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VOLUNTARY ORAL NICOTINE INTAKE IN MICE DOWNREGULATES GLUR2 BUT DOES NOT MODULATE DEPRESSION-LIKE BEHAVIORS

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Abstract

Repeated exposure to nicotine induces adaptive changes in the central nervous system including the mesolimbic dopamine (DA) projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). These modifications can modulate nicotine reward and reinforcement, but also anxiety and depression-related behaviors. The development of addiction-related phenotypes is known to be modulated by regulation of glutamate receptors, as well as activation of transcription factors including cAMP response element-binding protein (CREB), in the NAc.

We investigated the effects of nicotine pre-exposure on nicotine preference and levels of Glur1/2 and CREB in the mesolimbic system in male mice C57BL/6J and BALB/c inbred mice. We also evaluated locomotor activity, anxiety-like and depression-like behaviors known to be affected by nicotine. There were few behavioral changes in mice subjected to chronic nicotine exposure, but there was a marked regulation of GluR2 in the mesolimbic system. Both treated and non-treated animals showed a significant preference for nicotine when facing a choice with a control solution.

These results suggest that voluntary nicotine drinking induces nicotine preference in mice, but does not alter a number of affective behaviors. In addition, alterations in CREB and GluR1 levels are not sufficient to explain preference for nicotine in a 2-bottle choice paradigm.

Keywords

nicotine; 2-bottle choice; behavior; AMPA receptors; CREB

Introduction

Tobacco use induces adaptive changes in the central nervous system that could lead to nicotine dependence [8]. Mesolimbic dopamine (DA) projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) are critical for drug reinforcement. Nicotine stimulates the DA system [21], thus resulting in nicotine reward and reinforcement, but also modulating anxiety and depression-related behaviors [1].

Addictive behavior also depends on the habit-forming process, mediated in part through glutamatergic modulation of the dopamine system and its projections [7]. For example, AMPA receptors in the shell of the NAc are critical for Pavlovian approach to drug cues and in cocaine-

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seeking behavior in rats [27]. The glutamatergic input to the striatum could affect neuronal plasticity [7,29], critical for the development of behaviors related to drug addiction [15,16]. Addictive disorders also depend on drug-induced regulation of transcription factors including cAMP response element-binding protein (CREB), which are necessary for stable changes in neuronal function induced by repeated drug exposure [16,28].

Like other drugs of abuse, nicotine is self-administered and can result in a conditioned place preference under specific conditions [11]. Administration of nicotine in the drinking water is a less stressful alternative to repeated injection for chronic exposure experiments [20]. This method is very different from rapid infusion methods, such as smoking or jugular infusion, but results in intermittent exposure only during the waking period, and is sufficient to cause neuronal adaptations, including upregulation of nicotinic binding sites, that are observed in the brains of human smokers [18,19,26]. Furthermore, the effects of oral nicotine exposure are increased in adolescent mice [2]. Finally, as with intravenous self-administration, animals can titrate their drug intake; thus, it is possible to test nicotine preference.

We investigated the effects of oral nicotine pre-exposure on nicotine preference and levels of the glutamate receptor subunits Glur1/2 and CREB in the mesolimbic system. We subjected mice to increasing concentrations of nicotine in the drinking water from adolescence onward, and assessed nicotine intake at different concentrations in adulthood. We also measured locomotor activity, anxiety-like and depression-like behavior. Because the initiation of nicotine self-administration and abuse can rely on genetic differences between individuals, these experiments were also carried out in C57BL/6J and BALB/c mice. These inbred strains were chosen because of their clear phenotypic differences, especially related to depression-like behavior [12,13].

Material and methods

Animals

Male mice of the C57BL/6J (C57) and BALB/cJ (BALB) strains were obtained at ~ 3 weeks of age with nursing mothers from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under standard conditions: 12:12 light/dark cycle, (lights on at 7:00 am), controlled room temperature ($22 \pm 1^\circ\text{C}$) with free access to food. All studies were conducted in accordance with the principles and procedures described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. 12 mice per group (for a total of 48) were used for behavioral experiments. 8 animals per group were randomly chosen for biochemical analyses.

