Parental THC exposure leads to compulsive heroin-seeking and altered striatal synaptic plasticity in the subsequent generation

Running title: Cross-generational effects of THC exposure

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Abstract

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.

Keywords: cannabis, cross-generational transmission, striatum, development, addiction, synaptic plasticity

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 THC, that supports the notion of a gateway effect for addiction risk (Fergusson *et al*, 2007; Frenois *et al*, 2005; Kandel, 1975). While 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 which occurred during the lifespan 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 e.g. 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 (e.g. (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 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

21 days old male and female Long-Evans rats and lactating Long-Evans females with litter were purchased from Charles River Laboratories, Inc. (Wilmington, Massachusetts). 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 to 49. To determine tissue content of THC and its main metabolites THC-OH and THCA, ~400 mg brain tissue and 2 ml 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, NIDA). 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 2 females to 1 male during PND64-68. Females were single housed throughout pregnancy. Gestational parameters such as maternal weight gain, pregnancy length, and fetal weights were recorded. After birth at ~PND2, 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-naïve surrogates, which were used as nursing mothers. F1 offspring were weaned at ~PND24 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 (~PND35) or adulthood (~PND62). Animal care and handling were performed by technicians unfamiliar with parental treatment history. Animals were anaesthetized with CO_2, decapitated, brains were frozen in isopentane, and stored at -80°C until subsequent experiments.

Jugular vein catheterization surgeries

At ~PND56, F1 male rats were surgically implanted with jugular catheters (Brian Fromant, Cambridge, United Kingdom) as previously described (Dinieri *et al*, 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 Inc., St. Albans, VT). Animals were food restricted (18 g chow/day) throughout the experiment. Rats were allowed 3-h daily access to heroin (30 µg/kg/ i.v. infusion diacetylmorphine-HCl; NIDA 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 x 40.6 cm; MED Associates Inc., St. Albans, VT), equipped with the Versamax activity monitor system (AccuScan Instruments, Inc., Colombus, OH) and a dim flashing light source in the front side of the chamber. Locomotor activity was recorded for 30 min and data 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, Inc., see Supplementary Materials and Methods).

Quantitative reverse transcription PCR (qRT-PCR) analyses

Striatal and cortical brain regions were dissected from frozen adolescent (PND35) and adult (PND62) brains of rats with a 15-gauge sample punch on a cold block at -25ºC. 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 LightCycler480 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 PCR reactions: *Cnr1,* Rn00562880_m1; *Grin1,* Rn01436038_m1; *Grin2A,* Rn00561341_m1; *Grin2B,* Rn00680474_m1; *Gria1,* 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 via the ΔΔCT method (Livak and Schmittgen, 2001), and expressed as mRNA levels relative to the F1 VEH group.

Western blotting and NMDA receptor binding assays

Dorsal striatum was dissected from frozen adult (PND62) brains. Bilateral tissue punches were sonicated in PBS buffer containing protease inhibitor cocktail (Roche) for 5 min and then centrifuged at 21000 g for 10 min. A portion of the homogenate (60%) was processed for protein extraction and western blotting, while 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# 114-011 and 244-103, SYnaptic SYstems); GluA1 and GluA1 (cat# 1967284 and NG1900693, Millipore); Gapdh (cat# ABS16 and MAB374, Millipore). Blots were imaged on the Odyssey CLx infrared scanner (LI-COR). Proteins were analyzed using this instrument's Image Studio software (LI-COR, Lincoln,

Nebraska) and normalized to Gapdh in a dual fluorescent staining on the same gel. Further details are described in the Supplementary Material and Methods.

Electrophysiology

An electrophysiological approach for field population spikes (PS) recordings with a protocol previously optimized to induce 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 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, PSs 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 prior to 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 in 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 since 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 (three 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 sec 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 (Figures 2C and D) and in offspring that did not undergo heroin self-administration (Figures 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-naïve 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.

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 (PND35) and young adult (PND62) 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 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 to 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 (mPFC) and orbitofrontal cortex (OFC), 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), Fig. 4A), as well as GluN2B (*Grin2b*'s protein product; (F(1,14)=6.58, p=0.02), Fig 4B). Membrane-bound AMPA receptor subunits GluA1 and GluA2 remained unchanged (Fig. 4C and D). Intriguingly, the number of available membrane-bound NMDA receptor binding sites (identified via 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), Fig 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 long-term depression (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 *et al*, 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 to 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 due to 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 since 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, down-regulation of synaptic plasticity-related genes (e.g. *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.

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 if 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-naïve 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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Supplementary information is available at the *Neuropsychopharmacology* website.

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Figure legends

Fig. 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.

Fig. 2. F1 offspring of THC-exposed parents develop abnormal behaviors during a period of acute heroin withdrawal. (A) Open field horizontal activity three 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 three 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.

