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Parental THC Exposure Leads to Compulsive Heroin-Seeking and Altered Striatal Synaptic Plasticity in the Subsequent Generation

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Recent attention has been focused on the long-term impact of cannabis exposure, for which experimental animal studies have validated causal relationships between neurobiological and behavioral alterations during the individual's lifetime. Here, we show that adolescent exposure to Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis, results in behavioral and neurobiological abnormalities in the subsequent generation of rats as a consequence of parental germline exposure to the drug. Adult F1 offspring that were themselves unexposed to THC displayed increased work effort to self-administer heroin, with enhanced stereotyped behaviors during the period of acute heroin withdrawal. On the molecular level, parental THC exposure was associated with changes in the mRNA expression of cannabinoid, dopamine, and glutamatergic receptor genes in the striatum, a key component of the neuronal circuitry mediating compulsive behaviors and reward sensitivity. Specifically, decreased mRNA and protein levels, as well as NMDA receptor binding were observed in the dorsal striatum of adult offspring as a consequence of germline THC exposure. Electrophysiologically, plasticity was altered at excitatory synapses of the striatal circuitry that is known to mediate compulsive and goal-directed behaviors. These findings demonstrate that parental history of germline THC exposure affects the molecular characteristics of the striatum, can impact offspring phenotype, and could possibly confer enhanced risk for psychiatric disorders in the subsequent generation.
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INTRODUCTION

Marijuana (*Cannabis sativa*) continues to be the most commonly abused illicit drug by teenagers and young adults of childbearing age with significant social and public health implications (SAMHSA, 2011). Indeed, adolescents now smoke cannabis to a greater extent than cigarettes (Johnston *et al*, 2012). Despite of the perceived risk of cannabis use being relatively low in society, there is growing clinical awareness about the spectrum of behavioral and neurobiological disturbances associated with direct cannabis exposure such as anxiety, depression, psychosis, cognitive deficits, and social impairments (Crean *et al*, 2011; Leweke and Koethe, 2008; Malone *et al*, 2010; Morris *et al*, 2011). Furthermore, a variety of studies have documented enhanced drug-taking and drug-seeking behavior in humans and animals following adolescent exposure to cannabis or Δ^9-tetrahydrocannabinol (THC), that supports the notion of a gateway effect for addiction risk (Fergusson *et al*, 2007; Frenois *et al*, 2005; Kandel, 1975). Although significant research efforts have begun to characterize the long-term behavioral and neurobiological consequences of exposure to cannabis during an individual's lifetime, the possible impact on the progeny of marijuana users has not been examined.

Until recently, it was believed that the neurobiological disturbances that occurred during the life span of an individual were reprogrammed across most of the genome during the early phases of embryonic development from parent to offspring, thereby establishing a new 'state' for the next generation (reviewed, eg, in Cantone and Fisher (2013); Feng *et al* (2010)). This dogma has been challenged by several recent studies showing that the effects of environmental toxins (eg, Kundakovic and Champagne (2011); Nilsson *et al* (2012); Skinner *et al* (2008)) or drugs of abuse such as alcohol (Govorko *et al*, 2012), cocaine (Vassoler *et al*, 2013), and opiates (Byrnes *et al*, 2011; Byrnes *et al*, 2013) were inherited through the germline from parent to child. The relationship between developmental cannabis

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exposure and psychiatric vulnerability, as well as the important contribution of the endocannabinoid system to gamete function, raised the question as to whether transmission of the cannabis-induced epigenetic milieu could contribute to the development of neuropsychiatric disorders in subsequent generations. In this study, we examined behavioral phenotypes and neurobiological characteristics of F1 offspring with parental germline THC exposure using an animal model.

MATERIALS AND METHODS

Drugs

Δ^9-THC (50 mg/ml in ethanol solution) was evaporated under nitrogen gas, dissolved in saline (0.9% NaCl) containing 0.3% Tween 80, and diluted with saline to a concentration of 0.75 mg/ml (Dinieri and Hurd, 2012). Vehicle solution (VEH) was saline containing 0.3% Tween 80.

Animals

Twenty-one-day-old male and female Long-Evans rats and lactating Long-Evans females with litter were purchased from Charles River Laboratories (Wilmington, MA). Care and handling procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Local Animal Care and Use Committee. For details, see Supplementary Materials and Methods.

Cross-Generational THC Animal Model

A schematic of the paradigm is shown in Figure 1a. Male and female rats arrived at the facility at PND 21 and they were housed in same sex groups of four. The animals were carried through our established adolescent THC treatment protocol (Dinieri and Hurd, 2012; Ellgren *et al*, 2007; Tomasiewicz *et al*, 2012). Briefly, rats were exposed to either a moderate dose of THC (1.5 mg/kg i.p.) or VEH, one injection every third day during PND 28–49. To determine tissue content of THC and its main metabolites THC-OH and THCA, ~400 mg of brain tissue and 2 ml of trunk blood serum were analyzed by gas chromatography–mass spectrometry 16 and 28 days following the end of THC treatment (National Institute on Drug Abuse). No detectable levels of these compounds were evident (Supplementary Table S1). VEH-exposed females were mated with VEH-exposed males and THC-exposed females with THC-exposed males in a ratio of two females to one male during PND 64–68. Females were single housed throughout pregnancy. Gestational parameters such as maternal weight gain, pregnancy length, and fetal weights were recorded. After birth at ~PND 2, mixed litters were established combining an approximately equal number (12–14) of pups from THC- and VEH-exposed parents with a balanced proportion of males and females in each litter. The litters were cross-fostered to drug-naive surrogates, which were used as nursing mothers. F1 offsprings were weaned at ~PND 24, and groups of 3–4 animals were maintained without any drug treatment or behavioral testing on normal 12-h light/dark cycle with *ad libitum* access to food and water until adolescence (~PND 35) or adulthood (~PND 62). Animal care and handling were performed by technicians unfamiliar with parental treatment history. Animals were anesthetized with CO_2, were decapitated, their brains were frozen in isopentane, and were stored at −80 °C until subsequent experiments.