Experimental procedure: Nicotine preference test

Mice had *ad libitum* access to fluid for 2 hr a day from 10 a.m. to noon. Mice were randomly assigned to the control group or the nicotine exposed group. Each cage had two drinking bottles from the time of weaning to habituate the animals for the nicotine preference tests. During pre-exposure, both bottles were filled with nicotine bitartrate (Sigma) or a molar-equivalent of tartaric acid (Sigma) alone dissolved in 2% sucrose to increase palatability (pH adjusted to 7.0) [10]. The concentration of nicotine or tartaric acid was increased from 13 mg/L to 28 mg/L every 7 days to ensure the animals maintained their fluid intake (Table 1). Bottles were wrapped in foil to protect against photodegradation.

At adulthood, about 3 months of age, all animals were provided with 2 bottles, one with tartaric acid and another with nicotine (13 to 28 mg/L), to determine whether animals could titrate nicotine intake by increasing their nicotine drinking at low concentrations and decreasing at high concentrations. Consumption was measured daily by weighing bottles, and weight gain

was recorded each week. Bottles were randomly positioned to avoid any side preference (see Table 1 for experimental design).

Behavioral experiments

Mice were tested 2-4 hr after drinking. Each test was performed in the sequence described below, separated by 48 hr between tests.

Elevated plus maze—The elevated plus maze was performed as described [24]. The apparatus consisted of 2 open (4 mm-high edges) and 2 closed (black, 15 cm-high) arms (30 × 5 cm). Floors were white Plexiglas and the apparatus was elevated 50 cm above the floor. Each mouse was placed in the central square (5 × 5 cm) facing an open arm, and allowed to explore the maze for 5 min. The maze was thoroughly cleaned with water and dried with a cloth between sessions.

Locomotor activity measurement—Mice were placed in a clean cage (48 × 22 × 18 cm) for 20 min. Locomotor activity was recorded using the Optomax system (Columbus instruments, Columbus, Ohio, USA). Twelve parallel beams were equally distributed across the cage. Horizontal locomotor activity was derived from the number of beam breaks during the test session.

Tail suspension test—The tail suspension test was performed as has been described [14]. Mice were suspended by the tail with a paper clip attached with adhesive tape about 5-10 mm from the end of the tail. Time spent immobile was recorded over the 6 min test, along with number of bouts of immobility.

Novelty-suppressed feeding test—Mice were weighed and food was removed from the cage. The testing apparatus consisted of a clear Plexiglas enclosure (40 × 40 × 17 cm) with a lid. The floor was covered with 2 cm of bedding. A small piece of mouse chow was placed in the center on a piece of white, circular filter paper (diameter: 9.5 cm). At the start of the experiment, each animal was placed in a defined corner of the testing area, and the time to the first feeding event was recorded. Immediately after the mouse began to eat, the subject was removed and placed back in its home cage with a piece of chow. At the end of a 5-min period, the amount of food consumed was determined.

Passive Avoidance—Passive avoidance testing was performed using a chamber from Ugo Basile (Comerio, Italy). On Day 1, each mouse was placed in the light chamber and was allowed to explore both compartments for 5 min. On day 2, each mouse was placed in the light chamber and latency to enter the dark chamber was measured. Immediately after entry into the dark chamber, the door between compartments closed, and a 2-sec electric shock (0.2 mA) was administered through the grid floor. On Day 3, each mouse was placed in the light chamber, and time to enter the dark chamber was recorded.

Western blotting

At least two days after the end of behavioral testing, mice were rapidly decapitated (between 10 am and 1 pm), brains were removed, frozen immediately and kept at -80 °C until use. Brains were placed in phosphate buffered saline (PBS) and sliced in a plastic matrix at 4°C. Punches targeting the VTA, NAc shell (Sh) and NAc core (Co) were collected, sonicated in homogenization buffer (10% SDS, 50 mM TRIS (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF), then centrifuged at 14,000 × g (10 min). The time between thawing and homogenization did not exceed 1.5 minute.

Protein concentration was determined using a Lowry assay, 10 µg of protein per lane was loaded on a 10% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose membrane, rinsed in TRIS buffered saline (TBS), and blocked in 5% nonfat milk in TBS with 0.1% Tween (TBS-T) for 1 hr at room temperature. After rinsing in TBS-T, membranes were incubated overnight in primary antibody diluted in TBS-T.