Fig. 3. Dysregulation of striatal mRNA levels in adolescent and adult F1 offspring with parental THC exposure. Cnr1, glutamate and dopamine-related genes measured by qRT-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.

Fig. 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.

Fig. 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=4) was enhanced relative to the vehicle control group (open 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).

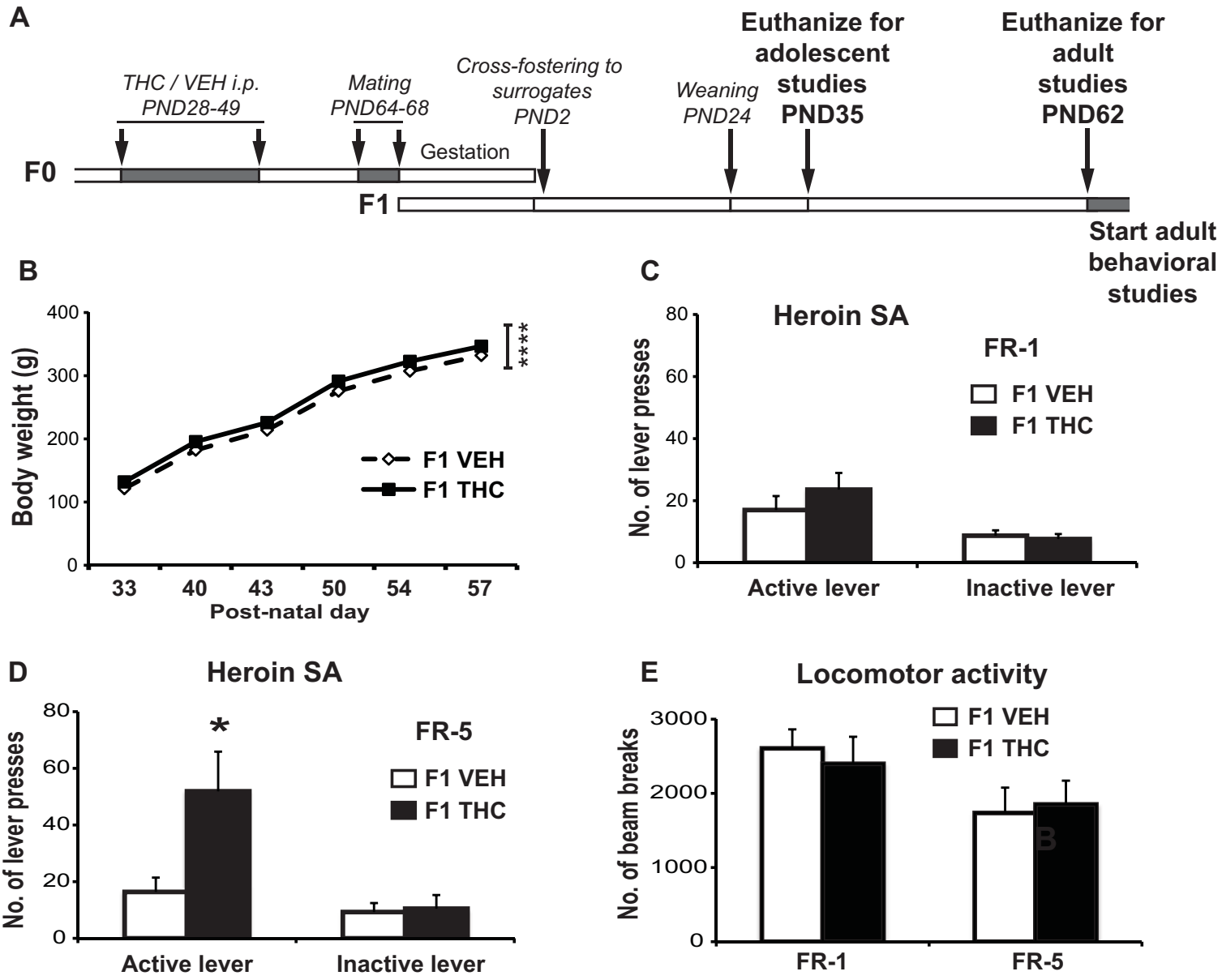
A
THC / VEH i.p.
PND28-49
Mating
PND64-68
Cross-fostering to
surrogates
PND2
Weaning
PND24
Euthanize for
adolescent
studies
PND35
Euthanize for
adult
studies
PND62
Gestation
F0
F1
Start adult
behavioral
studies
B
Body weight (g)
400
300
200
100
0
33
40
43
50
54
57
Post-natal day
****
F1 VEH
F1 THC
C
Heroin SA
FR-1
No. of lever presses
80
60
40
20
0
F1 VEH
F1 THC
Active lever
Inactive lever
D
Heroin SA
FR-5
No. of lever presses
80
60
40
20
0
*
F1 VEH
F1 THC
Active lever
Inactive lever
E
Locomotor activity
No. of beam breaks
3000
2000
1000
0
F1 VEH
F1 THC
B
FR-1
FR-5

