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Behavioral and Neurochemical Vulnerability During Adolescence in Mice: Studies with Nicotine

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People are very likely to start psychoactive drug use during adolescence, an earlier onset being associated with a higher risk of developing addiction later in life. In experiment I, *Pre-* (postnatal day (pnd) 23–35), *Mid-* (pnd 36–48), or *Post-* (pnd 49–61) adolescent mice underwent a restricted-drinking period (2 h/day for 12 days), one bottle containing water and the other containing nicotine (10 mg/l) or water. After this period, *Mid*-adolescents showed prominent exploration and reduced anxiety in the plus-maze. This ontogenetic profile was dampened by nicotine consumption. After 2 months, these mice were tested in a novel environment (30 min/day for 3 days). Locomotor-habituation profiles were specifically disrupted by nicotine consumption during *Mid*-adolescence, suggesting this age as a critical period. In experiment II, *Mid*-adolescent (pnd 35–44) and adult (pnd >70) mice were pretreated with nicotine (0, 0.03, 0.10, 0.30 mg/kg/day for 10 days). Acute nicotine administration had opposite effects on anxiety in adolescents and adults. At 2 months after pretreatment, we measured levels of AMPA GluR2/3 subunits, thought to be involved in the control of addictive behaviors. Nicotine exposure during *Mid*-adolescence dose-dependently downregulated these subunits in the striatum and hippocampus, but comparable exposure during adulthood had either opposite or no effects. NMDA NR2A/B subunits were affected by nicotine, but without age-related differences. The present data identified a nicotine-vulnerable age window, characterized by long-term disruption of locomotor habituation and downregulation of AMPA receptors. These findings support neurobiological vulnerability to drugs in adolescent humans. *Neuropsychopharmacology* (2004) **29,** 869–878, advance online publication, 10 December 2003; doi:10.1038/sj.npp.1300366

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INTRODUCTION

Adolescence is associated with increased sensation-seeking, reckless and risk-taking behaviors, low levels of harm avoidance and anxiety (Arnett, 1992; Wills *et al*, 1994). The age of first encounter with psychoactive compounds is critical, since a higher probability to shift from use to abuse and to develop addiction is associated with an early approach (Robins and Przybeck, 1985; for tobacco, see Taioli and Wynder, 1991; Anthony and Petronis, 1995; Breslau and Peterson 1996). Nearly one-third of worldwide adults are smokers, and the majority started this habit when adolescents (Mansvelder and McGehee, 2002). Adolescents also express symptoms of nicotine dependence after only few cigarettes (DiFranza *et al*, 2000).

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A suitable animal model is available for laboratory studies. Periadolescence is classically defined in rodents as the ontogenetic period including the week preceeding the onset of puberty and few days thereafter (Spear and Brake, 1983). During this period, brain areas show proliferation and maturation of axon terminals and synapses (Stamford, 1989; Teicher *et al*, 1995). Adolescent rodents show elevated levels of novelty seeking (Adriani *et al*, 1998), impulsivity (Adriani and Laviola, 2003), and risk-taking behavior (Macri' *et al*, 2002), as well as reduced response to stress (Adriani and Laviola, 2000). Oral intake of alcohol (Ferris *et al*, 1998) and nicotine (Adriani *et al*, 2002) is elevated. Recent studies indicate dramatic differences in response to nicotine between adolescent and adult rats (Faraday *et al*, 2001), enhanced vulnerability to develop nicotine addiction being found around puberty (Adriani *et al*, 2003). Experimental interest for the neural effect of adolescent nicotine exposure is growing (Trauth *et al*, 1999, 2001). Investigation of molecular consequences of early-onset nicotine addiction is important to elaborate intervention strategies, since tobacco use during adolescence could be a 'gateway' for other drugs of abuse (Kandel *et al*, 1992; Torabi *et al*, 1993).

The pathogenic process leading to addiction is so far not completely understood. The *rewarding power* of abused drugs is classically ascribed to meso-limbic dopaminergic projections (Wise, 1996; Robbins and Everitt, 2002). The onset of addictive behavior also implies alterations in the *habit-forming* process, which is thought to rely upon the nigro-striatal projections (Gerdeman *et al*, 2003). Glutamate receptors may play an important role in this regard (Gerdeman *et al*, 2003). In turn, nicotine is able to act on meso-limbic and nigro-striatal neurons that project to striatum, amygdala, and prefrontal cortex (Picciotto *et al*, 1998), thus modulating anxiety, behavioral (dis)inhibition, reward, and habit-forming processes. Nicotine may act not directly on dopamine cell bodies, but rather through glutamatergic inputs to dopamine neurons (Dajas-Bailador *et al*, 2000; Prendergast *et al*, 2001; Meyer *et al*, 2002; Mansvelder and McGehee, 2002). Recently, it was demonstrated that AMPA receptors play a key role in pavlovian approach to drug cues and in cocaine-seeking behavior in rats (DiCiano *et al*, 2001; DiCiano and Everitt, 2001; Sutton *et al*, 2003). Specifically, the glutamatergic input to the striatum might affect neuronal plasticity and habit-forming processes (Winder *et al*, 2002; Fasano and Brambilla, 2002; Gerdeman *et al*, 2003), which are key processes for the onset of drug addiction (Robbins and Everitt, 2002; Nestler, 2001, 2002). Even if molecular mechanisms are not yet clear, a pathological decrease of the AMPA glutamatergic input in the striatum might be considered an important hint for plastic changes leading to addictive habits.

