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Effects of chronic cannabinoid exposure during adolescence on reward preference and mPFC activation in adulthood

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Abstract

Cannabis is one of the most commonly used drugs among adolescents, with initial use beginning between the ages of 12 to 17. Although often perceived as a ‘soft drug’, both short- and long- term use have been associated with numerous adverse outcomes, including cognitive impairment, increased risk of substance abuse, and heightened risk of psychosis or schizophrenia in individuals with a predisposition. Further, the severity of these impairments is closely linked to initiation of use, i.e. earlier use increases risk. It has been suggested that adolescent vulnerability to the adverse consequences of cannabis use is due to ongoing brain development occurring during this time. Indeed, the adolescent brain continues to be remodeled well into adolescence and early adulthood, particularly in the prefrontal cortex (PFC). The medial prefrontal cortex (mPFC) has been implicated in reward processing and decision-making and alterations in mPFC development due to adolescent cannabis exposure could impair these functions. To model the effects of cannabis on mPFC function, we administered the synthetic cannabinoid WIN 55, 212–2 (WIN) to male and female rats from postnatal day 30–60. Once animals reached adulthood, we used a Probabilistic Reward (PR) choice task to elicit PFC activity and measure how patterns of activity to task-related events were modulated by adolescent WIN-treatment. Adult animals showed subtle effects of WIN-treatment on choice patterns. During task performance, mPFC activity elicited by lever press at the time of choices and reward delivery following choices were reduced in WIN-treated animals. This lasting effect of WIN suggests an impairment of the maturation of excitatory-inhibitory balance of signals in mPFC during adolescence, which may alter executive function into adulthood.

INTRODUCTION

Cannabis is one of the most commonly used drugs, with an estimated 181 million users worldwide (UNODC, 2015). Its global prevalence and popularity as a recreational drug has

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created a perception that it is less harmful than other illicit drugs; however, both short- and long- term use have been associated with numerous adverse outcomes, including memory impairment (Schweinsburg, Brown, & Tapert, 2008; Solowij et al., 2002), increased risk for addiction (Hall & Degenhardt, 2009; Lopez-Quintero et al., 2011), increased cognitive impairment (Meier et al., 2012), and heightened risk of psychosis or schizophrenia in individuals with a predisposition (Caspi et al., 2005). Additionally, the severity of these impairments is closely linked to initiation of use. Individuals who use cannabis regularly during adolescences are roughly 2–4 times more likely to exhibit symptoms of cannabis dependence within 2 years after first use (Chen, Storr, & Anthony, 2009).

Adolescents' heightened vulnerability to the adverse consequences of cannabis use are likely related to ongoing brain development during this time, particularly maturation of the endogenous cannabinoid (endocannabinoid) system. Cannabinoid receptors (CB1 and CB2) bind endogenous ligands (mainly anandamide and 2-arachidonoylglycerol) to modulate neural excitability. CB1 receptors are primarily expressed on glutamatergic and GABAergic synapses, where their activation results in suppression of either the excitatory or inhibitory outputs, respectively (Alger, 2002). During adolescence, glutamatergic inputs are selectively pruned, resulting in a net loss of nearly one-third of glutamate synapses across the brain (Insel, Miller, & Gelhard, 1990). At the same time, GABAergic synapses are largely spared from this pruning, leaving a refined network of inhibitory interneurons (Crews, He, & Hodge, 2007). Activation of CB1 receptors by exposure to cannabis during this period of refinement may disrupt functional development of those networks. Further, the endocannabinoid systems is known to mature slowly during development, with maximal CB1 receptor abundance during adolescence, thereby creating a 'window of vulnerability' when the adolescent brain is more sensitive to the effects of cannabis (Rodríguez de Fonseca, Ramos, Bonnín, & Fernández-Ruiz, 1993).

The prefrontal cortex (PFC) is particularly vulnerable to these effects. The finely tuned inhibitory balance that emerges as a result of glutamatergic synapse elimination and GABAergic synapse proliferation is particularly pronounced in the PFC (Huttenlocher, 1984), with synaptic remodeling continuing into late adolescence (Huttenlocher & Dabholkar, 1997). It has been suggested that this shift promotes the proper balance of excitation and inhibition critical for the acquisition of adult cognitive processes (Selemon, 2013).

PFC in general, and the medial subregion of prefrontal cortex (mPFC) specifically, have long been implicated in directing choices toward beneficial outcomes, with failures of such executive control strongly associated with impulsive and risky behaviors (Weafer & de Wit, 2014). Chronic activation of CB1 receptors by exogenous cannabinoids may disrupt the normal maturation of prefrontal inhibitory systems, altering cognitive and executive functions that rely on prefrontal signaling (Casey, Giedd, & Thomas, 2000). Indeed, long-term cognitive and social impairment have been consistently reported in rodents exposed to cannabis or synthetic cannabinoids during adolescence, even after 15 to 30 days without drug treatment (O'Shea, Singh, McGregor, & Mallet, 2004; Schneider & Koch, 2003, Schneider, Schömig, & Leweke, 2008).

