effects of treatment ($t_{(11)} = 0.238; P > 0.05$; and $t_{(11)} = 0.737; P > 0.05$, Fig. 5F,G respectively). Representative rastergrams comparing the spontaneous activity of VTA DA neurons in THC versus VEH pretreated rats are shown in Figure 5H. Thus, THC exposure during adolescence, but not...
during adulthood, selectively induces long-term hyper-DAergic activity in the VTA.

Adolescent THC Exposure Induces Dramatic Decreases in PFC Phosphorylated Protein Levels of GSK-3α/β, Akt Thr308, mTOR, p70S6 Kinase, and β-Catenin

We next analyzed PFC protein expression levels of the interconnected Wnt/Akt and mTOR signaling pathways (β-catenin, GSK-3, Akt, p70S6K, and mTOR), comparing adolescent VEH- versus THC pretreated rats. Western blot analysis of phosphorylated Akt-Thr308 (p-Akt-Thr308) revealed a dramatic decrease in adolescent THC versus VEH-treated rats (Fig. 6A). This decrease was so profound that quantification of protein levels in adolescent THC-treated samples was not possible within the linear range of the film nor could a band be detected even following over exposure (Fig. 6A). However, the density of protein expression of total Akt (t-Akt) was unchanged between groups (P > 0.05). We further found no significant difference in levels of phosphorylated Akt-Ser473 (p-Akt-Ser473), the expression of total Akt protein levels (t-Akt) or the ratio of p-Akt-Ser473/t-Akt between adolescent VEH- and THC pretreated rats (P > 0.05; Fig. 6B). Analysis of β-catenin protein expression revealed a significant decrease between adolescent VEH- versus THC pretreated rats (t10 = 1.77; P < 0.05; Fig. 6C). Western blot analyses revealed a significant decrease in levels of both phosphorylated GSK-3α and β (p-GSK-3α; (t10) = 3.73; P < 0.01 and t10 = 3.41; P < 0.01, respectively), the ratio of both p-GSK-3α/total-GSK-3α expression (t10 = 3.56; P < 0.01) and p-GSK-3β/total-GSK-3β expression (t10 = 2.87; P < 0.05) when comparing adolescent VEH- versus THC pretreated rats (Fig. 6D). Expression of total GSK-3β and GSK-3α protein levels (t-GSK-3β and t-GSK-3α, respectively) were unaffected (P > 0.05; Fig. 6D). Western blot analyses also revealed a significant decrease in levels of phosphorylated mTOR (p-mTOR) (t6 = 2.52; P < 0.05) and the ratio of p-mTOR/total mTOR expression (t6 = 2.077; P < 0.05) when comparing adolescent VEH- versus THC pretreated rats (Fig. 6E). Expression of total mTOR protein levels (t-mTOR) was unaffected (t6 = 0.33; P > 0.05; Fig. 6E). Finally, Western blot analyses revealed a significant decrease in levels of phosphorylated-p70S6K (p-p70S6K) (t6 = 17.68; P < 0.001), total p70S6K protein levels (t-p70S6K) (t6 = 11.65; P < 0.01; Fig. 6F), and the ratio of p-p70S6K/total-p70S6K expression (t6 = 6.92; P < 0.001) when comparing adolescent VEH- versus THC pretreated rats (Fig. 6F).

Adulthood THC Exposure Increases Phosphorylation of GSK-3α/β, Akt Ser473, and mTOR in the PFC