Jugular Vein Catheterization Surgeries

At ~PND 56, F1 male rats were surgically implanted with jugular catheters (Brian Fromant, Cambridge, UK) as previously described (Dinieri and Hurd, 2012; Ellgren *et al*, 2007). Catheter patency was confirmed by loss of muscle tone within seconds of i.v. infusion of Brevital. Rats that failed this test were eliminated from the study.

Heroin Self-Administration

The heroin self-administration study was conducted during the dark phase of the light/dark cycle according to established protocols (Ellgren *et al*, 2008; Ellgren *et al*, 2007) in operant equipment fitted with infrared beams to monitor locomotor activity (MED Associates, St Albans, VT). Animals were food restricted (18 g chow/day) throughout the experiment. Rats were allowed a 3-h daily access to heroin (30 μg/kg/i.v. infusion diacetylmorphine-HCl; National Institute on Drug Abuse Drug Supply) initially under a fixed ratio-1 (FR-1) reinforcement schedule, followed by increased work effort at FR-5, where five active lever presses resulted in a single-heroin infusion. Heroin was available in all self-administration sessions. Each drug infusion was associated with a conditioned stimulus 'cue' light that was illuminated after pressing the active lever while food pellet was delivered.

Open-Field Locomotor Behavior Testing

Rats were tested during the dark phase of the light/dark cycle in a standard squared plexiglass arena (40.6 cm × 40.6 cm; MED Associates), equipped with the Versamax activity monitor system (AccuScan Instruments, Colombus, OH) and a dim flashing light source in the front side of the chamber. Locomotor activity was recorded for 30 min and data were analyzed in 5-min time bins. Stereotypy and time spent in the front *vs* back of the chamber were defined using criteria in the VersaDat software (AccuScan Instruments; see Supplementary Materials and Methods).

Quantitative Reverse Transcription PCR Analyses

Striatal and cortical brain regions were dissected from frozen adolescent (PND 35) and adult (PND 62) brains of rats with a 15-gauge sample punch on a cold block at −25 °C. RNA was prepared from bilateral tissue punches using the RNAqueous-Micro Kit (Ambion) and cDNA was obtained with a first-strand synthesis kit (Quanta Biosciences). Quantitative real-time PCR was performed using the LightCycler 480 Probes Master reagent (Roche) and the TaqMan PCR program in a LightCycler 480 instrument (Roche). The following Taqman-based assays (Applied Biosystems) were used in triplicate PCRs: *Cnr1,* Rn00562880_m1; *Grin1,* Rn01436038_m1; *Grin2A,* Rn00561341_m1; *Grin2B,* Rn00680474_m1; *Gria1,*