Cannabis abuse during adolescence may alter the developmental trajectory of mPFC, potentially causing a long-term disruption of mPFC-mediated control, leading to poorer decision-making over the lifespan, even in populations that do not go on to develop such psychiatric disorders as schizophrenia or substance abuse. As such, adolescent cannabis use is poised to impair executive control of behavior across a broad population of users. To examine how perturbations of prefrontal cortex development by cannabinoid exposure during adolescence alter decision-making and its neural correlates, we recorded electrophysiologically from mPFC neurons as animals performed a Probabilistic Reward (PR) choice task following adolescent cannabinoid exposure. Adolescent rats received daily injections of WIN 55, 212–2 (WIN), a CB1/CB2 agonist, from postnatal day 30–60. We chose this developmental window to capture a broad window of adolescent development, beginning with puberty (PD 28) and ending as rats enter early adulthood (PD 63) (McCutcheon & Marinelli, 2009). Given that mPFC is known to facilitate modifications in choices based on reward outcomes, we expected adolescent WIN treatment to alter the normal development of mPFC responses to task-relevant events (cues, reward delivery) while rats performed the probabilistic reward choice task. While rats performed this task, mPFC neurons responded at the times of such task events as lever presses and receipt of reward, but showed generally suppressed in animals previously treated with WIN. This indicates that mPFC not only encodes, but also discriminates rewards to help guide behaviors toward optimal outcomes. Adolescent WIN treatment may impair this maturation, resulting in long-term alterations in decision-making.

MATERIALS AND METHODS

Animals

Male (n=17) and female (n=14) Long-Evans rats (Charles River Laboratory, Wilmington, MA) arrived in the laboratory on postnatal day 22 (PD 22). Rats were pair housed with same sex cagemates in large polycarbonate cages and provided lab chow (LabDiet 5012, Richmond, IN) and water *ad libitum* during development and surgery. During behavioral training and testing animals were moderately food restricted to no less than 90% free-fed weight. The colony was maintained on a 12-hour light/dark cycle (7:00AM–7:00PM) with behavioral testing conducted in a separate experimental room during the light phase of the cycle. Animals were treated in accordance with the guidelines put forth by the National Institutes of Health and under the approval of the Animal Care Committee of the University of Illinois at Chicago.

Estrous cycle

In female subjects, stages of the estrous cycle were determined cytologically by vaginal lavage immediately after daily behavioral testing (PD 95–110) as described previously by Goldman and colleagues (Goldman, Murr, & Cooper, 2007).

Drugs

Rats were treated with CB1/CB2 agonist WIN 55, 212–2 (WIN, Tocris Bioscience, Bristol, UK) from PD 30–60. WIN was dissolved in a mixture of 1% Tween 80 and 1% DMSO then diluted in saline (0.9%). WIN or vehicle control (VEH) was administered intraperitoneally

(i.p.) in a dose of 1.2 mg/kg. Injection volumes were 1 ml/kg and were administered at approximately 9am daily.

Behavioral testing apparatus

Behavioral testing was conducted in standard operant chambers (30.5cm × 24.0cm × 29.0cm) housed inside sound-attenuating boxes (Med Associates, St. Albans, VT). Each chamber was equipped with a house light, a central pellet dispenser for 45 mg sugar pellets (Bioserv), two retractable operant levers located on either side of the dispenser, and a cue light above each lever.

Probabilistic reward task

Animals performed a Probabilistic Reward (PR) choice task, in which they chose between small-certain and large-risky rewards by pressing a lever associated with either outcome. Before testing on the PR task, animals first underwent training beginning at PD 70 to lever press on a FR1 schedule until they met a criteria of 2 consecutive sessions with >70 presses. Animals were then trained on a daily Magnitude Discrimination (MD) task from PD 90–94, described previously (McMurray, Amodeo, & Roitman, 2015; Roitman & Roitman, 2010). In the MD task, the probability of large reward (3 sugar pellets) was fixed at 100% for large-risky lever presses. All presses of the small-certain lever resulted in delivery of 1 pellet. Following MD training, animals performed PR task from PD 95–110. In the PR task, the probability of receiving the large reward following presses of the large-risky lever varied from session to session, described below.

Each daily MD or PR session consisted of two blocks of trials: ‘forced response’ and ‘free choice’. In the first block of 24 ‘forced response’ trials, a single cue light above one lever was illuminated 2 seconds before the extension of the associated lever. For each rat, the designation of one lever as ‘small -certain’ and the other ‘large-risky’ was maintained throughout all MD and PR sessions, and counterbalanced across animals. Reward delivery following presses of the large-risky lever was determined randomly, without replacement, according to the session’s reward schedule to ensure unpredictable outcomes across a series of trials (i.e., for 33%, probability, 1 of every 3 risky choices was reinforced). During MD sessions, rats received a large reward of 3 sugar pellets for all large-risky trials. During PR sessions, rats received a large reward of 2, 3, or 6 sugar pellets for rewarded large-risky trials, depending on the session as described below, and zero for unrewarded. Small-certain lever presses were rewarded with 1 sugar pellet, 100% of the time (Figure 1A). The small-certain and large-risky levers were presented individually, in alternation, during the ‘forced response’ block.

Immediately following the forced response block, animals had a block of 76 additional ‘free choice’ trials. On each ‘free choice’ trial, the illumination of cue lights above both levers 2 seconds prior to lever extension indicated that both levers were active. Upon lever press, both cue lights extinguished and the levers retracted. In the MD sessions, all risky lever presses were reinforced with delivery of 3 sugar pellets. In the PR task, rats received a large reward of 2, 3, or 6 sugar pellets at the same probability as the ‘forced’ block for risky choices. For all certain choices in the MD and PR sessions, rats received 1 sugar pellet.