Figure 1

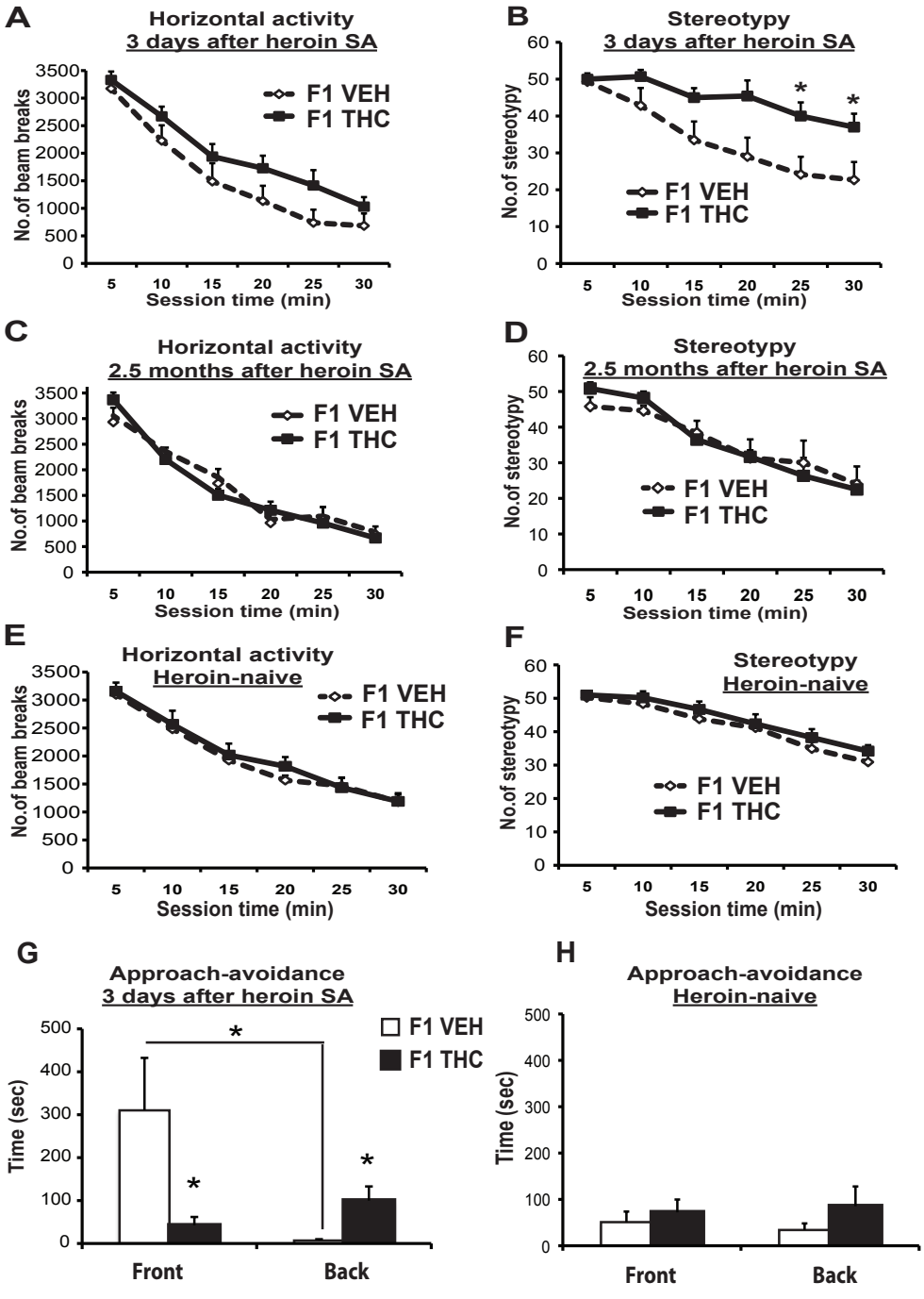
A
Horizontal activity
3 days after heroin SA
No.of beam breaks
Session time (min)
F1 VEH
F1 THC
B
Stereotypy
3 days after heroin SA
No.of stereotypy
Session time (min)
F1 VEH
F1 THC
C
Horizontal activity
2.5 months after heroin SA
No.of beam breaks
Session time (min)
F1 VEH
F1 THC
D
Stereotypy
2.5 months after heroin SA
No.of stereotypy
Session time (min)
F1 VEH
F1 THC
E
Horizontal activity
Heroin-naive
No.of beam breaks
Session time (min)
F1 VEH
F1 THC
F
Stereotypy
Heroin-naive
No.of stereotypy
Session time (min)
F1 VEH
F1 THC
G
Approach-avoidance
3 days after heroin SA
Time (sec)
F1 VEH
F1 THC
Front
Back
H
Approach-avoidance
Heroin-naive
Time (sec)
Front
Back