The present study focused on the effects of nicotine on anxiety and exploration in the elevated plus-maze. We also investigated the role of nicotine exposure during adolescence in the long-lasting neurobehavioral changes involved in vulnerability to addiction. Different aspects of vulnerability to nicotine during adolescence were assessed, using either a behavioral or a neurobiological approach. In a first experiment, we investigated the long-term behavioral effects of spontaneous nicotine intake during three age periods of adolescence (*Pre-* (postnatal day (pnd) 23–35), *Mid-* (pnd 36–48), *Post-* (pnd 49–61)). This experiment confirmed that vulnerability is specific of *Mid*-adolescence, and is not found at younger or older ages. Hence, in a second experiment, we assessed the long-term neurochemical consequences of nicotine exposure during *Mid*-adolescence, when compared to similar levels of drug exposure in already adult subjects. We demonstrated that the neurochemical effects of nicotine differ considerably depending on the age of exposure.

Some words must be spent about the two different nicotine-exposure paradigms adopted here. Both have their merits and possible shortcomes. The experimenter-imposed drug administration is a classical pharmacological tool, aimed at assessing the drug effects *per se*, independently of animal will. However, some stress associated with the handling and injection procedures is intrinsic and cannot be avoided. Conversely, the oral self-administration approach is perhaps more close to reality, in that animals can titrate the drug intake up to their individual satiation level. However, drinking levels can differ among subjects, and hence the interpretation of results may be difficult compared to 'pure' pharmacological assessments. The aim of the present work was not to directly compare the two paradigms, rather we adopted for each experiment the approach whose features were better fitting the actual purpose. Hence, to study nicotine effects on exploration and habituation, we chose oral intake, since satiation of drug appetite might be crucial to interfere with the natural drive toward exploration and novelty. Conversely, to investigate effects on glutamate receptors, a classical pharmacology approach was preferred, all animals receiving the same drug dosage. Indeed, a disomogeneous level of drug exposure might differentially affect receptor up/downregulation in single subjects, possibly leading to inconsistent results.

MATERIALS AND METHODS

Subjects, Breeding, and Rearing Conditions

Mice of the outbred CD-1 strain, without prior breeding experience, were purchased from a commercial breeder (Charles River Italia). On arrival, mice were housed in an air-conditioned room (temperature $21 \pm 1^{\circ}C$, relative humidity $60 \pm 10\%$), with a reversed 12-h light–dark cycle (light on at 2100). Water and food (Enriched Standard Diet, Mucedola, Settimo Milanese, Italy) were available *ad libitum*. After 2 weeks, breeding pairs were formed and housed in Plexiglas cages ($33 \times 13 \times 14$ cm), with metal tops and a sawdust bedding. After 2 weeks, the male was removed, and the females were housed individually. The day of delivery was considered as pnd 0. On pnd 1, litters were culled to four males and four females.

Plus-maze Apparatus and Testing (Experiments I and II)

The elevated plus-maze (Holmes and Rodgers, 1999) had two open arms ($27 \times 5 \times 0.25$ cm) and two closed arms ($27 \times 5 \times 15$ cm) extended from a common central platform (5×5 cm). The apparatus was made in Plexiglas (black floor, transparent walls) and was elevated to a height of 40 cm above the floor level. Open-arm exploration was encouraged by the inclusion of a slight raised edge (0.25 cm) around their perimeter.

For plus-maze testing, mice were gently placed on the central platform facing a closed arm and allowed to explore freely the entire maze for a single 6-min session. To avoid unnecessary distractions, the test was carried out under dim illumination and the experimenters were not present in the testing room. After each animal was tested, the plus-maze was thoroughly cleaned with wet and dry cloths. All sessions were recorded by a video-camera (positioned above and at approx. 60° to the maze). Videotapes were then scored by a highly trained observer, blind to group assignment of animals, using an ethological software (The Observer v3.0 for DOS, Noldus Information Technology, The Netherlands). The following measures were obtained: (1) latency to enter each arm; (2) number of entries in each arm; (3) time spent in each arm.

Experiment I—Spontaneous Nicotine Consumption

Mouse pups were weaned on pnd 21, three nonsibling subjects being housed in each Plexiglas cage ($33 \times 13 \times 14$ cm), according to sex. According to a split-litter design, three same-sex sibling animals coming from

the same litter were randomly assigned to undergo the fluid-exposure period (12 days) during one of three different age periods, namely *Pre*-adolescence (pnd 24–35), *Mid*-adolescence (pnd 37–48), or *Post*-adolescence (pnd 50–61). Litters were randomly assigned to either the Nicotine group ($n=9$) or the Water group ($n=5$).

During the specific age period of exposure, animals were adapted to restricted water availability (2 h/day from 0930 to 1130) as described previously (Adriani *et al*, 2002). Food pellets were always available *ad libitum*. For the daily drinking session, animals were gently placed individually in experimental cages of the same size and shape as home cage (standard cage tops accommodated two bottles and food pellets were provided on the floor of the cage). After a 2-day acclimation period (both bottles containing water), animals within each age group underwent the fluid-exposure period (12 days). Mice from the Water group were provided with two bottles containing water, while animals from the Nicotine group had a choice between water and a nicotine solution (10 mg/l). Daily fluid and drug intake were measured for each animal (see Adriani *et al*, 2002). Following the 12-day fluid-exposure period, water was again available *ad libitum* for all animals.

On the day of their last drinking session, animals of the specific age group underwent the plus-maze test. Immediately following the end of the drinking session, animals were transported from the animal room to the testing room, left undisturbed for at least 1 h, and then scored in the plus-maze. After the test, animals were returned to the animal room. When adults, 2 months after last nicotine exposure, all animals were tested for locomotor habituation to a novel environment in a 3-day test. Mice were transported from the animal room to the testing room, and left undisturbed for at least 1 h before the locomotor-habituation test.