Following the completion of MD testing, all animals began the PR task with an anchor condition in which probability of receiving a large reward of 3 pellets was set at 33% to ensure that all animals' first experience with a probabilistic reward schedule was matched. The probability of receiving the large reward on subsequent sessions varied across 5 sets of contingencies, presented in random order (Figure 1B). In the three 'Fixed Size' contingencies, large-risky reward size was fixed at 3 pellets and probability of reward was 16.7, 33, or 66.7%, such that expected value (EV; $EV = \text{size} * \text{probability}$) for risky presses was 0.5, 1, or 2 pellets, respectively. Two additional 'Fixed EV' contingencies were also tested, in which the expected values of the two levers were held constant (16.7% chance of 6 sugar pellets and 50% chance of 2 sugar pellets, both $EV=1$) to discourage reliance on only reward probability for choices. Each reward contingency was repeated for two consecutive days. Preference for the risky lever was measured as the percent of large-risky lever presses during the free choice block of the second test session only to exclude trials from the session in which animals were adapting to a new reward contingency.

We used three-way ANOVA to determine if risk-preference on free choice trials depended on reward probability, sex, or treatment, followed by post hoc Tukey tests where appropriate. We also examined whether performance adapted to reward contingencies across the course of each session by comparing the first half of free-choice trials (Early) to the second half (Late) using a paired samples t-tests.

Once animals completed the PR task, they began a four-day Extinction period. They received 1 sugar pellet for certain choices and no reward for risky choices (e.g. 0% risky payoff). Animals completed this task until they meet criterion (2 consecutive days of fewer than 25% risky presses during the free choice block) or until they reached four days. We used ANOVA to determine whether pressing the risky lever (now 0%) extinction depended on day, sex, or treatment.

Surgical procedure

During performance of the PR task, we recorded the activity of individual neurons in the mPFC. To perform electrophysiological recordings, custom-designed stainless steel Teflon insulated electrode arrays (MicroProbes Inc, Gaithersburg, MD), organized into 2 columns of 4 microwires (50 μ m diameter; tip separation 0.25mm), were stereotactically guided into the mPFC (AP +3.3, ML $\pm$ 1.2 relative to bregma, and -3.5 mm at a 10 degree angle relative to skull surface) under ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.) anesthesia once animals finished magazine training (approximately PD 80). Ground wires for each array were inserted into the brain at a distal location. Connectors were anchored to the skull via stainless steel screws and dental acrylic. Arrays were implanted 1 week prior to recording, allowing animals sufficient time to recover and ensuring normal behavioral performance.

Electrophysiological recordings

Electrophysiological recordings of mPFC activity were completed during performance of the PR task in adulthood (McMurray et al., 2015). The timing of trial events, such as cue and lever presentation, lever press, and sugar pellet delivery, were output from the Med

Associates equipment and time-stamped onto the neural spike data. During recording, individual waveform statistics, including principal components and inter-spike intervals, were used to identify waveforms belonging to individual neurons (PlexControl), which were then refined offline (Offline Sorter). The data were then exported to Matlab (Mathworks) for analysis as detailed below.

Electrophysiology analysis

Neural responses were analyzed to determine how they were affected by sex, treatment, and task-related factors (choice, reward size and probability). For each neuron, we first calculated firing rate in 0.5s bins across the entire session to measure its overall mean firing rate and standard deviation. Two-way ANOVA was used to determine if the baseline firing rate (–10s to –5s pre-cue onset) depended on sex or treatment group. We then calculated each neuron's average firing rate for all rewarded free choice trials in 0.5s bins, from –10s to +15s relative to lever press. The average for each neuron was z-normalized using its overall mean firing rate and standard deviation. Across the population of PFC neurons, principal component analysis (PCA) was used to identify the patterns that best accounted for variability. Each Principal Component (PC) identified one 'Type' of response as distinguished by its pattern of activity, which does not imply any particular anatomical property. Each neuron was then assigned to one of the 5 Types by comparing its response to each of the 5 PCs and determining which one it best matched using least squares fit models.

Once Type was assigned to each neuron, we compared whether the number of units assigned to each Type differed across sex and treatment groups using Chi-square, followed by z- tests for proportions, with Bonferroni-corrected p values. For each PC, we used repeated measures ANOVA to identify time epochs during which firing rate was significantly modulated from baseline. If significant modulation occurred before the lever press, average firing rate was calculated separately for small-certain and large-risky lever presses, and three-way ANOVA was used to determine whether firing rate was modulated by sex, treatment, or lever choice. If significant modulation occurred after the lever press, average firing rate was calculated separately for each reward outcome (Certain, Risky-gain, Risky-loss), and three-way ANOVA was used to determine whether firing rate was modulated by sex, treatment, or outcome/probability/EV (depending on conditions compared). Regression analyses were used to estimate the effect of these variables on firing rate during time windows of interest.