Figure 2

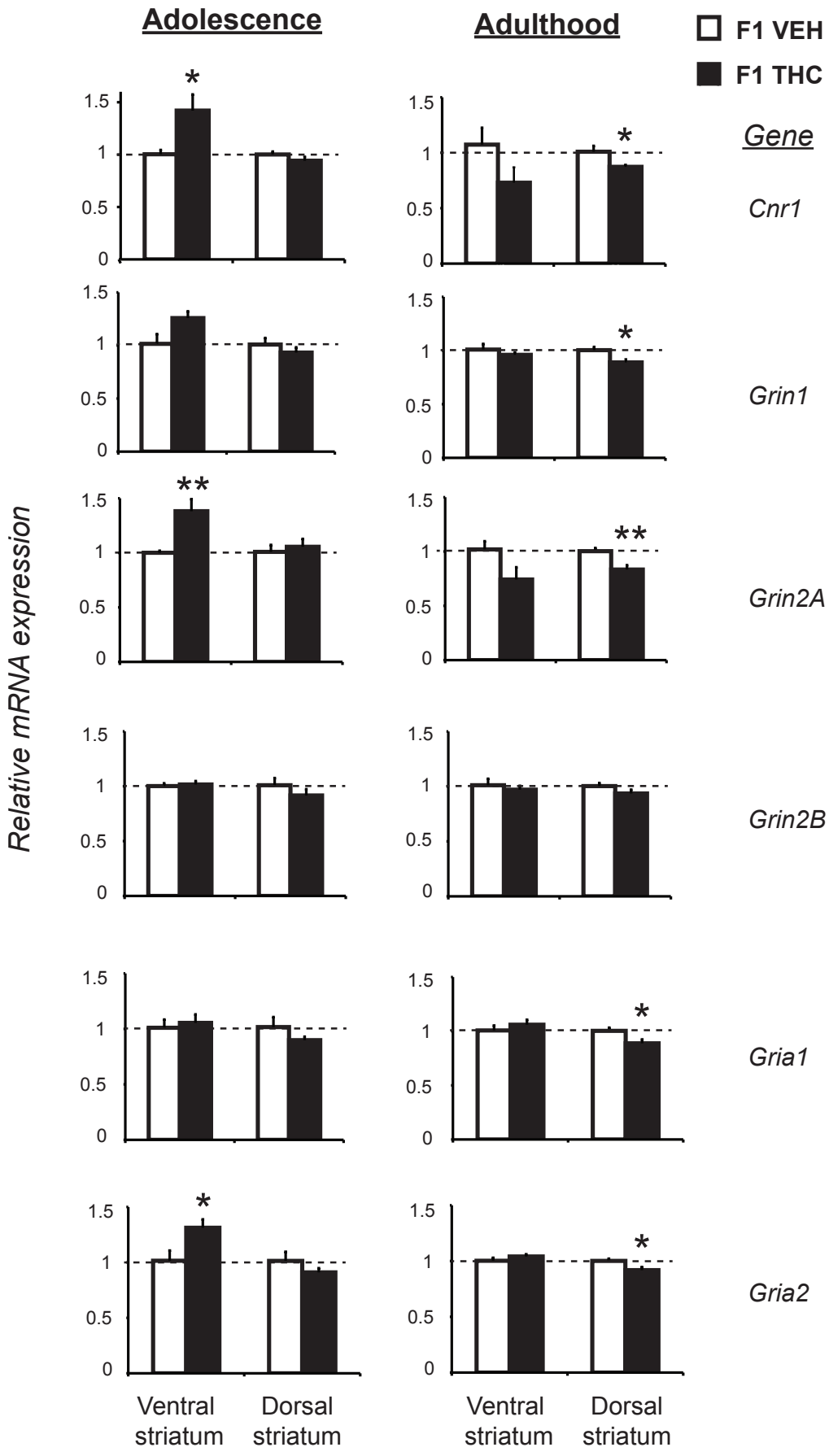
Adolescence
Adulthood
F1 VEH
F1 THC
Gene
Cnr1
Grin1
Grin2A
Grin2B
Gria1
Gria2
Relative mRNA expression
1.5
1
0.5
0
*
**
Ventral striatum
Dorsal striatum
Ventral striatum
Dorsal striatum

