Adolescent Exposure to Cannabinoids Induces Long-Lasting Changes in the Response to Drugs of Abuse of Rat Midbrain Dopamine Neurons

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Background: *Recent studies have raised concerns about subtle long-lasting neurobiological changes that might be triggered by exposure to* Cannabis *derivatives, especially in a critical phase of brain maturation, such as puberty. The mesolimbic dopamine (DA) system, involved in the processing of drug-induced reward, is a locus of action of cannabinoids and endocannabinoids. Thus, we compared the effects of repeated cannabinoid administration in adolescent and adult rats on DA neuronal functions and responses to drugs of abuse.*

Methods: *Single-unit extracellular recordings from antidromically identified mesoaccumbens DA neurons and from their target cells in the nucleus accumbens were carried out in urethane-anesthetized rats. Animals were pretreated during adolescence or adulthood, for 3 days, with the cannabinoid agonist WIN55212.2 (WIN) or vehicle and allowed a 2-week interval.*

Results: *In cannabinoid-administered rats, DA neurons were significantly less responsive to the stimulating action of WIN, regardless of the age of pretreatment; however, in the adolescent group, but not in the adult, long-lasting cross-tolerance developed to morphine, cocaine, and amphetamine.*

Conclusions: *Our study suggests that an enduring form of neuronal adaptation occurs in DA neurons after subchronic cannabinoid intake at a young age, affecting subsequent responses to drugs of abuse.*

Key Words: Cannabinoids, dopamine, adolescence, electrophysiology, morphine, cocaine

Hemp plant derivatives marihuana and hashish are among the most widely abused illicit drugs in adolescence. This widespread use is facilitated by the fact that these drugs are generally perceived by their users as relatively harmless (Primavera and Pascal 1986). Several studies were carried out to test the hypothesis that cannabinoids, especially when consumed at a preadolescent age, might represent a primer to more harmful drug use (Kandel 1975; Kandel and Faust 1975; Kandel et al 1992; Lynskey et al 2003). These studies suggest that premature use of cannabinoids is associated with a more frequent shift toward other drugs of abuse, as compared with later usage onset. Therefore, it can be speculated that subtle but long-lasting neurobiological changes might be triggered by exposure to these compounds, especially in a critical phase of cerebral maturation. A second major concern is whether cannabinoids possess psychotomimetic or psychotogenic properties. In fact, recent epidemiologic surveys indicate that exposure to Cannabis derivatives at a premature age is associated with a higher risk of schizophrenia, depression, and anxiety, as compared with matched control subjects, represented by subjects whose drug intake started at a later age (Arseneault et al 2002; Patton et al 2002; Zammit et al 2002).

In animals, studies have provided strong evidence of long-lasting behavioral and neurochemical effects of cannabinoids. Subchronic cannabinoid administration induces behavioral sensitization (Cadoni et al 2001; Rubino et al 2001, 2003) and cross-sensitization with other drugs of abuse (Pontieri et al 2001a, 2001b). On the other hand, several studies demonstrated that chronic cannabinoid administration can rapidly induce tolerance to behavioral and biochemical effects (Breivogel et al 1999; Maldonado and Rodriguez de Fonseca 2002); however, whether the age of the animals has any influence in the instatement of behavioral tolerance, sensitization, and other residual effects has not yet been determined. It has been shown that chronic exposure of immature rats to Δ^9-tetrahydrocannabinol (Δ^9-THC) caused more long-term residual effects on different behaviors than chronic treatment of mature rats (Stiglick and Kalant 1985). Furthermore, chronic pubertal but not chronic adult treatment with cannabinoids impairs sensorimotor gating, recognition memory, and performance in progressive ratio tasks (Schneider and Koch 2003).

The receptor for cannabinoids (CB1) belongs to the G_i/G_o-protein coupled receptor family, and, in mammalian brain, is densely diffused in regions involved in the processing of rewarding stimuli, habit formation, and higher cognitive functions (Herkenham et al 1990). Endogenous cannabinoids modulate neurotransmitter release in many brain regions via CB1 receptors (Morisset and Urban 2001; Wilson and Nicoll 2001, 2002; Wilson et al 2001). Accumulating evidence indicates that their peculiar mechanism of action as retrograde messengers is able to strongly influence both short-term and long-term forms of synaptic plasticity (Freund et al 2003; Kreitzer and Regehr 2002). It is, therefore, conceivable that intake of exogenous cannabinoids, especially in vulnerable developmental periods, such as the periadolescence, might induce residual effects. These effects might be in part responsible for the alleged facilitation of psychotic illness or escalating drug abuse in selected individuals (Degenhardt and Hall 2002). Indeed, recent findings suggest that a dysregulation of the endocannabinoids system might be associated with schizophrenia: endocannabinoid levels are higher in the cerebrospinal fluid of schizophrenic patients (Leweke et al 1999), and in postmortem studies CB1 receptor density is changed in the brain of schizophrenic patients (Dean et al 2001). Interestingly, changes in the dopamine (DA) transporter in the brain of schizophrenic patients are reverted by Δ^9-THC intake

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Received February 27, 2004; revised April 27, 2004; accepted May 6, 2004.

0006-3223/04/$30.00
doi:10.1016/j.biopsych.2004.05.006

(Dean et al 2003), thus further supporting the hypothesis of a cannabinoid role in psychosis as well as an interaction between the cannabinoid and the dopaminergic systems.