Locomotor-habituation Apparatus and Testing in Experiment I

Locomotor activity was measured in opaque Plexiglas rectangular boxes with smooth walls and floor ($20 \times 14 \times 27$ cm). Each box was provided with four infrared photobeams, placed on the wall a few centimeter above the floor. Beam interruptions were recorded by an IBM computer equipped with a specific custom-made software, and the *activity rate* (number of beam interruptions per second) was measured. Mice were exposed for a 30-min session on three subsequent days. Each 30-min session was automatically subdivided into 5-min intervals. The floor of the apparatus was cleaned after each animal was tested and the test was carried out under dim illumination. This procedure allowed the assessment of intra- and intersession habituation profiles of the locomotor response.

Experiment II—Experimenter-Imposed Nicotine Administration

Mouse pups were weaned on pnd 21, four same-sex pups coming from the same litter being housed in each cage ($33 \times 13 \times 14$ cm). Separate litters ($n=5$ per group) were randomly assigned to be repeatedly treated with nicotine either during adolescence (from pnd 35 to 44) or at adulthood (pnd >70). During the specific age period, the four sibling mice within each cage were injected with nicotine (0, 0.03, 0.10, and 0.30 mg/kg i.p. per day for 10 days).

On the first day of drug treatment, animals were also tested in the plus-maze for acute nicotine effects. Animals were transported from the animal room to the testing room, left undisturbed for at least 1 h, and then tested in the plus-maze 15 min after acute nicotine administration. After the plus-maze session, animals were returned to the animal room and kept in standard conditions. Then, 2 months following the 10-day nicotine treatment period, animals were killed by decapitation and brain samples were collected for neurochemical assessment.

Neurochemical Assessment in Experiment II

Brains were dissected on ice, the striatum, hippocampus, and prefrontal cortex were quickly removed from the brain and immediately stored at -80°C. Brain structures were weighed and homogenized in a 9 volume of homogenization buffer (50 mM Tris-HCl, pH 7.4, 10% sucrose, 5 mM EDTA) containing a protease-inhibitor cocktail (Sigma, USA). The homogenate was first centrifuged at 1000*g* for 10 min at 4°C to remove nuclei. Supernatants were immediately centrifuged at 34 000*g* for 20 min at 4°C. Then, the supernatants were discarded and the pellets were washed once, resuspended in 50 mM Tris-HCl, pH 7.4, assayed for protein content, using modified micro Lowry assay (Bio-RAD, USA) and stored at -80°C until analyzed.

We analyzed the levels of AMPA (GluR2/3) and NMDA (NR2A/B) glutamate-receptor subunits. Samples (10 μg/line) were diluted in Laemmli's samples buffer, loaded into precast 7.5% SDS-PAGE polyacrylamide (Bio-Rad, USA) gel at a quantity of 10 μg of total protein per line and ran at 125 V for about 1.5 h. Then proteins were transferred to Immobilon™ transfer membrane (Millipore, USA) overnight at room temperature in a Bio-Rad semidry transfer cell, using 40 mM Tris, 39 mM glycine, and 10% methanol as transfer buffer. Blots were blocked in 3% Carnation not-fat dry milk, TBS (100 mM Tris, 0.9% NaCI, pH 7.6) for 2 h. Following a brief wash with Tween-20 Tris-buffered saline (TTBS: 100 mM Tris, 0.9% NaCI, 0.05% Tween 20, pH 7.6), blots were incubated with rabbit polyclonal antibodies diluted to a concentration of 0.2 μg/ml (Chemicon, USA) for 2 h.

Blots included a range of protein standards (5–40 μg of the total solubilizate of mouse hippocampus or striatum). These standards were used to generate a protein standard curve. The linearity of the relationship between optical density and protein concentrations was verified and all samples matched to the linearity area. Rabbit anti-actin polyclonal antibodies (Chemicon, USA) were used as an additional internal control, mainly to avoid possible errors in protein concentrations. By looking at data on the actin internal standards, no evident interindividual variation emerged in sample loading within each group. Thus, actin data were not considered for correction within bands of interest.

Blots were washed three times in TTBS for 10 min each. Secondary antibodies (ImmunoPure, goat anti-rabbit, alkaline phosphatase conjugate, Pierce, USA) were applied for 1 h (dilution 1 : 5000) in TTBS. Blots were washed four

times in TTBS for 10 min each and developed in 1-Step™ NBT/BCIP substrate (Pierce, USA) during 10 min. Blots were scanned and digitized, and densitometric analysis performed using the Scion 'Image-Pro' Software (website http://www.scioncorp.com).

Drugs

All concentrations and doses are expressed as nicotine ([–]-1-methyl-2-[3-pyrityl] pyrrolidine) base. In experiment I, the drinking solutions were prepared by dissolving nicotine hydrogen tartrate (SIGMA, USA) in tap water. The drug concentration (10 mg/l) was selected on the basis of previous experience (Adriani *et al*, 2002). In experiment II, nicotine hydrogen tartrate was dissolved in saline (SAL, NaCl 0.9%). Doses were selected on the basis of literature data (Brioni *et al*, 1993; O'Neil and Brioni, 1994).

Design and Data Analysis

We adopted a split-litter design, each litter representing the statistical unit. This procedure is widely requested and adopted in studies dealing with immature animals, where sibling pups within each litter are not independent (Chiarotti *et al*, 1987). Separate analyses and *post hoc* comparisons (Tukey HSD) were performed when allowed. In experiment I, treatment was a between-litter factor, all other were within-litter factors. The general model was a 2 drug (Water *vs* Nicotine) × 2 sex (females *vs* males) × 3 age (*Pre- vs Mid- vs Post*-adolescents) model. For plus-maze latency measures, a two-level arm (closed *vs* open) factor was included in the ANOVA. For the locomotor-habituation test, the Water group was compared to the Nicotine group to assess the effects of developmental nicotine exposure. The design of the experiment included a 3 days × 6 repeated measures interaction. In experiment II, age was a between-litter factor, and all other were within-litter factors. The general model was a 2 age (adolescent *vs* adult) × 2 sex (female *vs* male) × 4 dose (0, 0.03, 0.1, 0.3 mg/kg) × repeated measures on the same subject.