In contrast to observations in adolescent treated rats, analysis of PFC protein expression levels in adult treated rats revealed no
significant difference in levels of phosphorylated Akt-Thr308 (p-Akt-Thr308), the expression of total Akt protein levels (t-Akt) and the ratio of p-Akt-Thr308/t-Akt between adult VEH- versus THC pre-treated rats (Ps > 0.05; Fig. 7A). In contrast, we found a significant increase in levels of phosphorylated Akt-Ser473 (p-Akt-Ser473) (t6) = 2.60; P < 0.05) and the ratio of p-Akt-Ser473/total-Akt (t6) = 3.88; P < 0.01) when comparing adult VEH- versus THC pre-treated rats (Fig. 7B). Expression of total Akt protein levels (t-Akt) was unaffected (t6) = 2.30; P > 0.05; Fig. 7B). Analysis of β-catenin expression revealed no significant changes between adult VEH- versus THC pretreated rats (t7) = 0.98; P > 0.05) (Fig. 7C). Western blot analyses revealed a significant increase in levels of both phosphorylated GSK-3α and β isoforms (p-GSK-3α/β) (t6) = 2.59; P < 0.05 and t6) = 2.39; P < 0.05;
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Figure 6. Long-term effects of chronic THC exposure during adolescence on PFC synaptic markers. (A) A dramatic decrease in phosphorylated Akt-Thr308 was found in adolescent THC versus VEH-treated rats. (B) Representative western blot for phosphorylated and total Akt expression in the PFC (left). No significant changes were found in phosphorylated Akt-Ser473, the total Akt and the ratio of phosphorylated to total Akt between adolescent VEH and adolescent THC pretreated rats. (C) Representative western blot for β-catenin expression in the PFC (left). A significant decrease in β-catenin expression is observed between adolescent VEH and adolescent THC pretreated rats. (D) Representative western blot for phosphorylated and total GSK-3α and β expression in the PFC (left). Densitometry analysis revealed a decrease in both phosphorylated GSK-3α and β isoforms and both the ratio of phosphorylated to total (GSK-3α and GSK-3β) in adolescent THC compared with adolescent VEH pretreated rats. No significant changes in total GSK-3α and β were found between groups. (E) Representative western blot for phosphorylated and total mTOR expression in the PFC (left). Densitometry analysis revealed a decrease in both phosphorylated mTOR and the ratio of phosphorylated to total mTOR in adolescent THC with compared with adolescent VEH pretreated rats. (F) Representative western blot for phosphorylated and total p70S6K expression in the PFC (left). Densitometry analysis revealed a decrease in phosphorylated p70S6K, the ratio of phosphorylated to total p70S6K in adolescent THC compared with adolescent VEH pretreated rats. No significant changes in total mTOR are observed. (G) Representative western blot for phosphorylated and total Akt expression in the PFC (left). No significant changes were found in total Akt mTOR or the ratio of phosphorylated to total mTOR in adolescent THC with compared with adolescent VEH pretreated rats. No significant changes in total mTOR are observed. (H) Representative western blot for phosphorylated and total p70S6K expression in the PFC (left). Densitometry analysis revealed a decrease in phosphorylated p70S6K, the ratio of phosphorylated to total p70S6K in adolescent THC compared with adolescent VEH pretreated rats. n = 4–6 rats, t-tests; ** indicated P < 0.01; * indicated P < 0.05. Error bars represent the standard error of the means (SEMs).

Discussion

The present study demonstrates that exposure to chronic THC during a specific window of adolescent neurodevelopment can induce enduring neuropsychiatric-like behavioral, molecular, and neuronal phenotypes, at the cortical and sub-cortical levels. Importantly, these effects were selectively limited to adolescent exposure as the majority of observed behavioral and neuronal alterations were not present following adulthood exposure. In addition, several of the observed molecular adaptations within the PFC occurred in opposite directions following adolescent versus adult THC exposure.

Behaviorally, rats receiving adolescent THC exposure displayed exploratory deficits in novel environments, which may be indicative of decreased motivational drive and/or flattened affect (Katz et al. 1981). In addition, THC treatment induced deficits in normal social interaction behaviors and cognition, which are well-established markers of prodromal and/or existing neuropsychiatric-related pathology (Marwick and Hall 2008; Green and Horan 2010). Interestingly, adult THC exposure induced deficits only in social memory, suggesting that both adolescent and adulthood exposure to chronic THC can induce social cognition deficits, consistent with previous reports indicating that chronic MJ exposure during adulthood may induce memory-related cognitive impairments (O’Shea et al. 2006; Schneider et al. 2008). Nevertheless, the finding that adolescent THC-treated rats demonstrated strongly decreased social interaction behavior is

respectively), the ratio of both p-GSK-3 α/total-GSK-3 α expression (t_{60} = 6.15; P < 0.01) and p-GSK-3 β/total-GSK-3 β expression (t_{60} = 3.51; P < 0.01) when comparing adult VEH- versus THC pretreated rats (Fig. 7D). Expression of total GSK-3 β and GSK-3 α protein levels (t-GSK-3 β and t-GSK-3 α, respectively) was unaffected (Ps > 0.05; Fig. 7D).