The mesolimbic DA system has been shown to be involved in the processing of several aspects of natural and drug-induced reward (see Schultz 2002 for a recent review), as well as in the neuropathology of psychoses (Laruelle et al 2003). It is also a locus of action of cannabinoid-related compounds; endogenous and exogenous cannabinoids have been shown to regulate both excitatory and inhibitory synaptic inputs to DA neurons (Melis et al 2004; Szabo et al 2002) and thus to physiologically affect their firing rate and pattern (French et al 1997; Gessa et al 1998).

In rodents, periadolescence has been defined as the ontogenetic period that includes the 7–10 days preceding the onset of puberty (at age 40 days) and the first few days thereafter (Spear and Brake 1983). This developmental period is characterized by specific neurobiological and behavioral features (Laviola et al 1999; Spear 2000; Stamford 1989; Trauth et al 1999, 2001). In the rat, the DA system shows considerable plasticity during postnatal life and does not complete maturation until the late adolescence and early adulthood (Benes et al 2000). If this system exhibits similar characteristics in the human brain, adolescence might represent a time window when abnormal interactions could be induced by exposure to drugs of abuse (Smith 2003).

On these bases, we hypothesized that mesolimbic DA neurons in the ventral tegmental area (VTA) and their targets in the nucleus accumbens (NAc) would be the ideal candidates to study whether cannabinoid administration, in an age-dependent manner, is able to induce long-lasting effects on neuronal functions and responses to subsequent administration of cannabinoids and different drugs of abuse.

Methods and Materials

Male Sprague-Dawley rats (Harlan, Milan, Italy) were housed in groups of three to six in standard conditions of temperature and humidity under a 12 hours/12 hours light/dark cycle (lights on at 7:00 AM), with food and water available ad libitum. After 3–4 days of habituation to the animal room, treatments began in two groups of animals of different ages: the "adolescent" group (aged 5–6 weeks) and the "adult" group (aged 8–9 weeks). Rats in each group received intraperitoneal (IP) injections twice a day for 3 consecutive days of increasing doses of the cannabinoid agonist WIN55212.2 (WIN) (first day, 2.0 mg/kg; second day, 4.0 mg/kg; third day, 8.0 mg/kg) or an equivalent volume of vehicle. This treatment regimen was chosen because it was previously demonstrated to be able to induce cross-sensitization with heroin (Pontieri et al 2001a) without inducing an overt spontaneous withdrawal syndrome (Aceto et al 2001).

Experiments, consisting of in vivo extracellular single-unit electrophysiologic recordings, were performed in anesthetized animals 14 days after the last cannabinoid injection.

All experiments were carried out in strict accordance with the guidelines for the care and use of animals approved by the American Physiological Society and European Economic Community Council Directive of November 24, 1986 (86/609). All efforts were made to minimize pain and suffering and to reduce the number of animals used.

Animals were anesthetized with urethane (1.5 g/kg, IP). Their femoral veins were cannulated for intravenous (IV) administration of pharmacologic agents and were placed in the stereotaxic apparatus (Kopf, Tujunga, California) with their body temperature maintained at 37°C ± 1°C by a heating pad. Thereafter, the scalp was retracted, and one burr hole was drilled above the VTA (anteroposterior [AP] +2.0 mm from lambda, lateral [L] .3–.6 mm from midline) or above the medial region of the NAc (AP +1.5 mm from bregma, L .8–1.3 mm from the midline) for the placement of a recording electrode. An additional hole was drilled for the placement of a Formvar-coated stimulating stainless steel bipolar electrode (250-μm tip diameter) above the "shell" of the NAc (AP +1.5 mm from bregma, L .8–1.3 mm from the midline, ventral [V] 6.5–7.0 mm), for antidromic activation of VTA→NAc neurons, or in the ipsilateral basolateral amygdala (BLA) (AP −3.2 mm from bregma, L 5.0 mm from midline, V 7.0 mm from cortical surface) for orthodromic stimulation of NAc neurons. Structures were localized according to the stereotaxic atlas of Paxinos and Watson (1997).

Extracellular Recordings

Single-unit activity of neurons located in VTA (V: 7.2–8.0 mm from the cortical surface) or in the medial part of the NAc ("shell") (V: 5.0–7.0 mm from the cortical surface) was recorded extracellularly with glass micropipettes filled with 2% pontamine sky blue dissolved in .5 mol/L sodium acetate (impedance 2–5 MΩ). Single-unit activity was filtered (bandpass 500–5000 Hz), and individual spikes were isolated by means of a window discriminator (NeuroLog System; Digitimer, Welwyn Garden City, Hertfordshire, UK), displayed on a digital storage oscilloscope (TDS 3012; Tektronics, Beaverton, Oregon) and recorded on video cassette. Experiments were sampled on line and off line by a computer connected to CED 1401 interface (Cambridge Electronic Design, Cambridge, United Kingdom).

VTA Experiments

Recording electrodes were slowly lowered into the VTA via a micromanipulator (Narishige, Tokyo, Japan).

Once a cell was detected, the position of the microelectrode was adjusted to maximize the spike amplitude relative to background noise.