RESULTS

Experiment I—Spontaneous Nicotine Consumption

Nicotine consumption. During the drug-exposure period, animals belonging to *Pre-*, *Mid-* or *Post*-adolescence age groups showed a mean daily nicotine intake of 1.39 ± 0.02 mg/kg (see also Adriani *et al*, 2002).

Latency to enter the arms. A main effect of Arm, $F_{(1,12)} = 48.7$, $p < 0.01$, was found, confirming that mice entered the closed arms with a shorter latency, when compared to the open arms. Age-related changes were evident, the Age by Arm interaction, $F_{(1,12)} = 5.04$, $p < 0.01$, and the Age by Arm by Drug interaction, $F_{(2,24)} = 5.58$, $p < 0.01$, being significant. As a whole, no significant or reliable changes emerged for latencies toward closed arms, whereas huge differences characterized the open arm. In particular, as shown in Figure 1 (upper left panel), *Mid*-adolescents from the Water group entered the open arms with a significantly shorter latency than subjects from the other ages ($p < 0.05$). This ontogenetic profile was profoundly altered in the Nicotine group (see Figure 1, upper right panel). In fact, *Mid*-adolescents that spontaneously self-administered nicotine showed a significantly higher latency to enter the open arm, when compared to their same-age controls ($p < 0.05$). Further, *Pre*-adolescents from the Nicotine group entered the open arm with a shorter latency than same-age controls ($p < 0.05$). The oral self-administration of nicotine did not affect the performance in the *Post*-adolescent group. Such a lack of effects could possibly reflect the development of tolerance due to chronic drug exposure.

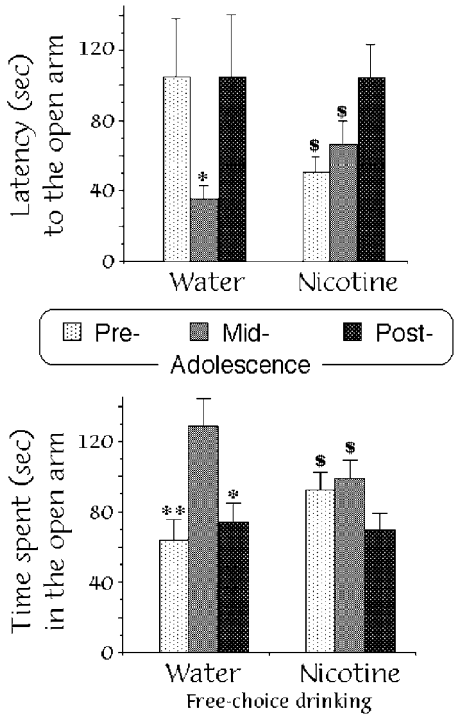

Figure 1 Upper panel: Mean (SEM) latency (s) to the first approach to the open arms. Lower panel: Mean (SEM) time (s) spent in the open arm (Experiment I). Mice during *Pre-*, *Mid-* or *Post*-adolescence underwent 12 days of free-choice drinking sessions, one bottle containing water and the other containing nicotine ($n = 18$) or water ($n = 10$). Mice were tested in the plus-maze at least 1 h after their last drinking session. In the absence of any effect of the sex variable, data from males and females have been collapsed. $^{*}p < 0.05$, $^{**}p < 0.01$ in multiple comparisons between age groups. $^{\$}p < 0.05$ in multiple comparisons between the Nicotine group and the Water control.

Time spent in open arms. As expected, animals spent in general about two-fold more time in the closed than in the open arms (data not shown). As for time spent in the open arm, a significant main effect of Age was found in the ANOVA, $F_{(2,24)} = 7.87$, $p < 0.01$, together with a significant Age by Drug interaction, $F_{(2,24)} = 3.24$, $p < 0.05$. An ontogenetic profile emerged within the Water group (see Figure 1, lower panel), with *Mid*-adolescents spending a larger amount of time in the unprotected area of the apparatus, when compared to animals from the other age groups ($p < 0.05$). Interestingly, age-related differences were no more evident within the Nicotine group (see Figure 1, lower panel), the amount of time spent by *Mid*-adolescents in the open arms of the maze being strongly reduced by the

oral self-administration of nicotine ($p<0.05$). Further, *Pre*-adolescents from the Nicotine group spent slightly more time in the open arm than controls ($p<0.05$). In other words, the characteristic age-related profile, which was consistent with previous work in our lab (Macri' *et al*, 2002), was actually dampened in animals allowed to drink nicotine.

Percent open-arm entries. Since the number of entries into each part of the apparatus is strictly related to levels of locomotor activity (ie an animal scoring high activity rates might enter each arm more frequently than another scoring lower), the open-arm entries were divided for the total number of entries. The profile of results for this variable was nicely overlapping to that described for time spent in the open arm (data not shown). Indeed, a main effect of Age, $F_{(2,24)}=7.97$, $p<0.01$, and an Age by Drug interaction, $F_{(2,24)}=5.55$, $p<0.01$, was found. An elevated proportion of open-arm entries was shown by *Mid*-adolescents within the control Water group, when compared to younger and older subjects. Again, a dramatic reduction of this parameter was exhibited by *Mid*-adolescents from the Nicotine group, when compared to their same-age controls ($p<0.05$). Hence, the regular ontogenetic profile was dramatically dampened in the Nicotine group.

Frequency of closed-arm entries. The number of closed-arm entries has been suggested as the best measure for locomotor activity in the plus-maze paradigm. No reliable or significant effects of Age and Drug variables were found (data not shown).

Body weight. As expected, body weight differed depending on the sex, $F_{(1,12)}=100$, $p<0.01$, and the age, $F_{(2,24)}=61.3$, $p<0.01$, of the subjects. No carry-over effects of prior drug intake were found on this measure (data not shown).