CREB (1:1000), pCREB (Ser133; 1:500) antibodies (Cell Signaling, Danvers, MA, USA), GluR1 (1:3000) and GluR2 (1:500) antisera (Abcam, Cambridge, MA, USA) were used. Blots were washed in TBS-T and incubated 1hr in 1:5000 affinity-purified fluorescent anti-rabbit or anti-mouse IgG (Rockland IRdye 800 and Alexafluor 680), washed in TBS-T then milliQ water, and scanned using an Odyssey Infrared Imager (LI-COR Biosciences). Results are expressed in arbitrary units derived from the absorbance measured $\pm$ SEM. Seven samples across the different groups were removed from the study due to abnormal variability (very faint, distorted and/or incomplete band) in Ponceau staining.

Statistical analysis

Data were analyzed by two-way analyses of variance (ANOVA) with Strain (2 levels), and Treatment (2 levels) as main factors, using StatView 5 (SAS Institute Inc, Cary, NC, USA). T-tests against the null hypothesis were used to determine nicotine preference. T-test post-hoc analyses (with Bonnferroni corrections if necessary) were performed when relevant. Significance level was set at 5%.

Results

Nicotine drinking paradigm

There were no significant differences in total volume of fluid ingested in the nicotine or vehicle groups, either during the forced consumption (see Table 1; data not shown: $F(1, 1148)=0.34$, $p=0.06$) or nicotine choice period (data not shown: $F(1, 1509)=1.23$, $p=0.27$). Although BALB mice drank more (nicotine and/or control solutions) than C57 mice both during the forced exposure period ($F(1, 1148)=7.43$, $p=0.006$) and in the choice period ($F(1, 1509)$, $p=42.78$, $p<0.0001$) no interactions were detected between strain and treatment.

During the nicotine choice period, mice titrated their nicotine intake as concentration increased to maintain a constant level (i.e. mice were drank more nicotine-based fluid when the concentration was low, and less when it was high (data not shown: $F(4, 481)=0.48$, $p=0.71$) independent of strain ($F(4, 481)=0.51$, $p=0.83$). No interactions between strain and treatment were detected ($F(4, 481)=0.15$, $p=0.10$).

During the choice paradigm, animals showed a marked preference for nicotine as compared to the control solution (Figure 1A; $t_{656}=10.22$, $p<0.0001$), although no differences were seen following pre-exposure to nicotine ($F(1, 645)=0.002$, $p=0.96$). A trend toward a strain difference was observed ($F(1, 645)=3.22$, $p=0.07$). There was no difference in preference between 13 mg/l and 25 mg/l ($t_{404}=-0.40$, $p=0.69$) but 18 mg/L was less preferred than 25 mg/l (Figure 1B; $t_{574}=-2.85$, $p=0.004$).

Behavioral tests

Forced nicotine intake did not result in significant behavioral differences in the paradigms studied, although strain differences were detected (data not shown): Elevated plus maze (time spent in the open arms): Treatment $F(1, 43)=0.48$, $p=0.49$, Strain $F(1, 43)=29.4$, $p<0.0001$, BALB>C57 in anxiety-like behavior; Locomotor activity in open field: Treatment $F(1, 43)=34.3$, $p=0.82$; Tail suspension test: Treatment $F(1, 41)=1.92$, $p=0.17$, Strain $F(1, 41)=111.28$,

$p < 0.0001$, C57>BALB; Novelty-suppressed feeding: $F(1, 44) = 0.33$, $p = 0.56$.); Passive avoidance: Treatment: $F(1, 44) = 0.17$, $p = 0.68$.

Biochemical analyses

No differences in GluR1 were detected between the different treatments ($F(1, 58) = 0.27$, $p = 0.87$) or genotypes (Figure 2). However, highly significant differences in expression were detected between brain regions, ($F(2, 58) = 90.53$, $p < 0.0001$). No interactions were detected between treatments and/or strain and/or brains regions.

No differences in GluR2 were detected overall across treatment and strain, although there were significant differences among brain areas and numerous interactions were detected (Figure 2). Treatment effects on GluR2 varied between BALB and C57 mice ($F(1, 130) = 8.86$, $p = 0.003$) and their respective brain loci ($F(2, 130) = 3.07$, $p = 0.04$). Posthoc analyses revealed that GluR2 levels were significantly reduced in the core ($t_{20} = 0.09$, $p = 0.0006$) and shell ($t_{22} = 0.38$, $p < 0.0001$) of the NAc and the VTA ($t_{22} = 0.38$, $p = 0.005$) of C57 mice but not BALB mice. No overall treatment effect on CREB or pCREB levels were detected, but total CREB levels differed between BALB and C57 mice ($F(1, 131) = 5.47$, $p = 0.02$), and their respective brain loci ($F(2, 131) = 6.59$, $p = 0.002$). No other interactions were detected (Figure 3).