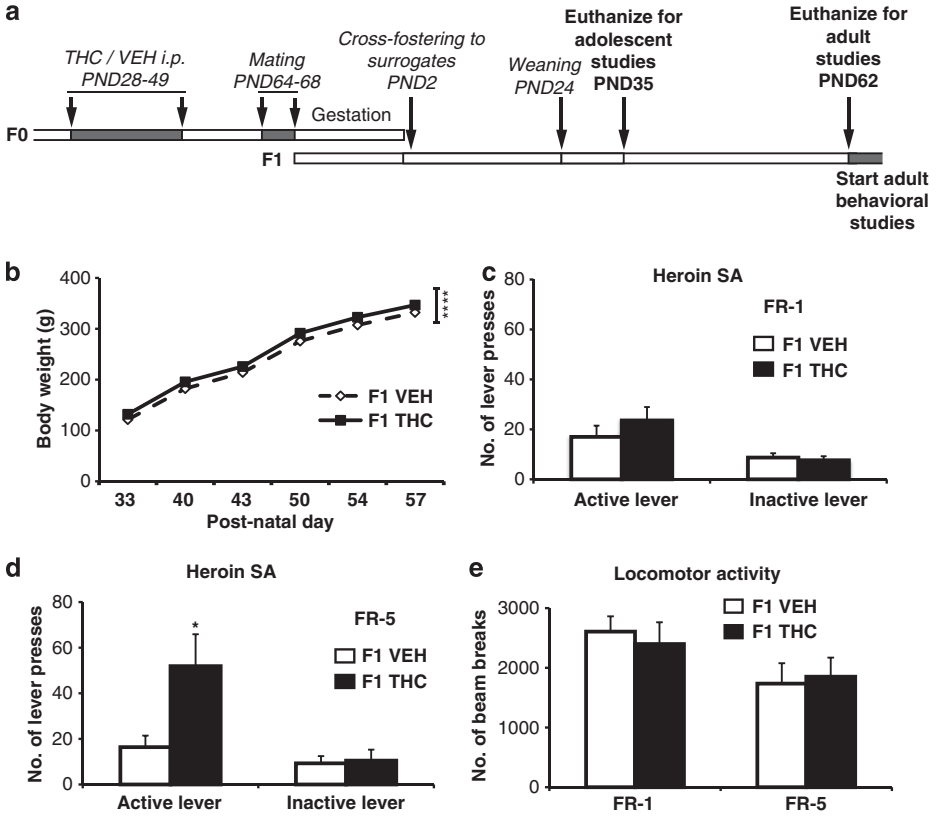

Figure 1 Parental germline THC exposure leads to increased work effort to self-administer heroin in adult F1 offspring. (a) Schematic overview of the experimental conditions used to study the cross-generational effects of parental germline THC exposure. (b) Body weight in three independent cohort of F1 offspring between early adolescence and adulthood. $N = 107$ (VEH group), $N = 92$ (THC group). (c) Heroin self-administration in adult F1 offspring under fixed ratio- (FR) 1 schedule. (d) Enhanced heroin intake with increased work effort at FR-5. (e) Locomotor activity during heroin self-administration sessions is not affected by parental THC exposure. $N = 6$–7 animals/group in heroin self-administration. Values are expressed as a mean ± SEM. * and **** indicate $p < 0.05$ and $p < 0.0001$, respectively, *vs* control (F1-VEH) subjects.

Rn00709588_m1; *Gria2,* Rn00568514_m1; beta-*2-microglobulin,* Rn00560865_m1). Each gene of interest was run in duplex with a reference gene (beta-*2-microglobulin*), data were normalized by the ΔΔCT method (Livak and Schmittgen, 2001), and were expressed as mRNA levels relative to the F1-VEH group.

Western Blotting and NMDA Receptor-Binding Assays

Dorsal striatum was dissected from frozen adult (PND 62) brains. Bilateral tissue punches were sonicated in PBS buffer containing protease inhibitor cocktail (Roche) for 5 min and then centrifuged at 21 000 *g* for 10 min. A portion of the homogenate (60%) was processed for protein extraction and western blotting, whereas the remaining 40% was used for NMDA receptor-binding assays using previously described conditions (Newell *et al*, 2013). Western blots were probed with primary antibodies against the following proteins: GluN1 and GluN2B (cat. nos. 114-011 and 244-103, SYnaptic SYstems); GluA1 and GluA1 (cat. nos. 1967284 and NG1900693, Millipore); Gapdh (cat. nos. ABS16 and MAB374, Millipore). Blots were imaged on the Odyssey CLx infrared scanner (LI-COR, Lincoln, Nebraska). Proteins were analyzed using this instrument's Image Studio software (LI-COR) and normalized to Gapdh in a dual fluorescent staining on the same gel. Further details are described in the Supplementary Materials and Methods.

Electrophysiology

An electrophysiological approach for field population spike recordings with a protocol previously optimized to induce long-term depression (LTD; 5-min-long train of 10 Hz stimuli) in striatal slices was used (Kreitzer and Malenka, 2007). Detailed description can be found in the Supplementary Materials and Methods.

Statistical Analyses

Statistical comparisons of the offspring with parental VEH- and THC exposure were performed by ANOVAs. Outliers were detected using the Grubbs' test. For heroin self-administration and gene expression studies, data were analyzed using one-way ANOVA, followed by Tukey-HSD *post hoc* comparisons when appropriate. Open-field locomotor activity experiments were analyzed using ANOVAs with repeated measures. Pearson correlations were calculated to assess the relationship between heroin self-administration and open-field locomotor activity data. For electrophysiological measurements, population spikes were averaged over 5-min intervals, and mixed model two-way

ANOVAs on the post stimulation responses were used to analyze the effect of germline THC exposure on LTD.

RESULTS

Rat Model of Cross-Generational THC Exposure

The phenotypic and neurobiological consequences of adolescent marijuana exposure in the F1 offspring were studied using the treatment regime described above. This regime was chosen to mimic the drug use pattern of male and female teenagers, who often engage in periodic recreational marijuana use in peer groups or as couples. Given that female rats were treated with THC before mating, it was important to establish that the developing fetus was not exposed to the drug *in utero* through the blood stream of the mother (Supplementary Figure S1).

Treatment of F0 parents with THC reduced the rate of pregnancy by ~40%, which is consistent with the documented effect on male fertility (Whan *et al*, 2006). There were no complications during pregnancy and no significant group differences in parental body weight before mating, in maternal weight gain during gestation, the length of pregnancy, in the total number of pups, and in the offspring male/female ratio in relation to parental THC exposure (data not shown). There was, however, increased body weight (Figure 1b) in the offspring of THC-exposed rats in the examined developmental period with a main effect of parental treatment ($F(1,197) = 16.79$, $p < 0.0001$). Statistical analyses showed no significant effects of offspring body weight with regards to the behavioral and molecular experiments described below, thus the influence of germline THC exposure on offspring weight was not examined further.

In this study, we focused on adult male offspring as our adolescent THC paradigm has been shown to induce a long-term increase in heroin self-administration behavior, as well as impaired striatal gene expression, in adult F0 males (Ellgren *et al*, 2008; Ellgren *et al*, 2007; Tomasiewicz *et al*, 2012). Three independent cohorts were used for the different experiments described below, with at least four litters and a maximum of two pups from the same litter represented within any group.