Figure 3

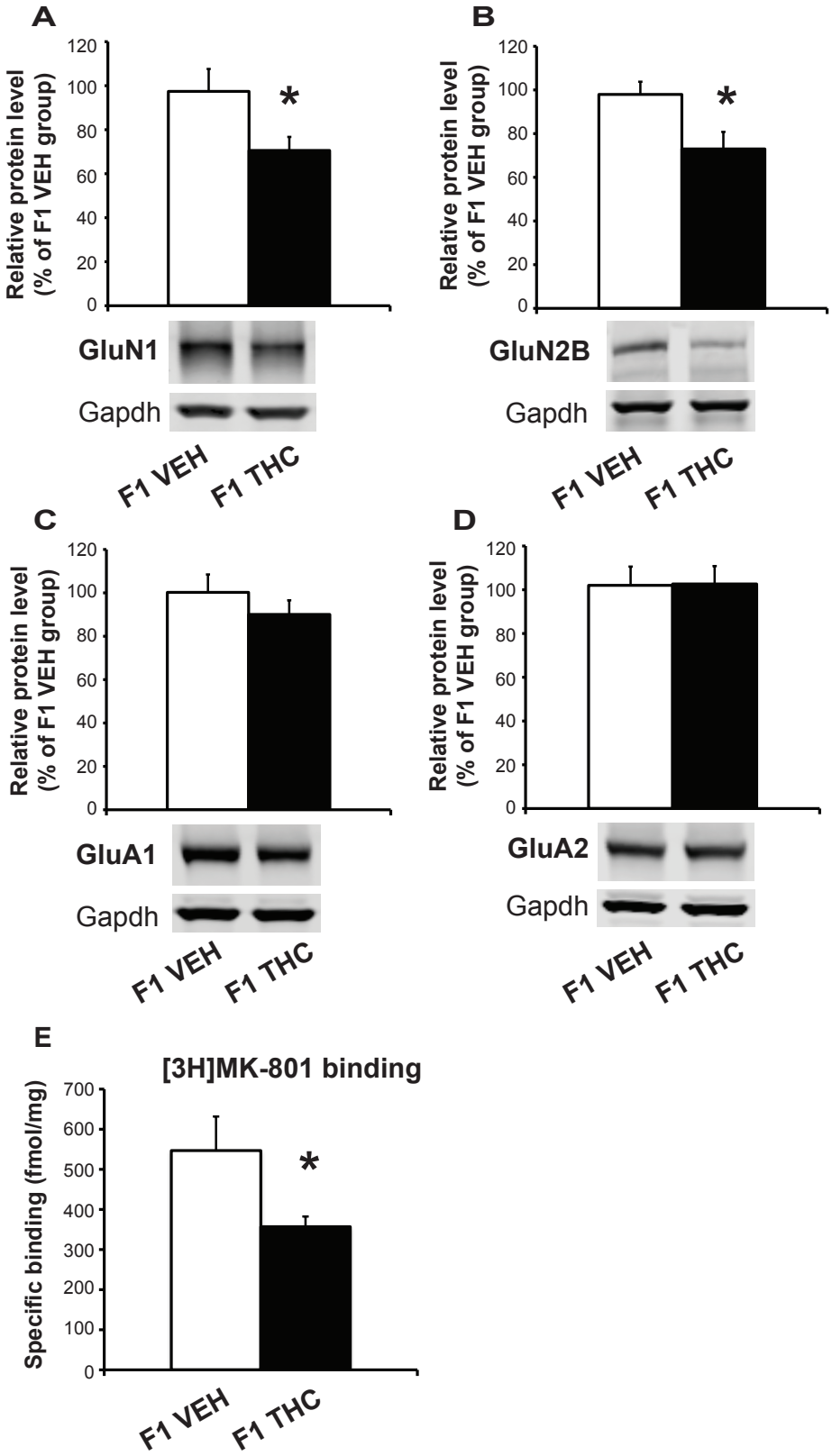
A
Relative protein level (% of F1 VEH group)
120
100
80
60
40
20
0
*
GluN1
Gapdh
F1 VEH
F1 THC
B
Relative protein level (% of F1 VEH group)
120
100
80
60
40
20
0
*
GluN2B
Gapdh
F1 VEH
F1 THC
C
Relative protein level (% of F1 VEH group)
120
100
80
60
40
20
0
GluA1
Gapdh
F1 VEH
F1 THC
D
Relative protein level (% of F1 VEH group)
120
100
80
60
40
20
0
GluA2
Gapdh
F1 VEH
F1 THC
E
[3H]MK-801 binding
Specific binding (fmol/mg)
700
600
500
400
300
200
100
0
*
F1 VEH
F1 THC