Single units were isolated and identified according to already-published criteria (Grace and Bunney 1983, 1984; Guyenet and Aghajanian 1978). Bursts were defined as the occurrence of two spikes at an interspike interval of <80 msec and terminated when the interspike interval exceeded 160 msec (Grace and Bunney 1983). Ventral tegmental area→NAc DA neurons were identified by antidromic spikes elicited by the stimulation of the shell of the NAc. An antidromic response was defined as the ability of evoked spikes to follow stimulation frequencies of >250 Hz, displaying constant latency and collision with spontaneously occurring spikes (Lipski 1981). Baseline firing rates were obtained for at least 5 min, and drugs were administered IV at exponentially increasing doses. After each dose, neuronal activity was recorded for a minimum of 2 min before a subsequent administration occurred. Only one cell was recorded per rat.

NAc Experiments

After the glass electrode had been positioned to the dorsal limit of the NAc, cells that responded to the stimulation of the BLA were searched. Stimuli (approximately 500 μA) were delivered to the BLA at 1-sec intervals while the microelectrode was lowered incrementally through the NAc. Once a cell was detected, the position of the microelectrode was adjusted to maximize the spike amplitude relative to background noise. Neurons that responded to BLA stimulation were identified by their robust excitatory response (latency range, 9–21 msec). Cells whose latency was longer than 26 msec after BLA stimulation

were not included in this study because they could exhibit a polysynaptic response component (Mulder et al 1998).

The experimental protocol was essentially that reported by Floresco et al (2001), with some modifications (Pistis et al 2002). Once a cell was isolated, stimulation currents were adjusted to half-maximal intensity, such as approximately 50% of electrical stimuli (1 Hz) in the BLA elicited an action potential in the recorded cell. Evoked spike probability was calculated by dividing the number of action potentials observed by the number of stimuli administered in 100-sec periods. Once stable levels of evoked spike probability were achieved (<10% changes over 10–15 min), drugs were administered IV, and spike probability was assessed every 100 sec. Changes in spike probability were an index of changes induced by the studied compounds over the excitation of NAc cells evoked by BLA stimulation. Only one cell was recorded per rat.

Drugs

WIN55212.2, morphine, amphetamine, and cocaine were purchased from Tocris-Cookson (Bristol, United Kingdom), S.a.l.a.r.s (Como, Italy), Sigma (Milano, Italy), and Akzo Pharmadivision Diosynth (Oss, Netherlands), respectively; SR141716A was a generous gift of Sanofi Researche (Montpellier, France). WIN55212.2 and SR141716A were emulsified in 1% Tween 80, then diluted in saline solution and sonicated. Morphine, amphetamine, and cocaine were dissolved in saline.

Histologic Assessment

At the end of each recording section, direct current (10 μA for 15 min) was passed through the recording electrode to eject pontamine sky blue, which allowed the identification of the recorded cells. Brains were removed and fixed in 8% formalin solution. The positions of the electrodes were microscopically identified on serial sections (60 μm) stained with cresyl violet.

Statistical Analysis

Drug-induced changes in spontaneous firing rate and pattern were calculated by averaging the effects for the 2 min after drug administration and normalized to the predrug baseline.

Data obtained were analyzed by two-way analysis of variance (ANOVA), one-way ANOVA, or Student *t* test, when appropriate. Post hoc multiple comparisons were made with the Dunnett test or Bonferroni test.

Results

A total of 107 DA neurons were included in this study. All neurons were identified by their well-established electrophysiologic features (see Methods and Materials) and by their antidromic response from the shell of the NAc. Additionally, all recording sites were located within the VTA, as confirmed by the histologic verification of pontamine sky blue dye. Treatment with cannabinoids and age of treatment had no effect on basal activity of DA neurons. Dopamine neurons in pooled vehicle-treated animals displayed a mean (± SD) firing rate of 2.66 ± .20 Hz (n = 47), with 15.6% ± 3.69% of action potentials fired in bursts. In pooled treated animals, these parameters were not significantly different (firing rate of 2.76 ± .18 Hz, p = .7; burst firing of 9.36% ± 1.9%, n = 60, p = .07). All cells were antidromically identified from their projection terminals in the shell of the NAc. Antidromic latency was 16.59 ± .78 msec in control animals and 16.96 ± .48 msec in cannabinoid-treated animals. These latencies are compatible with the conduction velocity of unmyelinated axons of DA neurons.