Histological verification of recording sites

Rats were deeply anesthetized with a lethal dose of sodium pentobarbital (100mg/kg; i.p.). To mark the placement of electrode tips, a 100- μ A current was passed through each electrode of the microelectrode array for 4s. Transcardial perfusions were then performed using physiological saline followed by 3% potassium ferrocyanide in a 10% formalin solution. After post-fixing and freezing, 50 μ m coronal brain sections were taken using a cryostat and mounted. The potassium ferrocyanide reacts with deposited iron in the electrodes to reveal a blue reaction product corresponding with the location of an electrode tip. The specific position of individual electrodes was assessed by visual examination of successive coronal sections (Paxinos & Watson, 2007) (Figure 2).

RESULTS

Weight gain across WIN treatment

During WIN administration (PD 30–60), adolescent rats exhibited typical increases in body weight across days ($F = 1088.3$, $p < 0.001$) which was greater in males than females ($F = 76.654$, $p < 0.001$) (Figure 3). During this period, there were no effects of WIN treatment on weight change ($F = 1.436$, $p = 0.24$). During adulthood (PD 70–115), when behavioral testing and electrophysiological recordings were conducted, weight increased across the days of testing ($F = 463.93$, $p < 0.001$) and was greater in males than females ($F = 42.871$, $p < 0.001$). The effects of day and sex on adult weight were also modulated by treatment ($F = 4.334$, $p < 0.01$), with males showing an interaction between treatment and day ($F = 3.312$, $p < 0.05$) that was not observed in females ($F = 1.5926$, $p = 0.20$). Overall, the magnitude of the weight difference due to WIN treatment was slight ($M = 9.8\text{g}$ for males, 16.4 for females) compared to the differences between sexes ($M = 148.7\text{g}$) and across the course of the experiment ($M = 217.9\text{g}$). During the days of behavioral testing and electrophysiological recordings in adulthood, all female rats – vehicle and WIN-treated – maintained regular estrus cycles.

Behavioral effects of adolescent cannabinoid exposure

Performance on the probabilistic reward task—During adulthood, rats performed daily sessions of the probabilistic reward task in which they chose between two lever options, each associated with either small-certain or large-risky reward. Reward size and probability of reward receipt on risky choices were manipulated to alter the value of the two options. The probability of reward receipt following a risky choice was held constant during each daily session, but varied pseudo-randomly across 5 contingencies.

Before testing on the PR task, we first showed that rats preferred the large reward over the small in the magnitude discrimination task. On the last day of MD testing, there were no differences in preference for the large reward across groups (Male VEH = $76.5 \pm 4.9\%$, Male WIN = $81.5 \pm 11.8\%$, Female VEH = $75.0 \pm 8.1\%$, Female WIN = $73.8 \pm 8.9\%$; $p = .930$). Training on the MD task ensured that the reward magnitudes used in the PR task were discriminable. In these sessions, both levers resulted in reward delivery on every press, and rats continued to sample both options with a preference for the larger reward.

In the *fixed EV* sessions of the PR task (i.e. 1 pellet at 100% vs. 6 at 16.7%, 3 at 33%, or 2 at 50%), rats' choices did not depend on the probability/size of large-risky reward ($F(2,81) = 1.27$, $p = .286$). The overall average risk-preferences for 16.7, 33, and 50% sessions were 48.4 ± 0.09 , 41.86 ± 0.10 , and $54.1 \pm 0.09\%$ respectively (Supplemental Figure 1A). Risk preference depended on both sex ($F(1,81) = 5.88$, $p = .018$) and treatment ($F(1,81) = 7.27$, $p = .009$), which appears to be due to the higher preference in the female control animals compared to the other groups ($F(3,81) = 4.43$, $p = .007$). The overall difference in risk-preference across the groups did not depend on the probability of large reward (group $\times$ probability: $F(6,70) = 0.53$, $p = .782$). Further, rats did not select the large-risky lever at different rates from the first to second half of each fixed EV session ($F(1,131) = 0.06$, $p = .805$; Supplemental Figure 1B). When neither lever choice was objectively more valuable,

the pattern of choices indicated that rats choose the large- risky lever at a stable rate, roughly evenly distributed across options, which did not depend on reward size/probability nor adapt over the course of a session.

When the expected value of the two available options in the PR task differed - due to a differences in the probability of the large-risky reward with a fixed size of 3 pellets (i.e. 1 pellet at 100% vs. 3 at 16.7%, 3 at 33%, or 3 at 66.7%) - rats adapted their behavior to select the objectively more valuable option more frequently. In contrast to the fixed EV sessions, rats' risk preferences depended on the probability of *fixed size* large-risky reward ($F(2,81) = 18.0$, $p = .000$) regardless of experimental group ($F(3,81) = 1.3$, $p = .280$). Overall risk-preferences for 16.7, 33, and 66.7% sessions were 37.7 ± 0.07 , 41.86 ± 0.10 , and $70.3 \pm 0.07\%$ respectively (Figure 4A). In the fixed size sessions, risk preference on 66.7% sessions was higher than 33% ($p = .0001$) and 16.7% ($p = .0000$), but 16.7 and 33% did not differ ($p = .8381$). A trend toward higher risk preference in females did not reach statistical significance ($F(1,81) = 3.82$, $p = .055$). In addition to a main effect of the probability of large reward delivery, risk preference depended on an interaction between probability and treatment ($F(2,70) = 3.49$, $p = .036$). The lowest level of risk-preference was observed in male control animals during 16.7% sessions, and this differed from all groups' risk-preferences on 67% sessions (Tukey post-hoc, designated 'a'). The highest levels of risk-preference were observed for WIN-treated animals during the 67% sessions. Here, both male (designated 'b') and female ('c') WIN-treated animals risk-preference was higher than male and female controls, and male WIN-treated animals in the 16.7% sessions.