Figure 4

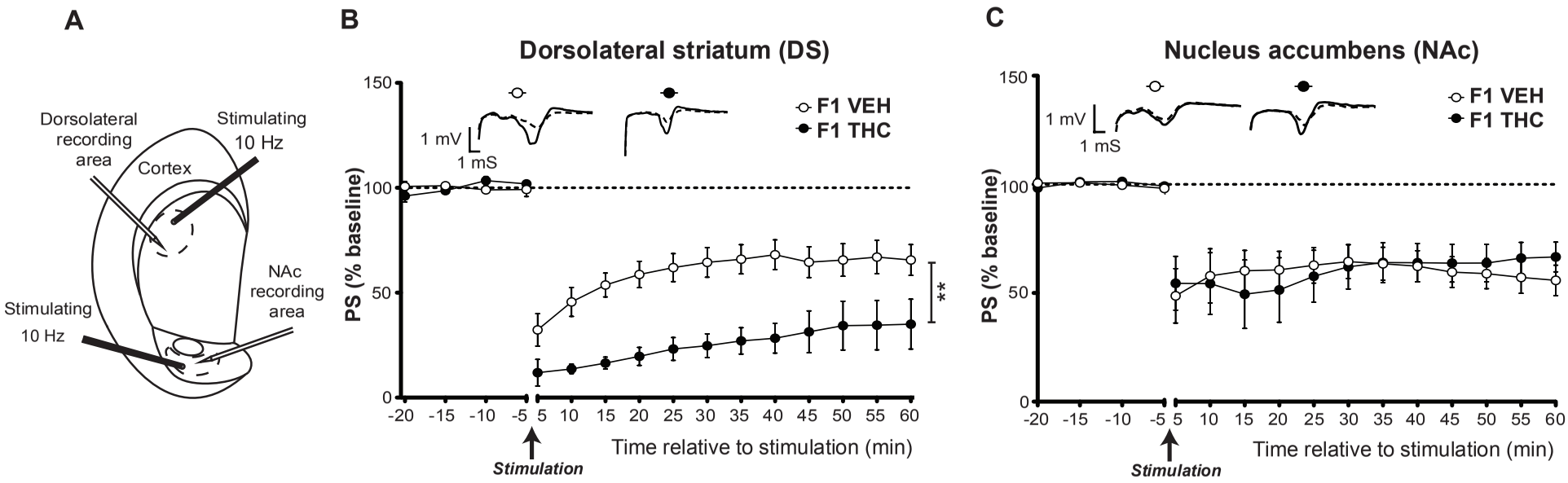
A
Dorsolateral recording area
Stimulating 10 Hz
Cortex
NAc recording area
Stimulating 10 Hz
B
Dorsolateral striatum (DS)
1 mV
1 mS
F1 VEH
F1 THC
PS (% baseline)
150
100
50
0
-20 -15 -10 -5 5 10 15 20 25 30 35 40 45 50 55 60
Time relative to stimulation (min)
Stimulation
**
C
Nucleus accumbens (NAc)
1 mV
1 mS
F1 VEH
F1 THC
PS (% baseline)
150
100
50
0
-20 -15 -10 -5 5 10 15 20 25 30 35 40 45 50 55 60
Time relative to stimulation (min)
Stimulation

Figure 5

Supplemental Materials and Methods

Animals

21 days old male and female Long-Evans rats, weighing 40-50 g, were purchased from Charles River Laboratories, Inc. (Wilmington, Massachusetts). They were housed in a humidity- and temperature-controlled environment on normal 12-h light/dark cycle with *ad libitum* access to food and water, were allowed to acclimate in the facility for one week, and were handled bi-weekly. Lactating Long-Evans females with litter were also obtained from Charles River Laboratories, Inc., and were allowed to acclimate to their new environment one week before used as surrogates to cross-foster neonate F1 generation pups. All 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.

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 x 40.6 cm; MED Associates Inc., St. Albans, VT), equipped with the Versamax activity monitor system (AccuScan Instruments, Inc., Colombus, OH) and a dim flashing light source in the front side of the chamber. Locomotor activity was recorded for 30 min and data 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, Inc.). Stereotypy is defined when the animal breaks the same beam or set of beams repeatedly; this typically occurs during grooming, head bobbing, etc. Number of stereotypy corresponds to the number of times the monitor observed stereotypic behavior in the animal. A break in stereotypy of one second or more is required to separate one stereotypic episode from the next. Time spent in front in the

proximity of the flashing light source is the total time that the animal spent in the two 8 cm x 8 cm corners of the chamber. Time spent in back was determined similarly.