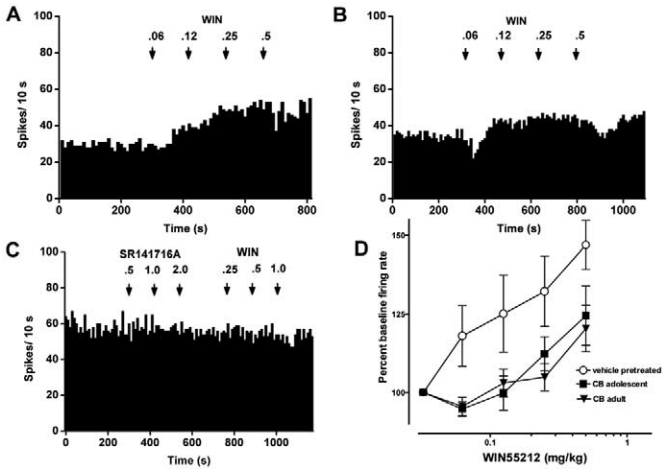

Figure 1. Long-lasting tolerance to stimulating effects of the CB1 receptor agonist WIN55212.2 (WIN) on the firing rate of mesoaccumbens dopamine (DA) neurons. Ventral tegmental area (VTA) DA neurons recorded from WIN-pretreated animals, regardless of the age of treatment, are less responsive to the stimulating action of this compound 2 weeks after last injection. Exemplificative firing rate histograms of antidromically identified DA neurons recorded from **(A)** a control animal and **(B)** an animal treated during adolescence show the effects on firing rate of systemic administration of WIN. **(C)** A representative firing rate histogram displays the lack of effect per se of the CB1 receptor antagonist SR141716A on the firing rate of a VTA DA neuron recorded from an adolescent pretreated animal. This compound, however, prevented the actions of subsequent injections of WIN. Arrows indicate time of administration; numbers above arrows indicate cumulative doses, expressed in mg/kg. **(D)** Dose–response curves displaying the effects of systemically administered cumulative doses of WIN (logarithmic scale, abscissa) on the firing rate of DA neurons recorded from the different groups of animals: rats that had vehicle injections (vehicle pretreated, n = 13), or injections of escalating doses of WIN during adolescence (CB adolescent, n = 13) or during adulthood (CB adult, n = 7). Firing rate is expressed as a percentage of baseline levels. There is a highly significant difference between treatment groups [two-way analysis of variance, F(treatment)(3,150) = 6.48, p = .0004; F(dose)(4,150) = 8.49, p < .0001; F(treatment × dose)(12,150) = .52, p = .9]. Data are expressed as mean ± SEM. CB, cannabinoids.

Consistent with previous reports (French et al 1997; Gessa et al 1998), acute administration of WIN at exponentially escalating doses (.0625–.5 mg/kg, IV) induced a dose-dependent increase in firing rate and burst firing in vehicle-treated animals (Figure 1A, D). Because no difference was observed between adolescent and adult control groups, either in the baseline firing rate (2.68 ± .22 Hz, n = 6, and 2.63 ± .33 Hz, n = 6, respectively) or in the effect of WIN, data from control animals were pooled. Maximum firing enhancement was 146.9% ± 7.7% of baseline at WIN .5 mg/kg (n = 12). Conversely, in treated animals, regardless of the age of treatment, firing rate was significantly less enhanced by acute WIN administration, as compared with control animals (Figure 1B, D), thus indicating age-independent, long-lasting tolerance. The actions of WIN were reverted or prevented by the cannabinoid receptor antagonist SR141716A (.25–2.0 mg/kg, IV), which, per se, was without significant effect, either in vehicle- or in cannabinoid-treated animals (Figure 1C, n = 3 for treated and control animals, respectively, p > .05, two-way ANOVA).

We also carried out experiments on principal efferent neurons in the shell of the NAc, the γ-aminobutyric acid (GABA)ergic medium spiny neurons, which are the target of antidromic identified DA→NAc neurons. Structures that send excitatory efferents to the NAc, such as the BLA (Katona et al 2001), the hippocampus, and the prefrontal cortex (Mailleux et al 1992;

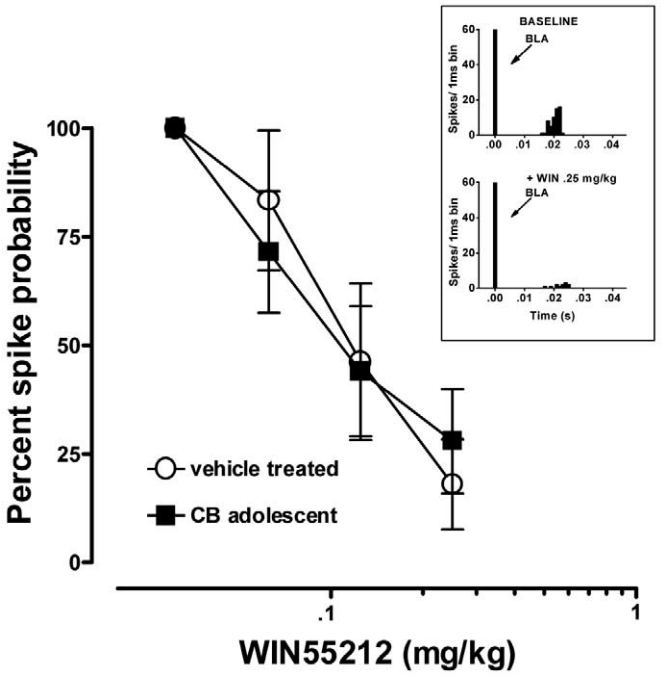

Figure 2. Cannabinoid (CB) pretreatment does not induce long-term tolerance on the excitability of neurons in the nucleus accumbens (NAc). WIN55212.2 (WIN) dose-dependently reduced the spike probability of NAc neurons, as compared with baseline, with no significant difference between groups (vehicle pretreated, $n = 6$; CB adolescent, $n = 6$) [two-way analysis of variance, F(treatment)(1,41) = .01, $p = .9$; F(dose)(3,41) = 14.78, $p < .0001$; F(treatment × dose)(3,41) = .26, $p = .85$]. Data were normalized to the baseline spike probability (100%). Inset: peristimulus time histograms display the typical response recorded from an NAc neuron to the stimulation of the basolateral amygdala (BLA). This neuron showed a baseline spike probability of .57 (57 spikes per 100 stimuli). After administration of WIN (.25 mg/kg), the spiking probability of the neuron was strongly decreased. Arrow indicates the stimulation artifacts. Data are expressed as mean ± SEM.