Parental Germline THC Exposure Leads to Increased Heroin Self-Administration Behavior in Adult Male F1 Offspring

A history of adolescent THC exposure has been associated with the development of addiction disorders later in life (Frenois *et al*, 2005). To investigate whether THC exposure of F0 male and female parents induces altered response to a drug of abuse, we examined heroin self-administration (30 μg/kg/infusion i.v.) behavior in adult F1 offspring. There were no differences related to parental THC exposure on stable self-administration behavior using FR-1 schedule reinforcement (Figure 1c). Interestingly, when the work effort to obtain the drug was elevated to an FR-5 schedule, the F1 progeny of parents with adolescent THC exposure exhibited significantly increased work effort to obtain the opiate ($F(1,11) = 4.98$, $p = 0.047$, Figure 1d). No difference in locomotor activity was observed during either FR-1 or FR-5 sessions (Figure 1e), suggesting that the increased active lever pressing under FR-5 schedule reflected a behavioral response specific to drug-seeking.

F1 Offspring with Parental Germline THC Exposure Develop Altered Stereotyped and Approach-Avoidance Behaviors Following Heroin Self-Administration

Withdrawal from drug addiction is known to be associated with symptoms that can promote drug-seeking behavior and make it difficult for the individual to maintain drug abstinence (Dacher and Nugent, 2011; Frenois *et al*, 2005). To address the influence of germline THC exposure on F1 offspring behavior associated with withdrawal, open-field behavior was assessed following a short (3 days) and prolonged (2.5 months) abstinence period after the final heroin self-administration session (Figure 2). F1-THC offspring tended to show moderately increased locomotor activity (Figure 2a) and exhibited significantly increased repetitive movements ('stereotyped behaviors') during the acute opioid withdrawal period, which is well characterized to be a stressed state (Figure 2b). For the stereotypy, two-way ANOVA analysis with repeated measures showed a significant effect of parental treatment ($F(1,11) = 7.38$, $p = 0.02$). *Post hoc* analysis indicated significant differences in the number of stereotyped movements at 20, 25, and 30 s during the testing session ($p < 0.05$). No correlation was observed between heroin intake and number of stereotyped movements throughout the self-administration experiment ($r = 0.24$, $p = 0.81$) or specifically during FR-5 schedule ($r = 0.24$, $p = 0.46$), suggesting that stereotypy is likely to develop in association with parental THC exposure and is not simply a consequence of the amount of heroin consumed. No differences in motor activity or stereotypy were detected after a prolonged period of extended abstinence (Figure 2c and d) and in offspring that did not undergo heroin self-administration (Figure 2e and f).

To screen for approach-avoidance behaviors associated with a novel sensory environmental stimulus, we took advantage of a modified open-field setup that contained a flashing light source on one side (front) of the chamber, creating distinct environmental stimuli in different quadrants (front *vs* back). Interestingly, this stimulus induced marked differences in the F1 offspring during the period of acute heroin withdrawal when the animals also showed increased stereotypy (front: $F(1,11) = 7.45$, $p = 0.02$; back: $F(1,11) = 6.72$, $p = 0.03$; Figure 3a). Enhanced approach behavior to the front side was evident in the acute heroin withdrawal period in control animals without parental THC history (F1-VEH front *vs* back: $F(1,10) = 8.44$, $p = 0.02$), but absent in F1-THC offspring (Figure 2g). There were no parental THC-related differences observed in the heroin-naive offspring in approach behavior to the light source (Figure 2h).

Taken together, the abnormal behaviors observed in F1 offspring emphasize cross-generational behavioral disturbances in association with parental history of THC exposure. Furthermore, heroin self-administration by the F1 generation tends to exacerbate THC-related alterations, leading to the expression of abnormal behaviors shortly after the acute cessation of heroin intake.