Sample preparation for western blotting and NMDA receptor binding assay

Dorsal striatum was dissected from frozen adult (PND62) brains of rats with a 15-gauge sample punch on a cold block at -25ºC. Bilateral tissue punches from each brain were sonicated in ice cold PBS containing protease inhibitor cocktail (Roche) for 5 min (30 sec ON/30 sec OFF) at medium power using a Bioruptor instrument (Diagenode). 60% of the homogenate was processed further for protein extraction and western blotting. For western blot analysis, samples were centrifuged at 21000 g for 10 min at 4ºC, pellets were resuspended in RIPA buffer (Thermo Scientific) containing protease inhibitors, sonicated for 2.5 min using the conditions described above, incubated for 60 min at 4ºC with agitation, and centrifuged at 21000 g for 10 min at 4ºC. Protein concentration in the supernatant was quantified and these membrane-bound protein extracts were used for western blotting as described in the Main Text. For NMDA receptor binding assay, 40% of the sonicated tissue homogenate was centrifuged at 21000 g for 10 min at 4ºC, pellets were resuspended in 30 mM HEPES buffer (pH 7.5) containing 100 μM glycine, and 1 mM EDTA, sonicated for 2.5 min, followed by two washes and centrifugations at 21000 g for 10 min at 4°C, with a 30 min incubation at 37°C in between. Finally, pellets were resuspended in 30 mM HEPES buffer (pH 7.5) containing 100 μM glycine, 1 mM EDTA and membrane solutions were frozen at 80ºC until further use.

NMDA receptor binding assay

Membrane preparations from 0.5 mg tissue per assay in triplicate reactions were incubated with gentle shaking at room temperature for 2.5 hours in 30 mM HEPES buffer (pH 7.5) containing 100 μM glycine, 100 μM glutamate, 1 mM EDTA, and 2 nM [3H]-MK-

801. Non-specific binding was determined by incubating membranes in triplicate reactions with [3H]-MK-801 in the presence of 20 μM "cold" MK-801. Following incubation, all samples were deposited onto Whatman GF/B filters (Brandel, Inc.) and washed 5 times in ice-cold 30 mM HEPES containing 1 mM EDTA (pH 7.5) using an M-24 Cell Harvester (Brandel, Inc.). The radioactivity of the filters was detected by liquid scintillation spectrometry.

Electrophysiology

Adult rats were anesthetized with isoflurane and decapitated. The brain was quickly removed and placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 118 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 24 $NaHCO_3$, and 15 glucose, bubbled with 95% O_2/5% CO_2. 400 μm-thick slices of the striatum were made on a vibrating blade microtome in ice cold ACSF bubbled with 95% O_2/ 5% CO_2. The slices were maintained in a submersion chamber (ACSF and humidified 95% O_2/5% CO_2 atmosphere) at 30°C for 30 min and then at room temperature for at least 1 h before electrophysiological recording. Recordings were performed with the slices resting on a nylon mesh in a submersion chamber, maintained at 30-32°C, and perfused with ACSF bubbled with 95% O_2/5 % CO_2. Monophasic 100 μs stimulus pulses were delivered at 0.033 Hz through a bipolar stainless steel electrode, and the field population spikes (PSs) were recorded with electrodes filled with ACSF (2-4 MΩ). Waveforms were collected and analyzed using pClamp 10.3 (Molecular Devices, Foster City, CA). The test stimulation for the experiment was set at the intensity inducing 50% of maximal response. LTD was induced by a 5 min-long train of 10 Hz stimuli delivered at an intensity that induced 100% of maximal response, after the PS had been stable for at least 20 min. Recordings in dorsal striatum were obtained from the dorsolateral region,

and nucleus accumbens recordings were obtained in the area immediately surrounding the anterior commissure. LTD was defined as a decrease in PS amplitude of greater then 10% from baseline, 1 hour after stimulation. All electrophysiological recordings and data acquisition were performed by operators blinded to the parental treatment history of animals.

Table S1. THC and its metabolites are undetectable in brain and blood serum of drug-exposed female rats at the time of mating. ~400 mg brain tissue and ~2 ml serum was analyzed from each subject. All animals were carried through our standard adolescent THC treatment and THC content analysis according to protocols described in the Main Text. Tissue samples from VEH-treated and THC-treated animals were examined on the last day of treatment as negative and positive controls, respectively. VEH, vehicle; THC-OH, hydroxy-THC; THCA, tetrahydrocannabinolic acid.