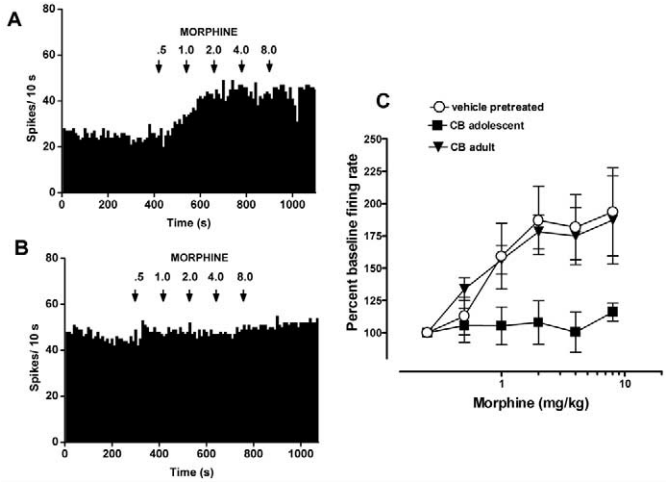

Figure 3. Cannabinoid (CB) administration during adolescence profoundly affects the responses of ventral tegmental area dopamine (DA) neurons to morphine. Typical rate histograms of antidromically identified DA neurons recorded from **(A)** a vehicle-treated rat and **(B)** a rat that received injections of cannabinoids during adolescence. Arrows indicate time of administration; numbers above arrows indicate cumulative doses, expressed in mg/kg. **(C)** Graphic depiction of the dose–response curve of intravenously administered morphine (logarithmic scale in the abscissa) and the firing rate, expressed as a percentage of baseline. There is a highly significant difference in the dose response to morphine between the control group (vehicle pretreated, $n = 9$) and the adolescent pretreated group (CB adolescent, $n = 6$) [two-way analysis of variance (ANOVA), F(treatment)(1,67) = 18.97, $p < .0001$; F(dose)(5,67) = 2.42, $p < .05$; F(treatment × dose)(5,61) = 1.76, $p = .13$]. This was not true for the difference between the adult pretreated group (CB adult, $n = 6$) and control animals, which is not significant [two-way ANOVA, F(treatment)(1,64) = 0, $p = .9$; F(dose)(5,64) = 6.07, $p = .0006$; F(treatment × dose)(5,64) = .14, $p = .98$]. Data are expressed as mean ± SEM.

Tsou et al 1998), show moderate to strong CB1 receptor levels. Nucleus accumbens neurons have a very low level of spontaneous activity; therefore, glutamatergic afferents are crucial to induce the generation of action potentials in these neurons (Pennartz et al 1994). Drugs of abuse, such as ethanol, nicotine (Criado et al 1997; Hakan and Eyl 1995; Hakan et al 1993), and cocaine (White 1990), inhibit the activity of NAc neurons, spontaneous or driven by the stimulation of excitatory afferents or glutamate microiontophoresis. Additionally, chronic cocaine elicits a long-lasting depression of excitatory synaptic transmission in the NAc, which might contribute to behavioral sensitization and addiction (Thomas et al 2001).

All neurons recorded from the NAc had a very low level of spontaneous activity (<.1 Hz) or were quiescent. Basolateral amygdala stimulation evoked firing in NAc neurons, with a mean latency of 15.8 ± 1.6 msec (range, 10.8–20.7 msec), which is compatible with a monosynaptic direct component (Mulder et al 1998). The average baseline spike probability was .46 ± .01. We previously found that cannabinoids strongly reduce firing responses of NAc neurons after electrical stimulation of the BLA (Pistis et al 2002). Accordingly, administration of .0625–.25 mg/kg IV of WIN in vehicle-treated animals dose-dependently inhibited evoked firing in NAc neurons ($n = 6$) (Figure 2). No significant difference from control animals was observed in the effect of WIN on NAc neurons recorded from cannabinoid-pretreated animals, which suggests that tolerance to cannabinoid-stimulating actions on DA neurons is not associated with changes of NAc neurons' excitability.

Several studies suggest an interaction between the cannabinoid and opioid systems in the central nervous system, especially after chronic treatment with either drug (see Discussion), which is responsible for cross-tolerance or cross-sensitization. Hence, we tested whether tolerance to acute cannabinoid administration is paralleled by a cross-tolerance to opioids on VTA DA neurons. Interestingly, acute morphine administration (.5–8.0 mg/kg, IV) had no effect on firing rate of VTA DA neurons recorded from the adolescent group within WIN-treated animals 2 weeks after the last cannabinoid administration (100.5% ± 15.6% of baseline at morphine 4.0 mg/kg, $n = 7$) (Figure 3B, C). On the other hand, morphine dose-dependently potentiated electrical activity of DA neurons in both control animals ($n = 9$) and WIN-pretreated adult animals ($n = 6$) (Figure 3A, C). The effect of cannabinoid pretreatment in the dose-response to morphine was highly significant (Figure 3C). The observation of this peculiar lack of responsiveness of DA neurons to WIN and morphine in cannabinoid-administered adolescent animals prompted us to investigate whether these neurons would display altered responses to other drugs of abuse, such as cocaine or amphetamine, which have one of their central loci of action in the DA system. It is well established that both cocaine and amphetamine inhibit DA neurons, presumably via increased somatodendritic DA acting on D2 autoreceptors (Einhorn et al 1988). Indeed, we found that in vehicle-treated animals, cocaine (.125–4.0 mg/kg, IV, $n = 6–8$, Figure 4A, C) and amphetamine (.0625–2.0 mg/kg, IV, $n =$