Western blot analyses also revealed a significant increase in levels of phosphorylated mTOR (p-mTOR) (t_{60} = 2.92; P < 0.05) and the ratio of p-mTOR/total mTOR expression (t_{60} = 3.67; P < 0.05) when comparing adult VEH- versus THC pretreated rats (Fig. 7E). Expression of total mTOR protein levels (t-mTOR) was unaffected (t_{60} = 0.35; P > 0.05; Fig. 7E). Finally, Western blot analyses revealed no significant changes in levels of phosphorylated-p70S6K (p-p70S6K), total p70S6K protein levels (t-p70S6K), and the ratio of p-p70S6K/total-p70S6K expression (when comparing adult VEH- versus THC pretreated rats [Ps > 0.05; Fig. 7F]).
associated with a persistent decline in working memory function (Earleywine 2007), further suggesting that adolescent THC exposure may induce neuropsychiatric-like cognitive deficits (Schneider et al. 2008; Stopponi et al. 2014). However, it is important to note that baseline differences between early versus later stages of adulthood may have an influence on behavioral outcomes. Sensorimotor gating deficits represent another core cognitive feature of schizophrenia (Braff and Geyer 1990). Using a standard prepulse inhibition (PPI) test, we observed selective PPI deficits indicative of disturbances in intrinsic motivational drive for social interaction, a phenomenon commonly reported in schizophrenia (Green and Horan 2010; Lee et al. 2013). Together, these findings demonstrate that chronic THC exposure specifically during adolescence leads to deficits in social motivation, consistent with clinical studies showing that heavy adolescent cannabis use is associated with a persistent “amotivational syndrome” principally defined by lethargy, inactivity, and social withdrawal (Looby and Earleywine 2007). In addition, consistent with the present findings, recognition memory in rodents is defined as the ability to discriminate the familiarity of recently encountered stimuli and is a functional index of working memory (Mumby 2001; Schneider et al. 2008). Importantly, working memory dysfunction is a core feature of schizophrenia and other neuropsychiatric disorders (Bowie and Harvey 2006), further suggesting that adolescent THC exposure may induce neuropsychiatric-like cognitive deficits.

We also observed significant increases in trait anxiety levels following adolescent THC exposure, consistent with other preclinical and clinical studies (Renard et al. 2014). Anxiety-related symptoms are a common co-morbidity in schizophrenia and other neuropsychiatric conditions (Achim et al. 2011). Interestingly, we observed several baseline differences in measures of anxiety and social interaction behaviors when testing occurred at early versus later stages of adulthood. Several possible reasons may account for these differences. One possibility is the fact that younger versus older rats have been shown previously to demonstrate different baseline levels of anxiety in similar behavioral assays, with older rats showing relatively lower levels (Slawecki 2005; Lynn and Brown 2010). Furthermore, our findings with social interaction behaviors are similar to a previous report (Quinn et al. 2008) wherein it was found that adult vehicle control groups demonstrated lower levels of baseline social interaction relative to adolescent treated groups. Thus, the present results are consistent with previous studies showing both anxiety and social interaction/cognition abnormalities specifically following adolescent THC exposure and tested at early adulthood (O'Shea et al. 2004; Quinn et al. 2008; Schneider et al. 2008; Stopponi et al. 2014).
following adolescent, but not adult THC exposure. PPI is commonly used to assess the ability to filter extraneous information from relevant salient environmental sensory information. Disruptions of this filtering mechanism have been postulated to underlie sensory overstimulation, psychosis, and attentional deficits (Braff and Geyer 1999). Indeed, PPI impairments are widely accepted as an endophenotype of schizophrenia with high translational validity (Braff et al. 2001). Functionally, PPI is thought to be regulated by cortico-limbic-striatopallidal circuitry in which DA transmission plays a central role (Koch 1999; Swerdlow et al. 2001). Disruptions in PFC DAergic transmission have been reported to induce disturbances in normal sensory filtering (Koch and Bubser 1994). Furthermore, drugs that stimulate DA release such as amphetamine (AMPH) cause profound disruptions in PPI (Geyer et al. 2003). Thus, the presently observed deficits in PPI are consistent with the hyper-DAergic phenotype observed in adolescent THC-treated rats and point to a potential mechanism that may account for deficits in cognitive filtering following selective adolescent THC exposure.