Locomotor Activity and Habituation Profiles in Experiment I

Long-term effect of nicotine exposure during adolescence. The ANOVA yielded a main effect of Day, $F_{(3,36)}=4.39$, $p<0.01$, and Time, $F_{(5,60)}=8.64$, $p<0.01$. Specifically, adult mice showed a clearcut intra- and intersession habituation profiles (see Figure 2). Interestingly, these profiles were also a function of the developmental age of drug exposure, a significant three-way Age by Day by Time interaction, $F_{(30,360)}=8.64$, $p<0.01$, being found. To better depict this interaction, separate analyses were performed within each age group. For *Pre*- and *Post*-adolescents, a main effect of Time, $F_{(5,60)}=20.3$ and 11.9, respectively, $p<0.01$, confirmed the presence of a clearcut within-session habituation profile. No carry-over effects of nicotine exposure emerged, although a nonsignificant tendency toward lower activity rates was apparent for mice exposed to nicotine when *Post*-adolescents. Interestingly, within *Mid*-adolescents, the Drug by Day interaction, $F_{(3,36)}=4.27$, $p<0.05$, and the Drug by Time interaction, $F_{(5,60)}=8.64$, $p<0.01$, were significant. Specifically, in mice from the control Water group, locomotor activity decreased during the course of the session and over subsequent days. Conversely, such a classical habituation profile was not evident at all in adult mice that were exposed to nicotine during *Mid*-adolescence. Rather, the latter subjects exhibited a clear deficit of habituation and were associated with abnormally elevated activity levels, when compared to controls (see Figure 2).

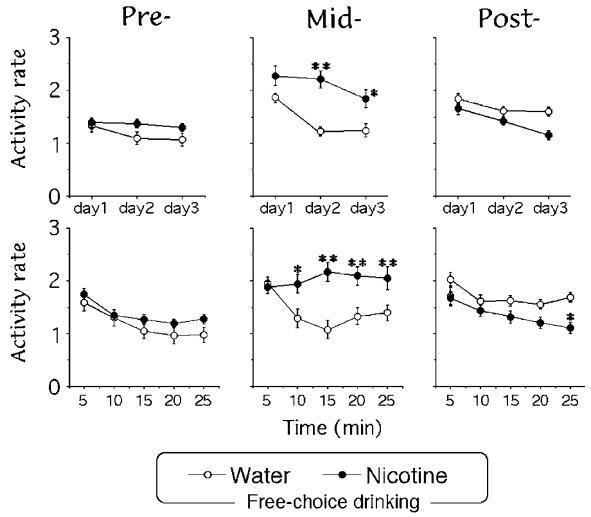

Figure 2 Mean (SEM) activity rate (number of beam interruptions per second) shown in the test for locomotor response to novelty (Experiment I). Mice during *Pre*-, *Mid*- or *Post*-adolescence underwent 12 days of free-choice drinking sessions, one bottle containing water and the other containing nicotine ($n=18$) or water ($n=10$). After a 2-month nicotine-free period, mice were tested for profiles of intersession (upper panels) and intrasession (lower panels) locomotor habituation, following exposure to a novel environment (a 30-min session for 3 days). In the absence of any effect of the sex variable, data from males and females have been collapsed. Multiple comparisons: $^{*}p<0.05$, $^{**}p<0.01$ between Water and Nicotine groups.

As a whole, these results revealed that both the intra- and intersession habituation profiles were specifically disrupted at adulthood by nicotine exposure during *Mid*-adolescence. Comparable exposure during other periods of adolescence was completely devoid of a similar effect (or tended to produce an opposite effect in the case of *Post*-adolescents). These findings may be interpreted as evidence of a narrow window of peculiar vulnerability to nicotine, as far as its long-term sequels are concerned.

Experiment II—Experimenter-imposed Nicotine Administration

Latency to enter the open arm. Data regarding the latency to enter closed arms were not included in the analysis, with all values being very close to zero. The ANOVA yielded a main effect of Age, $F_{(1,27)}=10.3$, $p<0.01$, indicating that adults entered the open arm with a higher latency than adolescents (92 ± 13 *vs* 39 ± 8 s, respectively). No significant or reliable effects of other variables were found.

Time spent in the open arms. As expected, time spent in the closed arms was about twice the time spent in the open arms (data not shown). As for time spent in open arms, the ANOVA was significant for the Age factor, $F_{(1,30)}=10.2$, $p<0.01$, and for the Age by Dose interaction, $F(3,30)=4.02$, $p<0.05$. Specifically, adult animals injected with nicotine

0.10 mg/kg spent much more time in the open arms, when compared to controls (see Figure 3, left panel). Conversely, upon the same dosage, adolescent animals tended to show a reduction of time spent in the open arm. *Post hoc* analysis revealed a significant difference in the response to the 0.10 mg/kg dose between the two ages ($p<0.05$). In contrast, no differences emerged at all other dosages.

Percent open arm entries. The ANOVA was significant for the Age factor, $F_{(1,30)}=10.2$, $p<0.01$, and for the Age by Dose interaction, $F_{(3,30)}=3.56$, $p<0.05$. Specifically, adult animals injected with nicotine 0.10 mg/kg showed much more open arms entries, when compared to controls (see Figure 3, right panel). Conversely, upon the same dosage, adolescent animals showed a tendency toward a reduction of open arm entries. *Post hoc* analysis revealed a significant difference between adolescents and adults only upon the 0.10 mg/kg dose. Again, the two ages did not differ at all other doses.