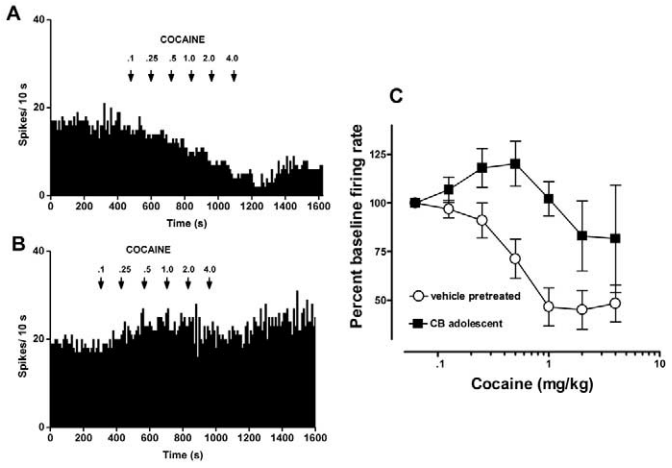

Figure 4. Pubertal cannabinoids (CB) reduce inhibitory responses of ventral tegmental area (VTA) dopamine (DA) neurons to cocaine. Representative rate histograms of antidromically identified VTA DA neurons recorded from **(A)** a rat that received vehicle injections during adolescence or **(B)** an age-matched animal that was injected with cannabinoids. Arrows indicate time of administration; numbers above arrows indicate cumulative doses, expressed in mg/kg. **(C)** Graphic depiction of the dose–response curve of intravenously administered cocaine (logarithmic scale in the abscissa) on the firing rate, expressed as a percentage of baseline. There is a highly significant difference in the dose response to cocaine between the two groups: the treated adolescent (CB adolescent, $n = 6$) and age-matched control animals (vehicle pretreated, $n = 6$) [two-way analysis of variance, F(treatment)(1,71) = 24.37, $p < .0001$; F(dose)(6,71) = 4.89, $p = .0003$; F(treatment × dose)(6,71) = 1.51, $p = .2$]. Data are expressed as mean ± SEM.

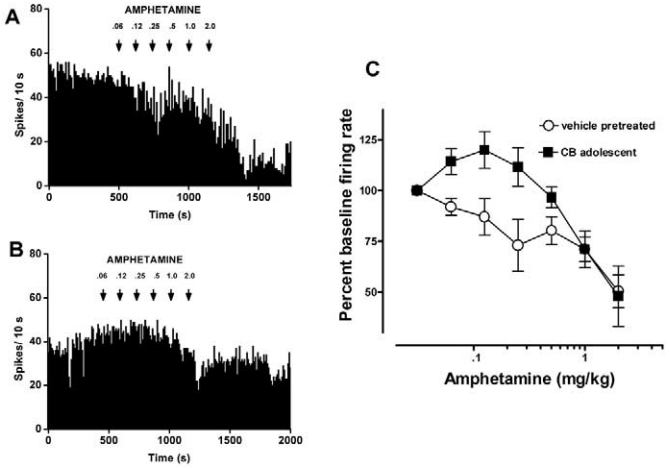

Figure 5. Adolescent cannabinoid (CB) exposure alters the response of midbrain dopamine (DA) neurons to amphetamine. Representative rate histograms of antidromically identified ventral tegmental area (VTA) DA neurons recorded from **(A)** a rat that received injection of vehicle or **(B)** cannabinoids during adolescence. Arrows indicate time of administration; numbers above arrows indicate cumulative doses, expressed in mg/kg. **(C)** Graphic depiction of the dose–response curve of intravenously administered amphetamine (logarithmic scale in the abscissa) on the firing rate, expressed as a percentage of baseline. There is a highly significant difference in the dose–response curve to amphetamine between the two groups, rats that experienced adolescent exposure to cannabinoids (CB adolescent, $n = 6$) and age-matched control animals (vehicle pretreated, $n = 9$) [two-way analysis of variance, F(treatment)(1,77) = 13.24, $p = .0005$; F(dose)(6,77) = 12.85, $p < .0001$; F(treatment × dose)(6,77) = 2.24, $p = .05$]. Data are expressed as mean ± SEM.

9, Figure 5A, C) dose-dependently inhibited firing rate of DA neurons. On the other hand, DA neurons recorded from WIN-treated animals displayed significantly less inhibition after cocaine ($n = 6$, Figure 4B, C) and amphetamine ($n = 6$, Figure 5B, C) administration, as compared with control animals. Interestingly, in these animals both cocaine and amphetamine at the lowest doses tested consistently induced a small increase in firing rate, which, however, was not statistically significant (t test, $p > .05$).