Tissue	Treatment	Analysis time point post-treatment (days)	THC	THC-OH	THCA
			Concentration in brain (ng/g)		
Brain	VEH	0	<0.1	<0.1	<0.5
Brain	VEH	0	<0.1	<0.1	<0.5
Brain	VEH	0	<0.1	<0.1	<0.5
Brain	THC	0	5.08	12.77	5.78
Brain	THC	0	9.66	16.52	6.49
Brain	THC	16	<0.1	<0.1	<0.5
Brain	THC	16	<0.1	<0.1	<0.5
Brain	THC	16	<0.1	<0.1	<0.5
Brain	THC	28	<0.1	<0.1	<0.5
Brain	THC	28	<0.1	<0.1	<0.5
Brain	THC	28	<0.1	<0.1	<0.5
			Concentration in serum (ng/mL)		
Serum	VEH	0	<1	<1	<1
Serum	VEH	0	<1	<1	<1
Serum	VEH	0	<1	<1	<1
Serum	THC	0	2.96	3.50	24.50
Serum	THC	0	2.42	2.75	13.90
Serum	THC	16	<1	<1	<1
Serum	THC	16	<1	<1	<1
Serum	THC	16	<1	<1	<1
Serum	THC	28	<1	<1	<1
Serum	THC	28	<1	<1	<1
Serum	THC	28	<1	<1	<1

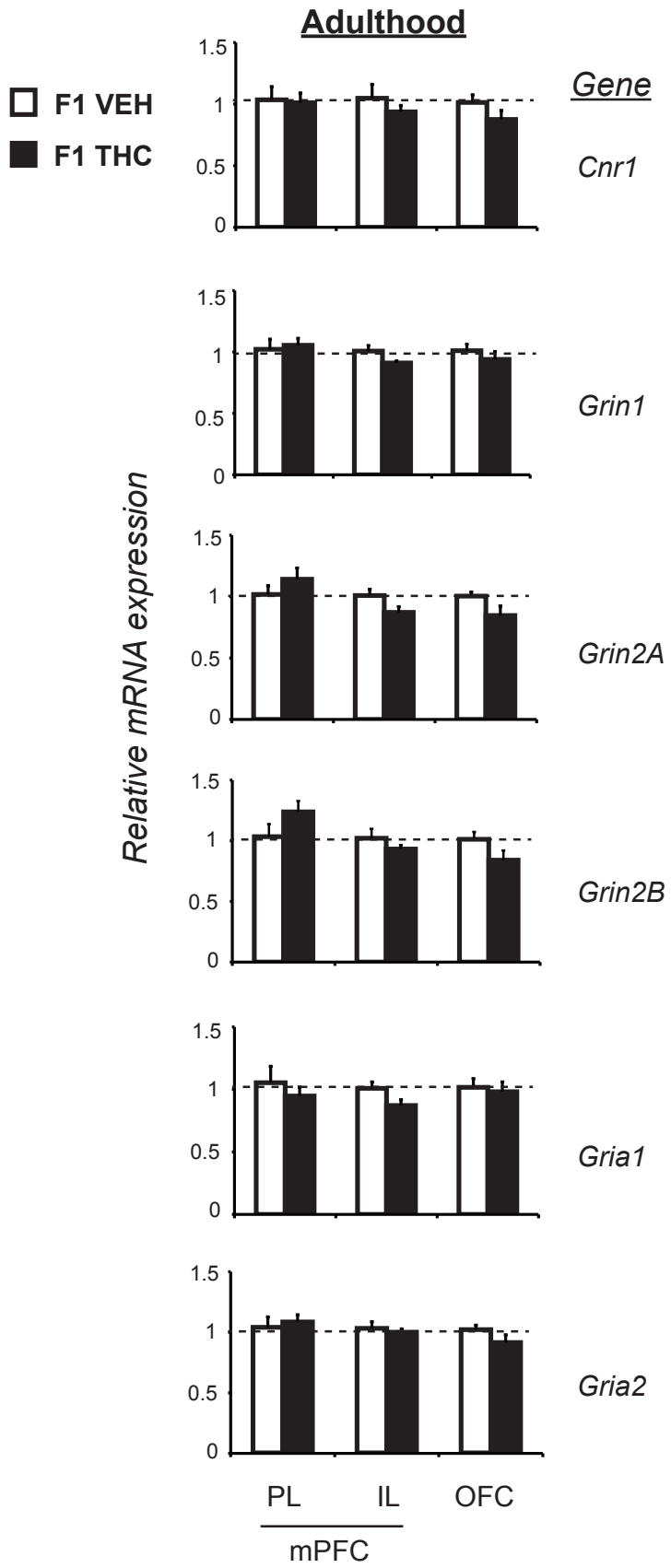

Figure S1. Cortical mRNA levels are unaffected in adult F1 offspring with parental THC exposure. mRNA levels were quantified using qRT-PCR and the ΔΔCT analysis method (see Main Text) and are expressed relative to the F1 VEH group ± SEM. N=7-8 animals/group. mPFC, medial prefrontal cortex; PL, prelimbic area; IL, infralimbic area; OFC, orbitofrontal cortex.