Frequency of closed arm entries. The ANOVA was significant for the Age factor, $F_{(1,30)}=5.35$, $p<0.05$, suggesting that adult animals showed much less closed arm entries, when compared to adolescents (see Table 1). However, this age-related profile was blunted by nicotine administration, since closed arm entries were somewhat increased in adolescent animals and conversely decreased in adults. Indeed, *post hoc* analysis confirmed a significant difference between ages only within the control SAL-injected group ($p<0.05$).

Neurochemical Assessment of Glutamate Receptors in Experiment II

Data revealed that even a short period (10 day) of nicotine administration produces long-lasting changes of immunoreactivity for AMPA glutamate receptors in the striatum and hippocampus of mice. These alterations were age- and dose-dependent. As for striatum, the ANOVA revealed a main Age effect, $F_{(1,15)}=8.63$, $p<0.01$, and a Drug by Age interaction, $F_{(3,45)}=19.5$, $p<0.001$. The immunoreactivity of GluR2/3 subunits of AMPA glutamate receptor dose-dependently decreased in the striatum of mice exposed when adolescent (Figure 4, upper right panel), whereas in the brain of animals exposed when adult exactly the opposite effect was observed (Figure 4, lower right panel). As for hippocampus, the ANOVA revealed a main Drug effect, $F_{(3,45)}=11.5$, $p<0.001$, and a Drug by Age interaction, $F_{(3,45)}=6.48$, $p<0.01$. In the hippocampus of mice exposed when adolescent, GluR2/3 immunoreactivity decreased in a dose-dependent manner (Figure 4, upper left panel). Animals exposed at adulthood demonstrated no significant changes in hippocampal GluR2/3 levels (Figure 4, lower left panel).

As for the NR2A/B subunit of NMDA receptors in the striatum, the ANOVA yielded a main effect of Age, $F_{(1,15)}=251$, $p<0.001$. At both ages, nicotine exposure induced a decrease of NR2A/B immunoreactivity for females and conversely no effect for males, Drug × Sex interactions, $F_{(3,24)}=3.63$ and $F_{(3,21)}=3.20$ for adult and adolescent exposure, $p<0.05$. However, this dose-dependent effect was similar at both ages. Hence, we may conclude that nicotine effects on NMDA receptors were gender-specific (see Table 2) but not age-dependent. Similarly, no reliable or significant differences due to sex or age of exposure were found for levels of NR2A/B immunoreactivity in the hippocampus.

Assessment of neurochemical parameters in the prefrontal cortex did not reveal any significant or reliable nicotine-induced change in either GluR2/3 or NR2A/B immunoreactivity. All these data confirm that NMDA receptors do not seem to play a significant role in age-related vulnerability to nicotine.

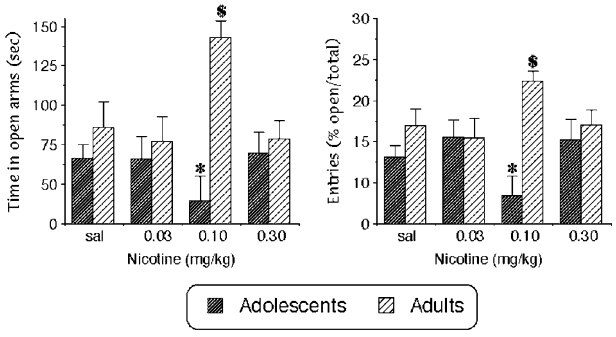

Figure 3 Left panel: Mean (SEM) time (s) spent in the open arm. Right panel: Mean (SEM) entries (% of total) in the open arm. Adolescent (pnd 35) and adult (pnd >70) mice were tested in the plus-maze 15 min after acute nicotine injection (Experiment II). In the absence of any effect of the sex variable, data from males and females have been collapsed ($n=10$). *$p<0.05$ in multiple comparisons between age groups. \$$p<0.05$ in multiple comparisons between a given nicotine dose and the SAL-injected control.

DISCUSSION

Data from the present study can be summarized as follows:

(1) Mice at *Mid*-adolescence showed low anxiety with an elevated exploratory drive, which was dampened after 12 days of spontaneous nicotine consumption. After 2 months, the regular profile of locomotor habituation in response to novelty was markedly disrupted by nicotine exposure. This change was specific for *Mid*-adolescent animals.

Table 1 Mean (±SEM) Frequency of Closed-arm Entries in the Plus-maze, as a Function of Age and Acute Nicotine Treatment in Experiment II

Nicotine dose (mg/kg)	Adolescents (pnd 35)	Adults (pnd >70)
SAL	22.3±2.0*	11.9±1.0
NIC 0.03	16.4±0.9	15.9±2.0
NIC 0.10	18.0±2.3	16.8±1.4
NIC 0.30	18.1±1.1	15.3±1.4

In the absence of significant or reliable gender differences, the two sexes have been pooled ($n=10$). *$p<0.05$ in *post hoc* comparisons between ages.