Discussion

In this report we provide evidence for a long-lasting tolerance to acute cannabinoids in VTA DA neurons after administration of a cannabinoid agonist. When administered in periadolescent juvenile rats, this tolerance was not restricted to cannabinoids, but cross-tolerance developed to other drugs of abuse (morphine, cocaine, and amphetamine).

The hypothesis of differential effects of drugs of abuse exposure during adolescence or adulthood has been poorly investigated. However, evidence suggests that when drugs of abuse are administered to immature animals, both short- and long-lasting effects are different when compared with mature animals (see Smith 2003 for a recent review). For example, alcohol exposure during adolescence has a lasting impact on sensitivity to ethanol-induced motor impairments (White et al 2002) and induces persistent electrophysiologic changes (Slawecki 2002) and long-lasting alterations in $GABA_A$ receptor functions (Grobin et al 2001). Additionally, there is rather strong evidence that the immature and mature brain are differentially vulnerable to nicotine: exposure to this drug during adolescence induces different long-term neuroadaptive and behavioral responses to nicotine than in adult animals (Schochet et al 2004; Trauth et al 2000), increases the IV self-administration of nicotine, and alters the expression of distinct subunits of nicotinic (Adriani et al 2003) or serotonin (Xu et al 2002) receptors in adult animals. Moreover, behavioral sensitization to amphetamine is increased during adolescence (Laviola et al 2001). In this framework, studies on cannabinoids are still few but consistently show augmented sensitivity to long-term behavioral effects after adolescent cannabinoid exposure (Biscaia et al 2003; Schochet et al 2004; Stiglick and Kalant 1983). To our knowledge, our study represents the first demonstration that adolescent and adult mesolimbic DA systems show subtle differential sensitivity after repeated cannabinoid intake. Indeed, cannabinoid treatment induces long-lasting changes in the behavioral responses to acute challenges of the same compounds or to other drugs (Cadoni et al 2001; Pontieri et al 2001a, 2001b; Rodriguez de Fonseca et al 1994; Rubino et al 2001, 2003). The nature of these changes, which range from tolerance to sensitization, and their underlying cellular mechanisms, however, are still not clear. Age and gender of the animals, type and dose of cannabinoids, treatment protocol, and interval before assessment are confounding factors, which vary widely among different studies and might be responsible for dissimilar results.

It is well established that during chronic cannabinoid administration, tolerance to behavioral effects of subsequent cannabinoid administrations develops rapidly (<1 week) in animals (Maldonado and Rodriguez de Fonseca 2002). This might be accompanied by downregulation of CB1 receptors or disrupted G-protein signaling (Rubino et al 1997). Accordingly, functional tolerance to cannabinoids, cross-tolerance to opioids, and blockade of long-term depression was demonstrated in NAc synapses

after chronic cannabinoid administration (Hoffman et al 2003). In the majority of the above-mentioned studies, assessment was carried out shortly (≤24 hours) after last drug injection. As a result, it is not clear how long this tolerance lasts, whether a switch to sensitization eventually occurs or, more importantly, whether the age of the animals has an influence. In fact, both Cadoni et al (2001) and Rubino et al (2001) reported behavioral sensitization to Δ^9-THC and morphine in chronic Δ^9-THC-treated animals after 14–20 days of withdrawal. Additionally, a schedule of pretreatment with WIN similar to the one used in the present study was demonstrated to induce long-lasting (14 days) cross-sensitization with heroin (Pontieri et al 2001b). Here, we observed a reduced response to WIN in DA neurons recorded from cannabinoid-treated animals 14 days after last injection. This effect is not dependent on the age of the animal and suggests that both the immature and mature DA systems are vulnerable to repeated cannabinoid administration. Indeed, acute cannabinoids, like the majority of abused drugs, stimulate the firing rate of DA neurons in vivo (French et al 1997; Gessa et al 1998) and increase extracellular DA in their terminal regions (Chen et al 1990; Tanda et al 1997, 2000). It is therefore conceivable that forms of neuronal adaptations occur within this system, which might be responsible for the observed functional tolerance. Together with the mesolimbic DA neurons, the NAc has been considered a central locus of natural and drug-induced reward. For example, excitatory synapses in the NAc are exquisitely sensitive to cannabinoid-induced inhibition, both in vivo and in vitro (Hoffman and Lupica 2001; Pistis et al 2002; Robbe et al 2001). This effect is robust after acute cannabinoid administration, and a study found tolerance shortly after chronic cannabinoid administration (Hoffman et al 2003); however, in our study, long-lasting tolerance to cannabinoids was not found within the NAc. This suggests that diverse neuronal systems, which are a target of acute cannabinoid administration, might be differentially vulnerable to long-lasting effects induced by chronic or subchronic administration.