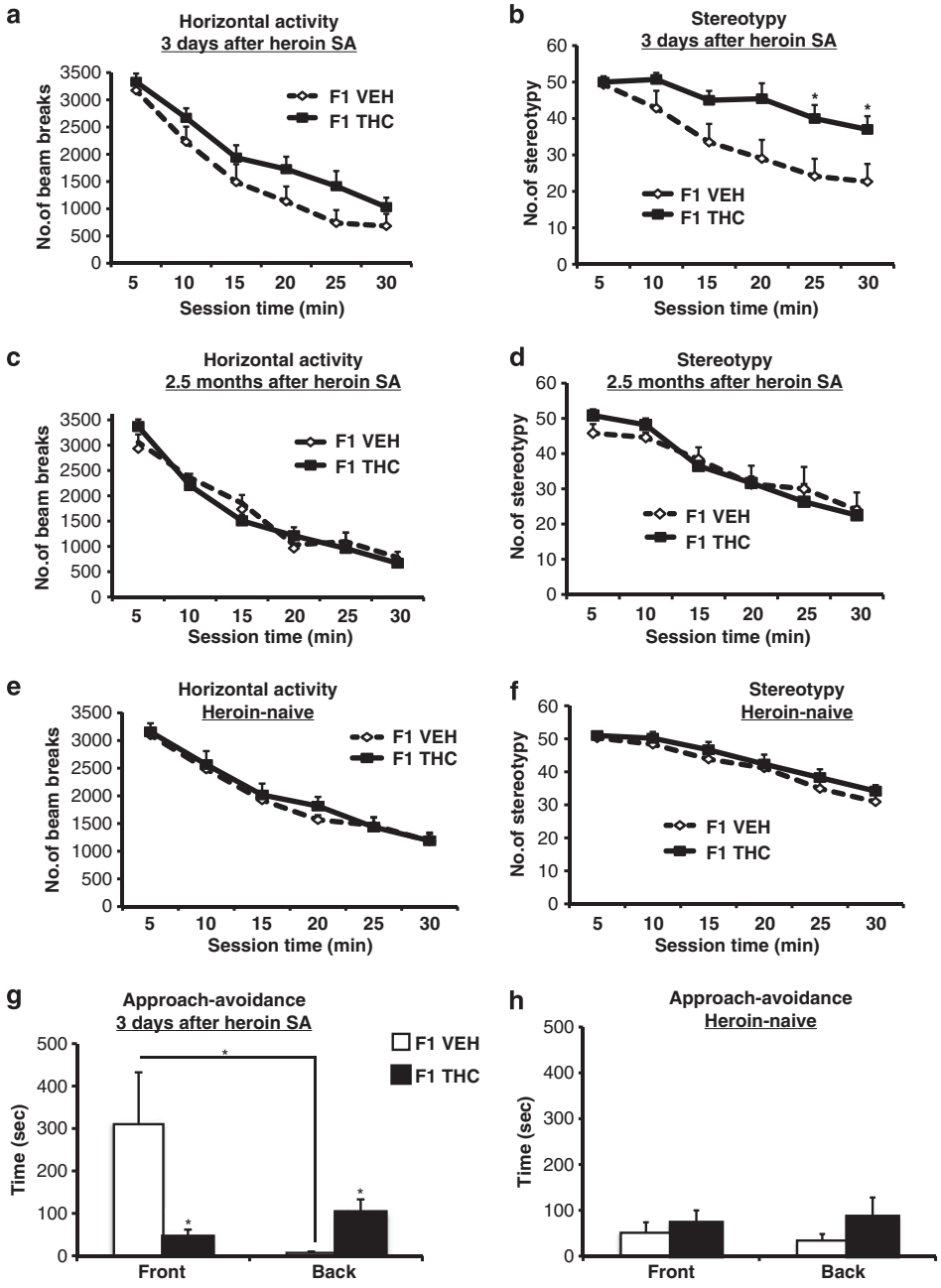

Figure 2 F1 offspring of THC-exposed parents develop abnormal behaviors during a period of acute heroin withdrawal. (a) Open-field horizontal activity 3 days following the final heroin self-administration session. (b) Increased stereotyped behavior following heroin self-administration. (c) Horizontal activity 2.5 months after final heroin self-administration. (d) No difference in stereotyped behavior 2.5 months after heroin intake. Horizontal activity (e) and stereotypy (f) was not altered in adult offspring without heroin exposure. (g) Offspring without parental THC exposure tend to approach and a novel light stimulus (in the front compartment of the open-field arena) tested 3 days following the final heroin self-administration session. (h) No difference in stimulus approach behavior in offspring without heroin exposure. Values are expressed as a mean ± SEM. *$p < 0.05$ *vs* control (F1-VEH) subjects. $N = 6$–7 animals/group.

Cross-Generational THC Exposure Leads to Molecular Abnormalities in the Striatum, Relevant to Synaptic Plasticity

Considering the central role of striatal circuitry in behaviors related to reward processing, motivation, emotion and motor activity (Everitt and Robbins, 2005; Girault, 2012; Koob and Volkow, 2010), we characterized relevant gene expression profiles of the dorsal and ventral striatum (nucleus accumbens; NAc). mRNA levels were measured by quantitative reverse transcription PCR in adolescent (PND 35) and young adult (PND 62) F1 animals. Given the importance of dopaminergic and glutamatergic inputs to the striatum with respect to addiction-related behaviors (Kalivas and Volkow, 2005), we investigated the expression of dopamine receptor subtypes (*Drd1* and *Drd2*) as well as several subunits of the NMDA and AMPA receptors. We also analyzed mRNA levels of the *Cnr1* gene that encodes for the CB1 receptor, a direct