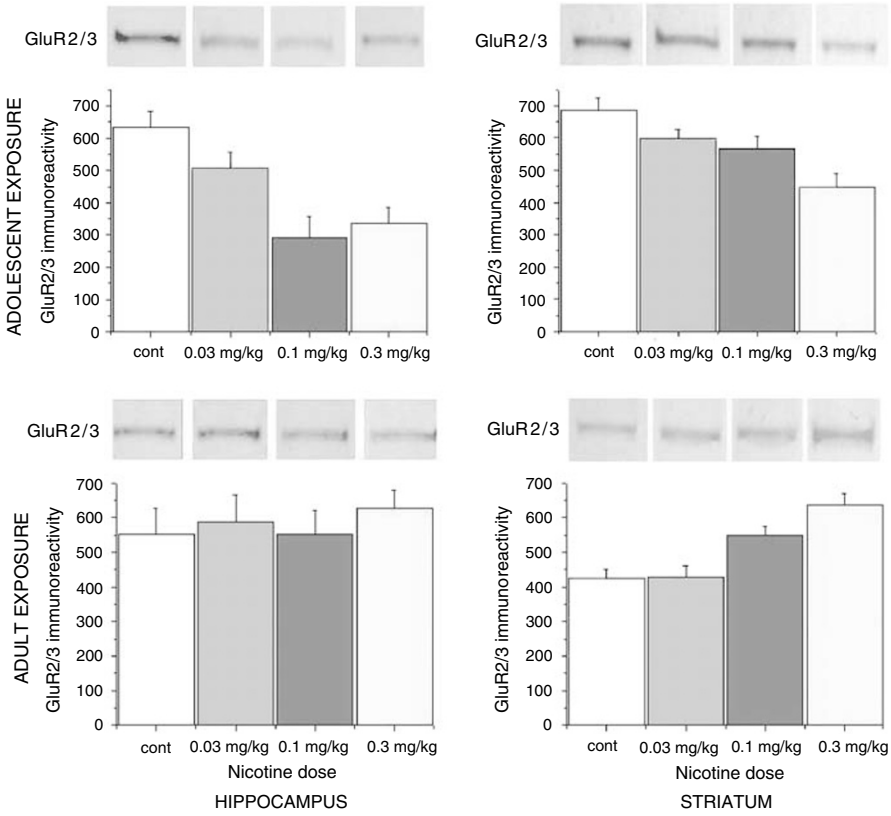

Figure 4 Optical density of the GluR2/3 subunit of AMPA receptors in the Western blots from striatum or hippocampus. Mice underwent 10 days of nicotine treatment during *Mid*-adolescence (pnd 35–44) or when adult (pnd >70). Animals were then killed 2 months after last drug injection (Experiment II). In the absence of any effect of the sex variable, data from males and females have been collapsed ($n = 10$).

Table 2 Mean (±SEM) Optical Density of the NR2A/B Subunit of NMDA Receptors in the Western Blots from Striatum of Mice from Experiment II

Nicotine dose (mg/kg)	Females	Males
SAL	620±47	672±47
NIC 0.03	497±58	520±81
NIC 0.10	337±40$	589±37*
NIC 0.30	280±56 $	564±61*

The two ages have been pooled ($n = 10$). * $p < 0.05$ in *post hoc* comparisons between sexes. $ $p < 0.05$ in *post hoc* comparisons between drug-exposed group and saline control.

(2) Acute nicotine administration (at the dose of 0.10 mg/kg) reduced anxiety in adults, whereas it tended to have an opposite effect in adolescent animals. A dose-dependent reduction of GluR2/3 immunoreactivity was found in the striatum and hippocampus 2 months after a pretreatment with nicotine during *Mid*-adolescence. A comparable pretreatment, but given in already adult subjects, dose-dependently increased striatal GluR2/3 receptors and had no effect in the hippocampus.

The present study was designed to characterize the spontaneous behavior expressed by mice at different phases of adolescence, as well as the effects of nicotine. The plus-maze test can provide information not only on anxiety (Brioni *et al*, 1993; O'Neil and Brioni, 1994; File *et al*, 1998) but also on the motivation to explore despite risky conditions and hence on risk-taking behavior (Holmes and Rodgers, 1999; Macri' *et al*, 2002). Classically, a clearcut avoidance for the open arms is reported at adulthood for both rats and mice (File *et al*, 1998; Holmes and Rodgers, 1999). Interestingly, an age-related discontinuity was revealed in Experiment I. Namely, mice showed elevated preference for the open and unprotected areas at *Mid*-adolescence (pnd 48), when compared to younger (pnd 35) and older (pnd 61) ages. Accordingly, these two latter age groups did not show differences when tested in Experiment II. Data from these two experiments confirm previous findings, suggesting an enhanced exploratory drive and/or reduced anxiety associated with risky situations during *Mid*-adolescence (Macri' *et al*, 2002; see Laviola *et al*, 2003).

Consistent with other reports in mice (Brioni *et al*, 1993; O'Neil and Brioni, 1994), the acute administration of nicotine (at the specific dose of 0.10 mg/kg) reduced anxiety in adults. Conversely, an opposite (ie anxiogenic) effect tended to appear in 35-day-old adolescent animals following acute nicotine administration (experiment II). In apparent contrast with a recent report in rats (Cheeta *et al*, 2001), no reliable sex differences apeared in the present mouse study. This discrepancy may perhaps be accounted for by a number of factors, including the animal species and experimental procedure. A prolonged 12-day period of spontaneous nicotine consumption during *Mid*-adolescence

strongly reduced the time spent in the open and unprotected areas of the plus-maze (experiment I). Noteworthy is the fact that the ontogenetic profile of *Mid*- adolescents, consisting of high exploratory drive and low anxiety, was abated by nicotine consumption. In other words, the possibility to self-administer nicotine during the peculiar phase of adolescence (otherwise characterized by novelty-seeking and risk-taking behaviors; see Laviola *et al*, 2003) produced more anxious animals, less prone to take risks. Due to nicotine drinking, *Mid*-adolescent mice possibly experienced an altered development of coping strategies toward novelty and risk, and this could lead to more persistent behavioral changes into adulthood.

In fact, the regular response of adult mice to novelty (ie locomotor habituation) was markedly disrupted, when measured 2 months after spontaneous nicotine consumption during *Mid*-adolescence. The consequences of nicotine exposure were highly age-specific, since nicotine exposure did not affect habituation in subjects consuming nicotine at younger or older ages. Rather, the effect (if any) of later drug exposure was in the opposite direction. Enhanced locomotor response to novelty is a largely validated index of vulnerability to the addictive properties of psychostimulants (Wise and Bozarth, 1987; Piazza *et al*, 1991). The present findings suggest that spontaneous consumption of nicotine during *Mid*-adolescence might be associated with a drug-vulnerable phenotype at adulthood. This would be highly consistent with very recent findings obtained in rats, in that nicotine exposure during adolescence was responsible for an increased vulnerability to develop nicotine addiction (Adriani *et al*, 2003), when compared to comparable exposure at adulthood. Altogether, these data obtained in animal models support the epidemiological reports of increased risk toward addiction in early-onset smokers (Taioli and Wynder, 1991; Breslau and Peterson 1996).