In addition to the different overall preferences for 16.7% and 66.7% fixed size large-risky options, behavior within these sessions showed that choices were guided by differences in reward contingencies. In these sessions, risk-preference depended on an interaction between probability and session half ($F(1,82) = 5.21$, $p = .025$). Risky lever responses were 10% lower in the second half of 16.7% sessions compared to the first ($t = -3.42$, $df = 23$, $p = .023$), but 11% higher in the second half of 66.7% sessions compared to the first ($t = 4.00$, $df = 20$, $p = .0007$, Figure 4B). As noted above, there was no change from first to second half in 33% sessions.

Across all session types – fixed EV and fixed size – animals were similarly engaged with completing the task. The number of trials completed for sessions under each contingency did not vary across the probabilities of large-risky reward delivery ($F(4,111) = .65$, $p = .630$), sex ($F(1,111) = .348$, $p = .065$), or WIN-treatment ($F(1,111) = .59$, $p = .446$). On average, rats completed 90.0 ± 1.4 trials per session (Supplemental Figure 2A). The total number of rewards earned increased by 57.47 pellets over the range probabilities tested for large-risky payoff ($F(4,111) = 40.09$, $p = .000$, Supplemental Figure 2B). Females earned 10.24 fewer pellets than males ($F(1,111) = 4.62$, $p = .034$) but there was no effect of WIN-treatment ($F(1,111) = 1.2$, $p = .277$).

Extinction—After the conclusion of probabilistic reward task test sessions, rats completed extinction sessions in which the large-risky lever was not rewarded (0%), while the small-certain continued to pay 1 pellet on 100% of choices. Over the course of extinction, the

reduction in risky lever responding was dependent on day ($F(2,55) = 37.56, p = .000$), but not sex ($F(1,55) = .41, p = .524$) or drug treatment ($F(1,55) = 2.45, p = .122$, Figure 4C).

In summary, rats' choices systematically varied with large-risky reward only when the EV's differed between the available options. Across the fixed EV sessions, rats' selected the large-risky option on 51.0% of overall trials (± 0.03). Female controls showed an elevated preference compared with the other groups, but maintained a constant level of preference across the probabilities/sizes of reward as well. Thus, in fixed EV sessions, there is not evidence that any of the task parameters caused rats to adapt their choice patterns. However, in the fixed size sessions, rats appropriately adapted their preference for the objectively more valuable option (small-certain for 16.7% and large-risky for 66.7%). There was a modest interaction between WIN-treatment and risk-preference, but behavior was most clearly controlled by the differing reward values of the two options. Thus, in the fixed size sessions, we are confident that animals were engaged in monitoring reward outcomes to direct behavior in the PR task, a function thought to elicit activity in PFC. The similarity in behavior between control and WIN-treated animals provides the opportunity to examine whether adolescent WIN-treatment affected task-related neural responses.

Patterns of medial prefrontal cortex activity during probabilistic reward task completion

Neural patterns are sensitive to lever press, reward—We recorded from 1315 neurons while rats performed the probabilistic reward task to examine how mPFC neurons respond to task-related events, and how this activity may be altered by adolescent WIN treatment. The average baseline firing rate across the population of recorded was higher in WIN treated animals ($F(1,1272) = 27.6, p = .000$), although the effect of WIN was larger in females (sex X treatment: $F(1,1269) = 30.32, p = .000$; Male VEH, $M = 3.48$ sp/s; Male WIN, $M = 4.28$ sp/s; Female VEH, $M = 3.36$ sp/s; Female WIN, $M = 4.24$ sp/s). We used principal component analysis to identify the patterns of activity (response Types) that accounted for the most variability in patterns of task-related responses across the population of neurons recorded (Figure 5). The Types capture such patterns as a sharp increase in activity at the time of lever press (Type 1), increased responding following reward delivery (Types 2 and 3), and reductions in activity following lever press (Types 4 and 5). Each neuron was categorized as one 'Type' by comparing its average response on rewarded trials to each PC and determining which it best matched using least squares fit models. The average responses for neurons assigned to each type are shown separately for rewarded and non-rewarded trials in Figure 5.

The assignment of recorded neurons to the different Types was not evenly distributed across the overall population (Chi-square = 428.39, $df = 4, p < 0.0001$), with more Type 2 and 4 neurons than expected by chance ($n = 473$ and 400, respectively), and fewer of Types 1, 3 and 5 ($n = 225, 91$, and 126). Types 1 and 2, with patterns of responses which together explained 51.7% of the variability of neural responses, were further analyzed for how they respond to different trial events. Type 2 neurons were evenly distributed across sex and treatment (Chi-square = 2.31, $df = 3, p = 0.5115$), but Type 1 units were not (Chi-square = 35.94, $df = 3, p < 0.0001$). Together, Types 3, 4, and 5 explained an additional 20.1% of variability. Neurons classified as Type 3 and 4 were not analyzed further due to a

disproportionately smaller number identified from female rats and Type 5 neurons were not further examined because they did not show differential responses between rewarded and non-rewarded trials.