The most intriguing finding of our study is the lack of effect by morphine on the firing rate of VTA DA neurons in animals treated during adolescence, as compared with the robust stimulating action in control animals and in the adult group. It is well established that opioids acutely potentiate DA neurotransmission by increasing the firing rate of DA neurons (Matthews and German 1984; Nowycky et al 1978) and DA release in their terminal areas (Di Chiara and Imperato 1988). A large number of studies have also demonstrated a functional interaction between the opioid and cannabinoid systems in brain reward circuits. For example, cannabinoids and opioids reciprocally influence self-administration in rodents (Fattore et a 2003; Navarro et al 2001) and monkeys (Justinova et al 2003) and modulate the expression of the withdrawal syndrome (Bhargava 1976a, 1976b; Hine et al 1975; Lichtman et al 2001; Navarro et al 1998). Other evidence supporting an interaction between opioid dependence and the cannabinoid system is that CB1 receptor knockout mice exhibit considerable decreases in both morphine self-administration and naloxone-precipitated morphine withdrawal (Ledent et al 1999); in proenkephalin knockout mice cannabinoid withdrawal is reduced (Valverde et al 2000); and μ opioid receptor knockout mice display reduced motivational properties of Δ^9-THC (Ghozland et al 2002). The mechanism of this interaction is not yet known. One possibility is that G-proteins or other downstream effectors in the intracellular cascade, common to CB1 and μ receptors, might be functionally altered after either chronic cannabinoid or opioid administration (Manzanares et al 1999). In our study, lack of response to morphine paralleled a decrease in the inhibitory effect of cocaine and amphetamine on VTA DA neurons. Cocaine and amphetamine are known to potently increase DA output both in the terminal areas of DA neurons (Hurd et al 1989; Sharp et al 1987) and in their somatodendritic regions (Kalivas and Duffy 1993). This dendritic release in turn is responsible for feedback inhibition of the DA neuron firing rate through somatodendritic D2-like DA autoreceptors (Einhorn et al 1988). Accordingly, in control animals both drugs dose-dependently decreased spontaneous firing of DA neurons. On the other hand, DA neurons from WIN-treated animals displayed considerable resistance to the inhibitory effect of these psychostimulants. The functional consequences of our findings in terms of released DA are not clear. It can be speculated that a lack of feedback inhibition on firing rate could result in enhanced neurotransmitter release in terminal regions, which can be responsible for the behavioral sensitization to amphetamine observed in cannabinoid-treated animals (Gorriti et al 1999; Lamarque et al 2001; Muschamp and Siviy 2002). However, because the latter studies were conducted on adult animals and shortly after cannabinoid abstinence, this hypothesis needs further appropriate testing.

The underlying mechanisms responsible for this multidrug tolerance are not currently known. Notably, CB1, μ, and D2 receptors share similar inhibitory G-protein systems and effectors. It is, therefore, likely that subchronic stimulation of CB1 receptors might dysregulate common intracellular cascades coupled to the activation of these diverse families of G_i-protein-coupled receptors (Yao et al 2003).

We have demonstrated that excitatory (Melis et al 2004) inputs to VTA DA neurons are modulated by both cannabinoids and endogenous cannabinoids, and this modulation contributes to functional changes of DA neuronal activity. Moreover, D2 stimulation triggers the release of endogenous cannabinoids, which dampen glutamatergic inputs to DA neurons (Melis et al 2004). Therefore, beside a direct effect of somatodendritic DA on D2 receptors, both amphetamine and cocaine might indirectly enhance the release of endocannabinoids, which act retrogradely, inhibiting glutamate release and the DA neuron firing rate. In this light, tolerance to presynaptic endocannabinoid actions might reverberate into reduced inhibitory actions by amphetamine and cocaine on the DA neuron firing rate. Therefore, it can be hypothesized that an additional mechanism by which chronic cannabinoid administration might affect DA neuronal activity is the disruption of endocannabinoid signaling and its short- and long-term regulation of synaptic inputs.

The reason for the age-dependence of the cross-tolerance to drugs of abuse remains to be established, but it might represent the result of differential sensitivity of an immature brain. The dopaminergic system undergoes extensive maturation and rearrangement until early adulthood: for example, DA innervation of terminal areas, such as the prefrontal cortex, is not completed until late adolescence in the rat (Benes et al 2000); reduced basal levels of DA and a reduced pool of readily releasable DA have been reported in periadolescent rats (Stamford 1989); and D1 and D2 receptor binding in the striatum undergoes robust changes during adolescence as a consequence of extensive pruning of dopaminergic synapses (Teicher et al 1995). Intriguingly, cannabinoids influence the expression of the neural adhesion molecule L1 in specific brain structures during the prenatal period (Gomez et al 2003). This protein plays a key role in cell

proliferation and migration, axonal elongation, and guidance, and it might also influence postnatal maturation of the DA system (Demyanenko et al 2001; Shults and Kimber 1992).

Our results add to the growing body of preclinical and clinical evidence that early exposure to drugs of abuse has atypical effects on behavioral responses or neurophysiologic and neurochemical functions (Smith 2003). The implications of our findings in relation to drug abuse have to be established in future studies. It could be speculated that cannabinoid-induced lacking, or blunted, responses of DA neurons to pharmacologic stimuli might reverberate into reduced responses to natural rewarding and motivational stimuli. In a specific time window like the adolescence, this might ultimately lead to enhanced vulnerability in selected individuals for the use of more harmful drugs of abuse.

This work was partially supported by the grants of Assessorato Igiene e Sanità (Regime Autonoma della Sardegna) and Cofin 2003 (Ministero dell'Istruzione, dell'Università e della Ricerca).

We thank Mr. Stefano Aramo for his skilled technical assistance and Dr. Le Fur (Sanofi Recherche) for the generous gift of SR141716A.

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