Nicotine Effects on Glutamate-receptor Subunits

In the present study, we demonstrated that even a short period of nicotine administration drastically changed the glutamate-receptor immunoreactivity in the striatum and hippocampus of mice, when measured 2 months later. As for NMDA receptors, long-term nicotine effects were sexually dimorphic, but no age-related differences were found. It is well known that the behavioral and neurochemical effects of NMDA-pathway activation differ between sexes (D'Souza *et al*, 1999). In agreement with the present data, a prolonged treatment with nicotine is reported to decrease NMDA-receptor density, but only under certain conditions of NMDA-receptor activation (Zhang *et al*, 1994). Hence, differences in basal function of NMDA system in males and females may perhaps account for the present data.

As for AMPA receptors, nicotine-induced alterations interacted with age of exposure and were dose-dependent. Specifically, a repeated nicotine treatment during adolescence was found to reduce striatal as well as hippocampal levels of the GluR2/3 subunit of the AMPA glutamate receptor. Conversely, comparable nicotine treatment, but given when mice were adult, had no effect or enhanced these levels. These results may provide an insight into the neurobiological mechanisms responsible for adolescents' vulnerability to addictive drugs. It is important to note that adolescent and adult mice had opposite patterns of GluR2/3 immunoreactivity in response to nicotine administration. These observations may prove the existence of different molecular mechanisms leading to opposite effects on the risk of developing nicotine addiction for adolescent and adult animals.

There is some evidence in the literature that glutamatergic pathways are involved in nicotine's mechanism of action (Wise, 1996; Mansvelder and McGehee, 2002). The focal *in vivo* administration of an NMDA-receptor antagonist within the VTA inhibits the nicotine-induced increases of DA release within the ventral striatum, suggesting nicotinic modulation of glutamatergic transmission (Schilstrom *et al*, 1998). AMPA and NMDA receptors modulate the locomotor activity induced by amphetamine in mice (Burns *et al*, 1994). Interestingly, AMPA glutamate receptors seem to be specifically involved in drug-seeking and vulnerability to drug addiction, in that increased levels of AMPA-receptor subunits were correlated to extinction during self-administration experiments (Sutton *et al*, 2003). In particular, the overexpression of AMPA-receptor subunits in the ventral striatum facilitates the extinction of cocaine-seeking responses, and conversely extinction from chronic cocaine self-administration induces experience-dependent increases in the subunits of AMPA receptors in the ventral striatum (Sutton *et al*, 2003). Taking into account these evidences, it seems possible to suggest that nicotine exposure may affect drug-reward and addictive habit-forming processes by modulating the striatal level of AMPA receptors. In other words, the glutamatergic control over synaptic plasticity within the striatum might be strongly involved in the development of addictive habits and compulsive-intake behaviors (Winder *et al*, 2002; Fasano and Brambilla, 2002; Gerdeman *et al*, 2003). The latter have been strongly involved in the development of addiction (Nestler, 2001, 2002; Robbins and Everitt, 2002). Noteworthy is the fact that mice exposed to nicotine during *Mid*-adolescence or when they were already adult demonstrate quite an opposite picture for AMPA immunoreactivity and hence in terms of behavioral vulnerability.

CONCLUSION

Addiction is thought to involve persistent neurobiological changes that facilitate relapse to drug use despite efforts to abstain. Epidemiological evidence warned us that a greater risk of developing addiction is associated with an early onset of drug use (see Introduction). However, the etiopathological mechanisms are so far poorly understood. Nicotine exposure is well known to produce long-term consequences on forebrain systems (for a review, see Balfour *et al*, 1998). Yet, only recently a growing interest became evident about the fact that neurobehavioral effects of nicotine exposure during adolescence differ from comparable exposure in adult subjects (Trauth *et al*, 1999, 2001; Adriani *et al*, 2003).

The present results provide a neurobiological basis for increased vulnerability to nicotine in the still plastic brain of adolescents. We found: (1) a specific age window for

nicotine-induced disruption of the locomotor response to novelty (a validated index of enhanced vulnerability to drugs); and (2) an opposite pattern of dose-dependent changes in glutamate-receptor immunoreactivity as a consequence of nicotine exposure during either adolescence or adulthood. According to literature (DiCiano and Everitt, 2001; DiCiano *et al*, 2001; Sutton *et al*, 2003), the up- or downregulation of AMPA receptors may suggest a better or worsened control, respectively, over pavlovian approach to drug-conditioned cues and over drug-seeking behavior. Nicotine pretreatment in *Mid*-adolescent mice, but not comparable exposure at adulthood, produced a profound and long-lasting downregulation of the AMPA-receptor pathway. This change could alter intrastriatal plasticity and habit-forming processes (Winder *et al*, 2002; Fasano and Brambilla, 2002; Gerdeman *et al*, 2003), which have been involved in potential vulnerability to addiction (Nestler, 2001, 2002; Robbins and Everitt, 2002).

In summary, the developing brain of adolescent seems to be highly vulnerable to nicotine (Slotkin, 2002). It appears that *Mid*-adolescent mice (and rats) undergo a specifically 'vulnerable' age period and may represent a suitable animal model for human adolescence, with enough face and construct validity (Spear, 2000), being able to show (1) behavioral features that resemble those found in human adolescents; (2) an elevated vulnerability to the consumption of drugs, compared to other ages; and (3) marked long-term vulnerability as a consequence of such exposure.

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