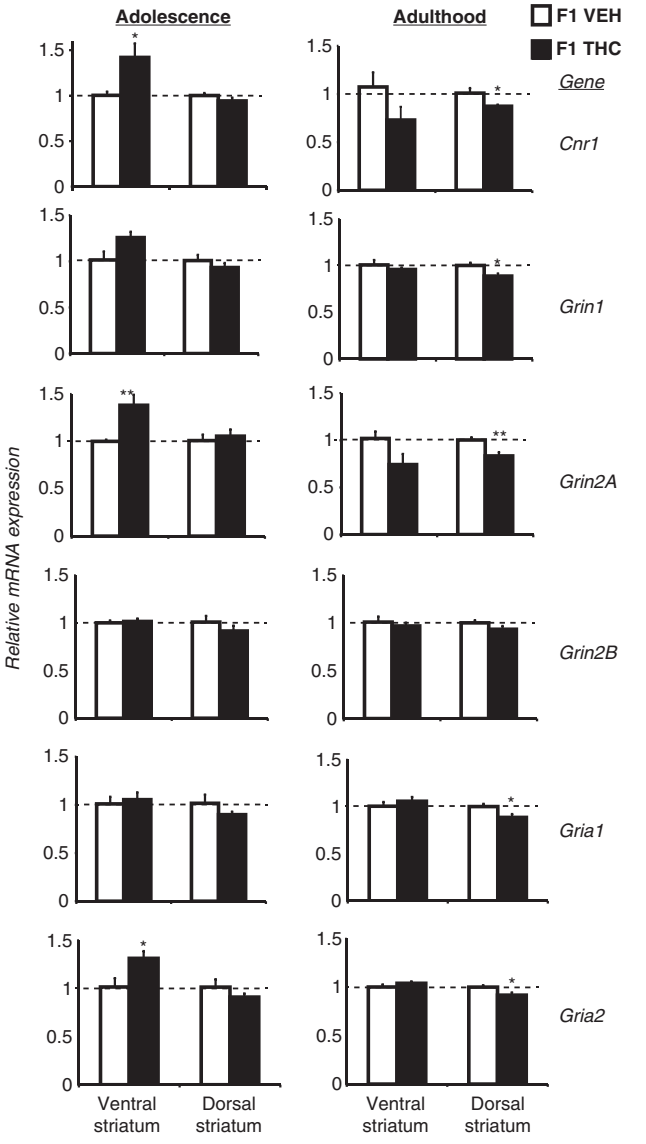

Figure 3 Dysregulation of striatal mRNA levels in adolescent and adult F1 offspring with parental THC exposure. Cnr1, glutamate, and dopamine-related genes are measured by quantitative reverse transcription PCR in the dorsal and ventral striatum. Values are expressed as percent of control ± SEM. * and ** indicate $p<0.05$ and $p<0.01$, respectively. N = 4–5 adolescent group; N = 7–8 adult group.

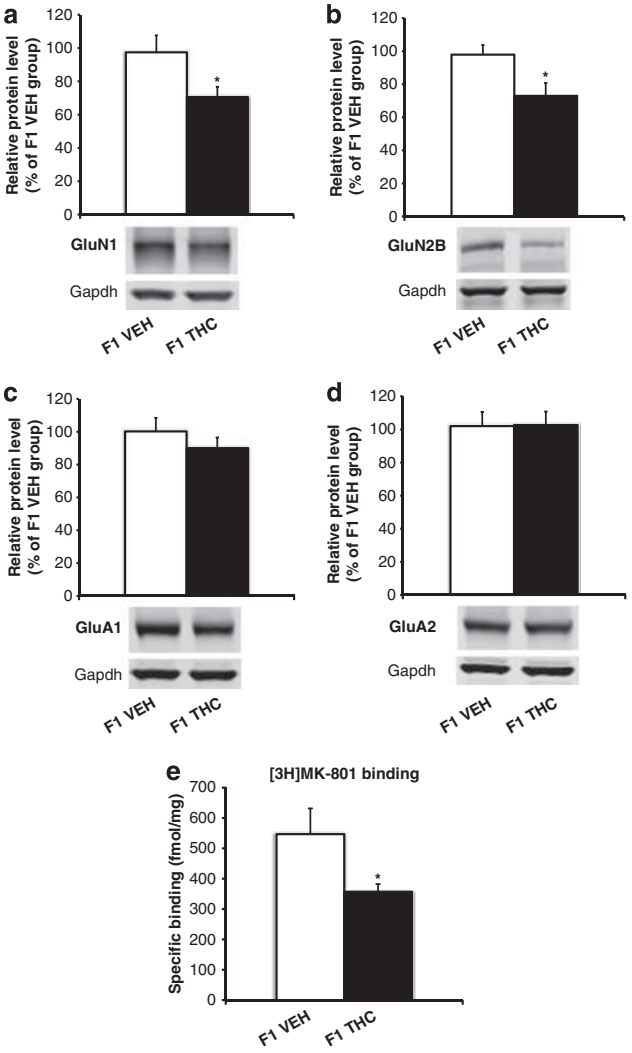

Figure 4 Abnormal NMDA receptor subunit levels and binding in the dorsal striatum of adult offspring with parental THC exposure. GluN1 (a), GluN2B (b), GluA1 (c), and GluA2 (d) levels, detected by western blotting. Images below each graph show representative western blot images. Gapdh served as loading control. N = 8 animals/group. (e) Reduced membrane-bound NMDA receptor availability, analyzed by [^{3}H]MK-801-binding assay. N = 5–8 animals/group. Values are expressed as a mean ± SEM. *$p<0.05$ *vs* control (F1-VEH) subjects.

target of THC (Cooper and Haney, 2008; Harkany *et al*, 2008; Mulder *et al*, 2008; Pertwee, 2008).

The results revealed significant alterations in striatal mRNA levels in the F1 progeny (Figure 3). Impairments of *Cnr1* and glutamate receptors were localized to the NAc (*Cnr1*: $F(1,8)=6.80$, $p=0.03$; *Grin2A*: $F(1,8)=11.42$, $p=0.009$; *Gria2*: $F(1,8)=6.24$, $p=0.04$) at the adolescent time point and expression was increased. In adult offspring, alterations were more prominent in the dorsal striatum and the mRNA levels were reduced as compared with F1-VEH adult animals (*Cnr1*: $F(1,14)=5.89$, $p=0.03$; *Drd2*: $F(1,13)=9.42$, $p=0.009$; *Grin1*: $F(1,14)=7.12$, $p=0.02$; *Grin2A*: $F(1,14)=11.35$, $p=0.005$; *Gria1*: $F(1,14)=6.14$, $p=0.03$; *Gria2*: $F(1,14)=5.84$, $p=0.03$). No significant impairments were found in the mRNA levels of the same genes studied in the medial prefrontal cortex and orbitofrontal cortex, brain regions that have direct connectivity with the striatum and have also been associated with addiction vulnerability (Supplementary Figure S1).