Prominent theories of schizophrenia suggest that dysregulated DAergic transmission and PFC dysfunction represent important underlying neuropathological features of schizophrenia (Perlstein et al. 2001; Laviolette 2007). We report for the first time that THC exposure specifically during adolescence induces a state of hyper-DAergic function in the mesocorticolimbic system, persisting into early adulthood. Cortical regulation of sub-cortical DA neuronal activity states is indicated both by anatomical and by functional evidence. For example, a recent report demonstrated persistent downregulation of the GABAergic marker GAD 67 in the PFC of adult rats chronically exposed to THC during adolescence (Zambrello et al. 2014). Furthermore, the PFC sends functional, excitatory descending projections to VTA neuronal populations (French et al. 1997; Gessa et al. 1998) which form excitatory synapses onto both DA and non-DA VTA neurons. Thus, cortical disinhibition induced by THC exposure may be sufficient to induce cortical overdrive onto VTA DA neurons, leading to a hyper-DAergic phenotype. Indeed, we have recently reported that acute CB1R activation within the PFC is capable of strongly activating VTA DA neurons and distorting emotional salience processing through functional interactions with recurrent, VTA→PFC DA receptor transmission (Draycott et al. 2014). Furthermore, hyper-DAergic states underlying schizophrenia have been associated with increased DAergic activity in both striatal and PFC regions (Lindström et al. 1999). For example, hyper-DAergic activity within the PFC is associated with schizophrenia-like cognitive disorganization (Murphy et al. 1996), and cognitive deficits associated with schizophrenia have been linked to dysregulations in PFC neuronal function (Perlstein et al. 2001). Finally, we have reported that overstimulation of PFC DA receptors can distort emotional memory processing and potentiate normally non-salient fear stimuli (Launon et al. 2013). Interestingly, we observed decreased exploratory locomotor behavior in adolescent THC versus VEH-treated experimental groups. While such a phenotype may seem counterintuitive to the hyper-DAergic state observed in the adolescent treatment group, 1 possibility is that these rats engaged in lower levels of exploratory locomotor activity as a consequence of increased levels of anxiety, as demonstrated in our anxiety testing behavioral assay.

The molecular underpinnings of schizophrenia-related psychopathology are complex and not entirely understood. Given our findings demonstrating a state of sub-cortical hyper-DAergic activity, we performed a series of molecular analyses within the PFC to determine how VTA DAergic outputs may alter DA-related signaling pathways at the cortical level. Interestingly, we observed several molecular alterations in the PFC that may be linked to a hyper-DAergic phenotype. Several signaling pathways have emerged as important players in schizophrenia-related etiology. For example, the mTORC1 signaling pathway is recognized as a critical integrator of synaptic plasticity, regulation of protein synthesis, and cognitive processing (Hoeffer and Klann 2010). Recent studies have also implicated the mTOR pathway in neurodevelopmental and neuropsychiatric disorders, including schizophrenia (Gururajan and Van Den Buuse 2014). GSK-3, the major downstream target of Akt, is critical for the regulation of transcription factors including β-catenin, which is an important component of the Wnt signal transduction pathway (MacDonald et al. 2009). GSK-3 also plays a central role in the regulation of the mammalian target of rapamycin complex 1 (mTORC1) (Ma and Blenis 2009), which includes mTOR and p70S6K.

Notably, we observed profound reductions in levels of phospho-GSK-3 α/β, mTOR, p70S6K, AKT (at threonine 308), and in total β-Catenin levels, following adolescent THC exposure. Thus, the persistent decrease in phosphorylated Akt-thr308 observed in the PFC of adolescent THC rats is mechanistically consistent with a decrease in levels of phosphorylated GSK3 and its downstream signaling molecules (β-catenin and mTORC1). Importantly, consistent with our observed behavioral and neuronal phenotypes, disturbances in these pathways have been linked both to neuropsychiatric disorders and to dysregulation of DAergic function. For example, previous reports have demonstrated significant downregulation of GSK-3 α/β, AKT1, and β-catenin in post-mortem PFC tissue samples from schizophrenia patients (Beasley et al. 2001; Kozlovsky et al. 2001). In addition to schizophrenia, substantial reductions in levels of p70S6K and mTORC1 have been observed in post-mortem PFC samples from mood disorder patients (Jernigan et al. 2011).