For further analyses, we focused on the responses of neurons during the sessions in which large-risky reward size was fixed at 3 pellets. It was during these sessions that animals reliably adapted their choice patterns according to reward contingencies. In addition, the comparisons between small and large reward size were matched across these sessions at 1 and 3 pellets, respectively. We hypothesized that adolescent WIN treatment would result in lasting alterations in mPFC signaling driven by task-related events in awake-behaving animals, and were interested in whether these differences would depend on sex as well.

Type 1 responds at time of cue and lever press—Type 1 units showed an elevation of firing rate from baseline levels during the period preceding, and during, the lever press (−2 to +1s relative to press; indicated by black bars in Figure 6). No significant differences were observed in baseline activity of these neurons (8 to 5s preceding press) across sex ($F(1,114) = .38, p = .540$) or treatment ($F(1,114) = .4, p = .529$). During the lever press epoch, the phasic increase in activity depended on sex ($F(1,411) = 5.5, p = .0195$) and treatment ($F(1,411) = 24.98, p = .000$), with higher activity in females than males (0.666 vs 0.443 sp/s (z)), and lower activity in WIN-treated animals compared with controls (0.357 vs 0.593 sp/s (z)). The reduction in activity associated with WIN treatment was larger in females than males (sex X treatment, $F(1,408) = 10.43, p = .0013$). The magnitude of the increase in firing rate at the time of the lever press did not depend on whether the certain or risky lever was selected ($F(1,411) = 0.22, p = .640$). Thus, mPFC neurons from females showed larger transient responses than males, and while adolescent WIN treatment resulted in a long-term reduction in this response, the effect was more pronounced in females.

Type 2 neurons encode reward outcome—The activity of Type 2 neurons modulated during the period associated with reward delivery compared with baseline activity (+2.5s to +15s post lever press; indicated by black bar in Figure 7A). No significant differences were observed in baseline activity of these neurons (8 to 5s preceding press) across sex ($F(1,220) = .114, p = .061$) or treatment ($F(1,220) = .67, p = .415$). We examined both the early (+2.5 to +8s post press) and late (+8 to +15s post press) periods following reward delivery to determine if responses were modulated by reward parameters. During the early epoch (Figure 7A **insets**, darker colored bars), average activity depended on outcome ($F(1,474) = 8.99, p = .0029$), with responses highest for risky-gains, intermediate for certain rewards, and lowest for risky-losses. These initial responses did not differ across sex ($F(1,474) = 3.86, p = .0502$) or treatment group ($F(1,474) = 2.93, p = .0874$). The post-reward activity that persisted into the late epoch (Figure 7A **insets**, lighter colored bars) continued to depend on outcome ($F(1,474) = 44.13, p = .0001$), and was modulated by both sex ($F(1,467) = 9.21, p = .0025$) and treatment ($F(1,467) = 6.91, p = .0089$). Late post-reward responses were higher in females than males, and were reduced in WIN-treated animals, with a larger reduction evident in WIN-treated females.

Post-reward activity of Type 2 responses was modulated by probability of reward as well as reward size. The risky-win trials represented in Figure 6A are an average of trials in which 3

pellets were delivered at 16.7, 33.3, and 66.7% probability. During the early part of the post-reward period, the response to risky-gains, the magnitude of the neural response on risky-win trials depended on an interaction between sex, treatment and probability of win ($F(2,205) = 4.05$, $p = .0189$; dark red bars in Figure 7B). During the late part of the post-reward period, the response to risky-gains depended on the probability of reward ($F(2,216) = 6.0$, $p = .0029$), with higher magnitude responses to less frequent wins, as well as treatment ($F(1,216) = 5.53$, $p = .0196$), with reduced responding in WIN-treated rats (light red bars). This pattern of activity shows modulation of mPFC neurons that depends on reward size and probability, suggesting that the response does not depend only on the size of the reward or the amount of time needed to consume it. This representation differs between male and female animals, and, like Type 1 neurons, is reduced following adolescent WIN treatment. This reduction in responsiveness is more prominent in female rats.

DISCUSSION

Here, we measured the lasting effect of adolescent cannabinoid treatment on behaviorally-driven activity in mPFC. Using a task in which animals chose between rewards of different sizes and probabilities, we were able to evoke neural activity during ongoing choice behavior and compare how these responses were altered by prior WIN-treatment during adolescence. The subtle effects on choice behavior were coupled with blunted mPFC activation.

In the PR task, there were two types of sessions: fixed EV, in which the objective value of the two options were equal, and fixed size, in which one lever was objectively more valuable ('certain' lever > 'risky' in 16.7% and 'risky' lever > 'certain' in 66.7%). When there was an objective difference in value between levers, the lowest levels of risk-preference were observed in control males in the 16.6% sessions and the highest levels of risk-preference were observed in WIN-treated animals in the 67% sessions. During these sessions, we found that animals continued to adapt their behavioral response pattern over the course of a session. During 16.7% sessions, where the certain lever has a higher expected value, animals pressed the risky lever less frequently during the second half of the session. Similarly, animals increased risky lever presses during the second half of 66.7% sessions, where the risky lever is the more valuable choice.