To assess abnormalities on the protein level, several glutamate receptor subunits were analyzed by western blotting in the dorsal striatum of an independent cohort of adult offspring. Membrane-bound protein level of the GluN1 subunit (the product of *Grin1* mRNA) of the NMDA receptor was decreased ($(F(1,14)=5.07$, $p=0.04)$; Figure 4a), as well as GluN2B (*Grin2b*'s protein product; $(F(1,14)=6.58$, $p=0.02)$; Figure 4b). Membrane-bound AMPA receptor subunits GluA1 and GluA2 remained unchanged (Figure 4c and d). Intriguingly, the number of available membrane-bound NMDA receptor-binding sites (identified by radioligand-binding assay using [^{3}H]MK-801 in the same animals) also showed a consistent reduction in

association with parental THC exposure ($F(1,11) = 6.76$, $p = 0.02$; Figure 4e). Overall, these results demonstrate that parental THC exposure leads to brain region-specific impairments in the striatum, raising the question as to the functional consequences of these abnormalities.

Parental Germline THC Exposure Leads to Increased LTD in the Dorsal Striatum of Adult F1 Offspring

Activity of medium spiny neurons in the striatum is regulated by glutamatergic input that contributes to forms of synaptic plasticity such as LTD (Gerdeman *et al*, 2002). Striatal LTD has been strongly associated with habitual behaviors and reinforcement learning (Kreitzer and Malenka, 2007; Singla *et al*, 2007). The finding that the expression of molecules known to be critical for striatal LTD, such as NMDA receptors as well as *Cnr1*, were reduced in the dorsal striatum of adult F1 offspring (Figures 3 and 4) suggested potential alterations of LTD in these animals. To investigate whether parental THC exposure induced functional changes in synaptic plasticity, we used an electrophysiological approach with a protocol optimized to induce LTD in striatal slices (Figure 5a). A train of monophasic stimuli (10 Hz for 5 min) of glutamatergic inputs to the striatum evoked striking differences in the ventral and dorsal subregions that related to parental germline THC history. LTD was most prominent in the dorsal as compared with ventral striatum, and LTD in dorsal striatum was significantly larger with a main effect of parental treatment ($F(1,11) = 10.5$, $p = 0.0079$) in offspring of THC-exposed parents (Figure 5b). No F1-THC-related effect was detected in the NAc (Figure 5c). These findings demonstrate that parental THC exposure leads to a significant cross-generational effect on dorsal striatal LTD in adult offspring.

DISCUSSION

The results from this study provide evidence that there are significant consequences of adolescent THC exposure beyond its direct influence on somatic cells in the individual organism. These effects are transmitted to unexposed offspring and impact their developing brain and their behavioral phenotype. Our findings also revealed important molecular and neurophysiological abnormalities related to neuronal systems linked to goal-directed behavior and habit formation.

A major question regarding THC exposure has revolved around its purported long-term effects as a predictor of subsequent addiction risk later in life (Fergusson and Horwood, 2000; Hall and Lynskey, 2005; Yamaguchi and Kandel, 1984). Despite the controversies in human studies because of their complex confounds, investigations have demonstrated that fetal and adolescent THC exposure leads to increased intake and behavioral sensitivity to drugs of abuse later on in adulthood, thus in support of the 'gateway' hypothesis (Biscaia *et al*, 2008; Ellgren *et al*, 2008; Ellgren *et al*, 2007; Tomasiewicz *et al*, 2012). Evidence garnered in our current study now imply a 'cross-generational gateway' state in F1 offspring. This is hypothesized based on the fact that animals with parental germline THC exposure exhibited enhanced heroin self-administration behavior that required increased work effort. Although control animals did not increase their heroin intake that required elevated work effort in the current testing environment, there was a significant group effect emphasizing that the conditions experienced by all animals dissociated a parental THC exposure-related sensitivity. Elevated morphine sensitivity was also described in offspring of rats exposed to a synthetic cannabinoid (Byrnes *et al*, 2012; Vassoler *et al*, 2013).

An interesting feature of the molecular markers studied in the NAc was that those significantly changed were evident at the adolescent time point, a developmental period characterized by risky behavior, which is associated with individual vulnerability to drug-seeking behavior. The differences in the NAc and the dorsal striatum, observed in disturbances of the *Cnr1*, *Grin1*, *Grin2A*, and *Gria2* genes in adolescence and adulthood, has additional implications as they emphasize the dynamic nature of the impact of germline THC exposure. These abnormal mRNA levels first in the NAc and later in the dorsal striatum mirror the transition from reward-oriented to habitual, compulsive drug-taking that normally typifies the progression from recreational drug use to addiction disorder (reviewed in Everitt and Robbins (2013); Gerdeman *et al* (2003)). Disturbances of the dorsolateral striatum and glutamatergic systems in this dorsal striatal subregion are well documented to influence stimulus response, habit learning, and compulsive behaviors (Arnold *et al*, 2004; Shmelkov *et al*, 2010; Vollstadt-Klein *et al*, 2010; Welch *et al*, 2007). Similarly to the observations in this study, downregulation of synaptic plasticity-related genes (eg, *Gria1*, *Drd2*, and other molecules that regulate glutamate receptor function) has been reported in the dorsal striatum of relapse-vulnerable rats following chronic cocaine self-administration (Brown *et al*, 2011).