Discussion

Sensitization to the psychostimulant properties of nicotine occurs following repeated intermittent exposure, potentially underlying the shift from drug use to abuse, when 'desire for the drug' might become sensitized independently from 'drug pleasure' [1]. In our study, pre-exposure to nicotine did not influence nicotine intake, although mice strongly preferred nicotine over control solution even when animals were nicotine naïve. In mice, paradigm differences can significantly affect nicotine intake [11]. For example, dose or water-deprivation schedule strongly influences mouse oral nicotine intake, and single-housing can modify stress levels in male mice, altering nicotine consumption [3,25]. Dopaminergic neurons of the VTA show enhanced responsiveness to AMPA receptor stimulation after chronic stimulant exposure, potentially mediated by increased expression of specific AMPA receptor subunits following chronic drug administration. This could contribute to heightened activity of the mesolimbic dopamine system, and drug sensitization [17]. Nicotine stimulates glutamatergic inputs to dopamine neurons [1]. AMPA receptors seem to be specifically involved in drug-seeking and in vulnerability to drug addiction [6,7,27], through cortical glutamatergic inputs to the striatum that might affect neuronal plasticity and habit forming processes. In our study, nicotine exposure down-regulated GluR2 in the NAc and the VTA of C57 but not BALB mice. This decrease in GluR2 expression has been observed previously in adolescent animals administered nicotine, whereas comparable exposure during adulthood had the opposite or no effect [1]. However, nicotine preference was similar in mice that did not receive nicotine pre-exposure, suggesting that while chronic nicotine use during adolescence can down-regulate GluR2, this does not translate into a shift of nicotine preference. Alternatively, because mice preferred nicotine at baseline, they might have already reached a threshold of nicotine consumption due to a ceiling effect.

None of the behaviors assayed showed significant differences between the treatment groups. This is unforeseen as nicotine/nicotinic agents can modify antidepressant-like and anxiety-like behavior, passive avoidance, and locomotor activation [9,10,14,21,22]. However, most previous studies used different routes of admission and/or acute treatment in adults. Adaptation following the oral nicotine pre-treatment period may lead to compensatory changes in systems regulating these behaviors, consistent with the lack of differences we found in CREB expression or activation. CREB is involved in depression-like and anxiety-like behaviors [4, 23] and is activated in the NAc following chronic drug use [5,17], behaviors unaffected in the

present study. However, adaptations associated with addiction may also occur outside of the mesolimbic dopamine system [17]. In addition, modulation by CREM could also account for some of the results we observed. However, further data are needed to investigate this possibility.

It is noteworthy that no differences in response to nicotine treatment could be detected in the two strains used here, and that both strains showed a lack of nicotine-induced behavioral effects after nicotine pre-exposure. Nonetheless, statistical analyses revealed strain differences in the different paradigms, similar to what has been reported previously [12,13]. For instance, BALB mice showed more anxiety-like behavior than C57 mice in the elevated plus maze, and exhibited less behavioral despair in the tail suspension test, suggesting that there is enough statistical power to detect differences in behavior, and that the two strains share at least some neurochemical and behavioral responses to adolescent nicotine exposure.

In summary, these results indicate that nicotine drinking with previous fluid deprivation is an efficient way to induce nicotine preference in distinct genetic backgrounds of mice. However, despite significant nicotine intake, mice did not show robust biochemical alterations beyond GluR2 downregulation. Thus, alterations in CREB and GluR1 are not sufficient to explain preference for nicotine in a 2-bottle choice paradigm.

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Figure 1. Nicotine consumption in C57BL/6J and BALB/cJ mice during the nicotine preference paradigm. **A:** Average preference per session for nicotine +/- SEM. **B:** Average nicotine intake per session expressed in mg/kg +/- SEM. n = 12/group.

Figure 2.

Western blotting for GluR1 and GluR2 in the NAc (core and shell) and VTA of C57BL/6J and BALB/cJ mice. Results are expressed in arbitrary units of absorbance $\pm$ SEM. Comparisons are made between nicotine and control groups. *** $p < 0.001$. $n = 7-8$ /group.

Figure 3.

Western blotting for CREB and pCREB in the NAc (core and shell) and the VTA of C57BL/6J and BALB/cJ. Results are expressed in arbitrary units of absorbance $\pm$ SEM. $n = 6-8$ /group.