Interestingly, the dramatic reductions in PFC GSK-3α/β and p70S6K phosphorylation levels that we observed following adolescent THC exposure are consistent with a hyper-DAergic phenotype and DA receptor stimulation within the PFC. Indeed, considerable evidence demonstrates that DA receptor transmission is capable of strongly modulating the phosphorylation states of both Akt and GSK-3 pathways within the mammalian brain. For example, pharmacological inhibition or genetic deletion of the DA D2 receptor increases phosphorylated Akt-thr308 and GSK3β in the striatum (Beaulieu 2011; Sutton and Rushlow 2012). In addition, transgenic mice lacking the DA transporter (DAT–KO) demonstrate persistent mesolimbic hyper-dopaminergic and reduced Akt phosphorylation at threonine 308, concomitant with a reduction in the phosphorylation of GSK3α/β (Beaulieu et al. 2004). A similar effect on Akt and GSK-3 has been observed following the administration of psychostimulants that increase extracellular DA concentrations, such as amphetamine (Poller et al. 2010). Furthermore, chronic administration of DA D2 agonists, (which would be expected to mimic the presently observed hyper-DAergic state induced by THC), has been shown to dramatically downregulate PFC levels of Akt and GSK-3α/β (Sutton and Rushlow 2012). Finally, pharmacological stimulation of D2 receptors potently downregulates levels of p70S6K (Fasano et al. 2008), consistent with the present findings. Thus, the observed molecular PFC adaptations observed following adolescent (but not adulthood) THC exposure might be associated with the known effects of a persistent hyper-DAergic state. These results demonstrate for the first time that adolescent THC exposure may lead to schizophrenia-like DAergic alterations in early adulthood which may in turn lead to a developmental cascade of cognitive and affective abnormalities in early adulthood,
consistent with schizophrenia-like symptomology. Although the present study focused exclusively on the PFC, the effects of VTA hyper-DAergic activity may be expected to impact molecular and neuronal activity parameters in other VTA DAergic output targets, such as the nucleus accumbens (NAC) and/or basolateral amygdala (BLA). Future studies are required to more fully investigate these possibilities.

Interestingly, in direct contrast to the effects of adolescent exposure, adult THC exposure selectively increased levels of phospho-GSK-3 α/β isoforms and Akt at serine 473 residue (Akt-ser473), while having no effect on the expression levels of other examined signaling pathways. Furthermore, since Akt-ser473 is a substrate of the mTOR Complex 2 (mTORC2) (Sarbasov et al. 2005), THC exposure during adulthood appears to have a selective impact on the mTORC2 signaling pathway. Thus, consistent with the dissociable effects of adolescent versus adulthood THC exposure on other behavioral and DAergic neuronal activity, these findings demonstrate that THC exposure during adolescence has a selective effect on modulating schizophrenia-related molecular, neuronal, and behavioral endophenotypes, which are not affected following adulthood exposure. However, it is important to note that while the present preclinical study revealed several intriguing neuronal, molecular, and behavioral phenomena in experimental animals consistent with clinical and neuropathological features of schizophrenia, these disturbances are observed also in other complex neuropsychiatric disorders, such as mood disturbances (Beaulieu et al. 2004; Gould et al. 2004; Jernigan et al. 2011). Caution is therefore warranted when applying these findings to potential side effects associated with adolescent MJ exposure.

Interestingly, recent reports have linked alterations in the mTORC2 pathway to neuronal plasticity and cytoskeletal regulation as opposed to mTORC1, which is closely linked to synaptic plasticity mechanisms (Hoeffer and Klann 2010). In addition, mTORC2 has been linked to DAergic neuroadaptations associated with chronic opiate exposure and decreased DA release within the mesolimbic pathway (Mazei-Robison et al. 2011). While we observed no changes in spontaneous DAergic neuronal activity following adulthood THC exposure, 1 intriguing possibility is that the observed alterations selectively in the mTORC2 pathway may relate to an adaptive response to repeated THC-induced DAergic modulation that is present in the adult brain, but absent during adolescent neurodevelopment. Future studies are required to more fully characterize the potential phenotypic effects related to these observed molecular adaptations following adulthood THC exposure.

In summary, consistent with a growing body of clinical evidence, the present findings demonstrate that adolescence represents a selective neurodevelopmental window of vulnerability wherein the developing brain is particularly sensitive to the effects of chronic THC exposure. Given the exponential global rise in cannabis use for both recreational and therapeutic purposes, these findings have critical implications for public health policy development related to regulating adolescent versus adulthood exposure to cannabis.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

Notes
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