The overall choice patterns suggest that animals were able to adapt behavior flexibly when the value between the options differed. WIN treatment did not impair animals' ability to flexibly respond to changes in reward contingencies, but it was associated with slightly elevated preference for the risky option across 16.7% and 66.7% sessions, perhaps indicating a greater tolerance of reward omission. In sessions with matched expected value between the two options, preference for the risky option remained stable, and choices did not adapt over the course of the sessions. It is important to note that in these fixed EV sessions, there was no optimal option, and individual animals resorted to consistent individual biases. Following the PR task, all animals successfully and similarly completed an extinction task, where the probability of reward delivery on the risky lever was 0%. Under conditions of certainty on both levers (1 vs 3 pellets in MD, or 1 vs 0 pellets in extinction), behavior was not affected by prior WIN treatment.

Behavior in the PR task has been shown to reliably drive neural activity in PFC. We used *in vivo* electrophysiology during task performance to record event-related single-unit activity in mPFC during task performance. The mPFC has long been implicated as being integral to decision-making under conditions of uncertainty by tracking outcomes to facilitate modifications in choice and behavior (Orsini, Moorman, Young, Setlow, & Floresco, 2015; St. Onge, Chiu, & Floresco, 2010). Furthermore, mPFC is known to be activated by receipt of rewards (Knutson, Fong, Adams, Varner, & Hommer, 2001; Knutson, Fong, Bennett, Adams, & Hommer, 2003; Kuhnen & Knutson, 2005), expectation of reward (Kuhnen & Knutson, 2005) and expected value of future rewards (Kable & Glimcher, 2007). Of the five dominant types of neurons our PCA revealed, we more closely examined populations that responded to key task features and were observed in all groups of animals.

Type 1 neurons were active in the seconds leading up to, and during, the lever press. Cannabinoid exposure during development has been linked to reduced transmission of GABAergic interneurons in mPFC (Cass et al., 2014; Uhlhaas & Singer, 2006), which manifests as a long-term increase in excitability. As a result, we expected to see activity that resembled that of a juvenile brain (Sturman & Moghaddam, 2011), with elevated excitability. Instead, we observed a blunted response in WIN-treated animals during lever press compared to Controls. One potential reason why we did not observe increased excitability is due to the nature of our experiment. Previous work that showed long-term excitability were achieved using ventral hippocampal stimulation in deeply anesthetized rodents (Cass et al., 2014). Here, mPFC activation was driven by task-events in awake-behaving animals, resulting in a greater complexity in inputs. In addition, our electrophysiological recordings did not allow for the biomolecular identification of the neurons belonging to this Unit Type, nor can we determine which layer of cortex we are recording from. If we were recording from pyramidal cells, and our hypothesis about CB1 stimulation reducing glutamatergic drive onto inhibitory interneurons is correct, then we should have seen increased activation. It is possible that we were recording from a more heterogenous population of mPFC cells receiving inputs from a wider range of structures, including ventral hippocampus, amygdala, and midbrain dopamine neurons.

The other dominant neural population was Type 2, which responded following reward delivery, presumably when the animal is able to process the outcome of its choice. These neurons responded according to the size and probability of delivered outcome. Following Certain presses, all groups (Male VEH, Male WIN, Female VEH, Female WIN) showed an increase in mPFC firing that persisted for several seconds. If the animal made a risky choice and was rewarded, the increase in mPFC responding was higher and persisted longer than responses to certain rewards. Lastly, if an animal pressed risky and reward was omitted, firing rate immediately returned back to baseline. These findings support previous research suggesting that mPFC is indeed sensitive to receipt of rewards (Knutson et al., 2001, 2003; Kuhnen & Knutson, 2005) and resemble findings from previous work in our lab that used a similar task during orbitofrontal cortex (OFC) recordings (McMurray et al., 2015). This work supported the well-established role of OFC in encoding the outcome of choices (during the post-press period), and our findings indicate that mPFC may perform a similar function. Indeed, previous work has indicated that a subpopulation of mPFC neurons may be outcome-selective to encode not only the receipt of reward, but also the value of it (Luk &

Wallis, 2009; Matsumoto, Suzuki, & Tanaka, 2003). The reduction in reward-driven activity may result in a less discriminable signal differentiating reward from omission. This reduction in discriminability between reward and omission may contribute to the modest increase in risk preference/reduction in avoidance of omission in WIN-treated animals.

Overall, we observed consisted reductions in task-dependent mPFC activity in animals treated with WIN in adolescence. However, these differences in activity did not translate into strong impairments in behavioral performance. Treatments such as temporary pharmacological inactivation of mPFC have been shown to result in increased preferences for risky outcomes of a larger magnitude than observed here (St. Onge & Floresco, 2010). The more muted behavioral effect in our WIN-treated animals may be attributed to fact that many of the neural structures that contribute to task performance mature earlier than PFC, and were thus potentially less affected by WIN-treatment. In addition, we selected a more moderate dose of WIN to mimic chronic, casual cannabinoid use more prevalent in typical adolescents (Monitoring the Future, 2017), rather than a higher dose associated with abuse. A consequence of this treatment regimen may be a more modest effect on behavior, which could be tested with higher doses of WIN in future studies.