Our findings are thus in line with these established relationships in several ways. First, the data showed that enhanced LTD associated with parental THC exposure was localized to the dorsal striatum. Second, the functional disturbances in LTD co-localized with abnormal mRNA expression of the CB1 receptor, as well as NMDA and AMPA receptor subunits. This suggests that parental germline THC exposure leads to cross-generational disturbances in dorsal striatal synaptic plasticity in adult offspring. Based on these results, an important direction of future studies will be to elucidate the specific form of LTD underlying these striatal alterations by use of pharmacological and molecular manipulations. Third, the molecular and synaptic plasticity alterations localized to the dorsal striatum could predict a system poised for enhanced compulsive behavior in adult animals. Indeed, germline THC exposure was associated with exacerbated stereotyped behavior in adult offspring during a period of acute opiate withdrawal (Contarino and Papaleo, 2005). Interestingly, chronic morphine exposure has been shown to induce stereotyped behaviors in mice, and this effect has been linked to disturbances in the regulation of glutamate receptors (Capone *et al*, 2008). Altogether, the observations presented in this study demonstrate that some of the molecular alterations induced by cross-generational effects of parental THC exposure might remain dormant, but can synergistically interact with environmental conditions directly experienced by F1 animals during their lifetime to alter individual vulnerability to psychiatric illnesses.

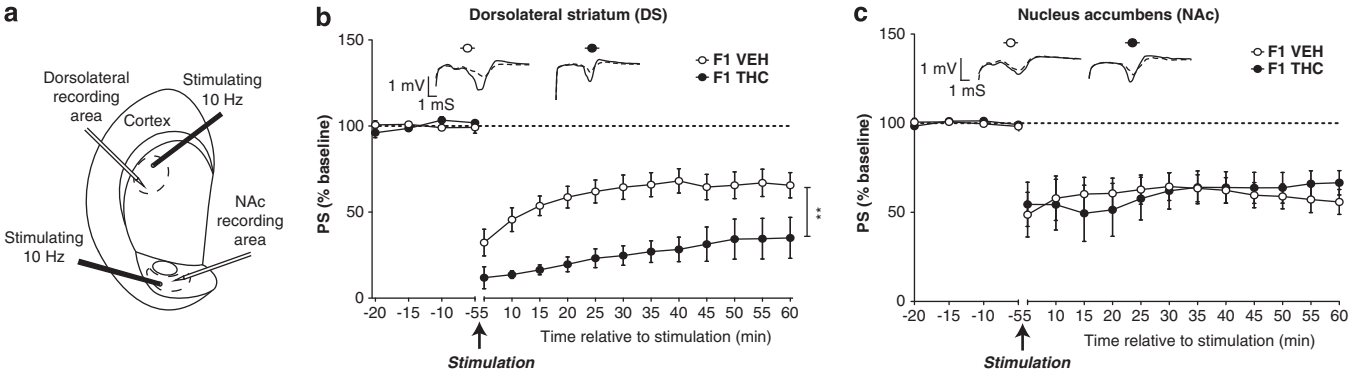

Figure 5 Parental THC exposure leads to increased LTD in the dorsal striatum of F1 offspring with parental germline THC exposure. (a) Schematic of a coronal striatal slice showing the general placement of the stimulating (filled symbol) and recording (open symbol) electrodes in the dorsolateral striatum (DS) and nucleus accumbens (NAc). (b) LTD in the dorsal striatum of rats in the THC group (filled symbols, $N = 9$; ** indicates $p < 0.01$ as a main effect of THC exposure; there was no significant interaction between time and group. Inset shows samples of superimposed averaged sample PSs recorded during the baseline period (solid line) and during the final 5 min of the experiment (dashed line). (c) LTD induced in NAc was unaffected by parental THC exposure ($N = 4$ THC group; $N = 9$ vehicle group). Sample traces as described in b.

A recent study that investigated the transgenerational effects of cocaine also provided evidence for behavioral and molecular disturbances in F1 offspring (Vassoler *et al*, 2013). The mechanisms that mediate the disturbances currently observed in association with parental germline THC exposure, along with the parental lineage that transmits the effects, remain to be determined. Moreover, insights into true, potentially epigenetic, inheritance will require further studies into subsequent generations to investigate whether the disturbances are maintained across multiple generations. In the present study, we focused on genes implicated in addiction-related disorders; however, a genome-wide approach has greater potential to identify novel molecular targets and pathways most linked to the cross-generational effects of THC. Another important consideration for future studies is the identification of discrete cellular localizations and circuits impaired by germline THC exposure. Such investigations will likely provide important knowledge regarding the link between specific neuronal pathways and behavioral phenotypes most sensitive to the cross-generational effects of marijuana.

In summary, our study provides evidence that parental germline THC exposure alters the molecular regulation of the striatum and changes the sensitivity to compulsive drug intake in THC-naive offspring. These findings, along with the growing use of cannabis among young people who may subsequently bear children, highlight the importance for further investigations into the long-term impact of drug exposure not only during the individual's lifetime, but also on future generations.

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