A flaw of the current study is that we did not assess the influence adolescent cannabinoid exposure had on sexual development and estrous cycle prior to completion of the risk task, which occurs approximately 60 days after cycling begins. There is significant evidence that there is a relationship between the endocannabinoid system and sex hormones (Pérez-López, Chedraui, & Cuadros-López, 2010). CB1 receptors are present in large quantities in the hypothalamus and are involved in endocrine system regulation, while sex hormones modulate endocannabinoid activity and function (Pérez-López et al., 2010), as evidenced by fluctuations in CB1 receptor density across the estrous cycle (Gonzalez et al., 2000; Rodríguez de Fonseca et al., 1993). In addition to the influence of endogenous cannabinoids, exogenous cannabinoids are known to affect the production of various hormones, including gonadal steroids, growth hormones, and glucocorticoids (Brown and Dobs, 2002). To obtain a more accurate picture of the interaction between the endocannabinoid system and estrous cycle, it will be useful to begin vaginal lavage as soon as female rats reach sexual maturity and begin cycling and to correlate estrus stage with behavioral performance and neural activity more systematically.

In summary, this work demonstrates that WIN-treatment during adolescence alters the development of mPFC such that neurons show reduced responses to behaviorally-relevant events during decision making. This blunted activity may result in subtle increases in preference for uncertain rewards, but not in a manner that is consistent across sexes and probabilities. Additionally, mPFC neurons encode reward value, a role that is more traditionally ascribed to OFC. These results provide insight into the lasting, functional alterations on choice behavior resulting from adolescent cannabis use. Adolescence is period of vulnerability to cannabis, and continuing this line of research to identify the precise role cannabinoid exposure *during* adolescence in mPFC is of utmost importance. Enhancing our understanding of the lasting effects of cannabis on the developing brain is more relevant than ever before, as decriminalization and legalization changes have made it more accessible and popular.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Adolescent cannabinoid (WIN) treatment altered choice patterns in adulthood
- WIN treatment had lasting effect on responses of neurons in mPFC
- Neural responses at time of choice were blunted by adolescent cannabinoid
- Reward-responsive neurons showed reduced activity following WIN

Figure 1. (A) Task schematic for the Magnitude Discrimination and Probabilistic Reward tasks. (B) Expected Values of large-risky lever according to probability and reward size, and maximum reinforcers earned if large-risky lever were selected on all free choice trials.

Figure 2.
Location of electrophysiological recording sites. Dots indicate approximate locations of each electrode tip.

Figure 3.

Body weight over the course of the experiment. Males and females differed in weight as expected, but no differences were observed due to adolescent WIN administration. During adulthood, female weight was not affected by treatment history. In males, there was an interaction between treatment history across days. The periods of the experiment were: drug treatment (WIN), Magazine Training (MT), Surgery (S), Magnitude Discrimination (MD), Probabilistic Reward task (PR), and Extinction (E).

Figure 4.

(A) Performance during the Fixed Size contingencies of the probabilistic reward task. Animals pressed 34.8% more frequently during the 66.7% contingency than the 16.7% contingency ($p = .000$). Further analysis also indicated that behavior is affected by a probability of large risky reward ($p = .000$), with higher preference for the large-risky option on 66.7% sessions compared with 33% ($p = .0001$) and 16.7% ($p = .0000$). Behavior also depended on an interaction between probability and drug ($p = .036$; post-hoc differences marked by 'a', 'b', and 'c'). (B) Performance on the probabilistic reward task during Fixed Size contingencies across a session. During 16% sessions, animals chose the large-risky option less frequently (-10%) during the Late half of the session compared to the Early ($p = .0023$). During 66.7% sessions, animals chose the large-risky lever more ($+11\%$) frequently during the Late half of the session compared to the Early ($p = .0007$). (C) Performance during extinction testing. All animals reduced presses on the non-rewarded lever across days ($p = .000$).

Figure 5.

For each neuron recorded ($n=1315$), normalized average firing rate was calculated for all rewarded trials aligned to the time of the lever press. Principal Component Analysis was used to identify the patterns of activity that explained the variance across the population of neural responses. The first 5 Principal Components (PCs) are shown as the dashed lines, with line weight proportional to the amount of variance explained. Each neuron recorded was identified as Type 1–5 based on the PC that best accounted for its pattern of activity on rewarded trials using least squares regression. The average responses for rewarded (red) and non-rewarded (black) trials for the units assigned to each PC are plotted with the PC. The arrow on each x-axis marks the time of first sugar pellet delivery in rewarded trials.

Figure 6.

Normalized activity patterns of Type 1 units grouped by trial outcome on free-choice trials for each treatment group. Numbers in the bottom corner of each graph indicate the number of units contributing to the average. Horizontal black bar at the bottom of each panel indicates temporal period in which response rates from baseline level of activity. All data are aligned to the time of lever press/choice (time = 0). Main effect of sex ($p = .0142$) and treatment ($p = .000$) were observed (note: y-axis is different in female VEH group). There was no effect of whether the certain or risky lever was selected on neural response ($p = .640$).

Figure 7.

Normalized activity patterns of Type 2 units grouped by trial outcome on free-choice trials for each treatment group. (A) Numbers in the bottom corner of each graph indicate the number of units contributing to the average. Horizontal black bars at the bottom of each panel indicate temporal periods ('early' and 'late') in which response rates differed from baseline level. All data are aligned to the time of reward delivery (time = 0). Insets show average firing rate for each outcome during the early (darker bars) and late (lighter bars) periods of the post-reward epoch. (B) For risky-gains (3 sugar pellets delivered), responses differed by the probability of risky reward in the session. Type 2 neurons in the mPFC respond differently to reward outcomes according to both size and probability in the probabilistic reward task.