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Early adolescent nicotine exposure affects later-life hippocampal mu-opioid receptors activity and morphine reward but not physical dependence in male mice

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Abstract

Rationale.—There is extensive literature regarding nicotine-opioid functional interactions. The possibility that use of nicotine products during adolescence might increase the risk of substance abuse such as morphine later in adulthood is particularly relevant to the current opioid crisis.

Objectives.—To investigate the effects of nicotine exposure for seven days during adolescence in mice on morphine reward as well as morphine physical dependence later in adulthood.

Methods.—Mice were exposed to nicotine in either early or late adolescence then evaluated for morphine reward and withdrawal symptoms in adulthood. A separate group of mice was exposed to nicotine during adolescent and tissue was evaluated for changes in MOR-mediated G-protein activity using [³⁵S]GTPγS binding assays.

Results.—We report that a 7-day exposure to a low dose of nicotine during early adolescence significantly enhanced morphine preference in the CPP test in adult mice. In contrast, the same treatment with nicotine had no effect on expression of somatic withdrawal signs in morphine-dependent adult mice. MOR-mediated G-protein activity in hippocampus, but not thalamus and striatum of adult mice, was significantly altered by adolescent nicotine treatment.

Conclusions.—Adolescence is a unique developmental stage during which nicotine has long-term effects on future drug-taking behavior. Further studies are needed to identify the neurotransmitters and mechanisms involved in increased vulnerability to drug abuse.

Keywords

Morphine; Nicotine; Adolescent; Conditioned place preference; Reward; Withdrawal

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Conflict of Interest

The authors have no conflicts of interest to disclose

1. Introduction

Despite an intense effort to publicize the risks of smoking, it remains a significant public health problem, especially among adolescents. In 2016, an estimated 63.4 million or 23.5 percent of Americans ages 12 and older were current (within the past 30 days) users of a tobacco product (SAMHSA 2016). These numbers differ by age group: in 2016, 15.5% (37.8 million) of U.S. adults were current cigarette smokers and 2.2% of middle school and 8% of high school students reported that they smoked cigarettes in the past 30 days (CDC, 2018). The rate of tobacco use among adolescents in Europe is overall higher with an average of average for all countries around 12% (Euro WHO, 2016). Interestingly, a majority (57.6 percent) of adolescents from ages 12 to 17 who smoked cigarettes in the past month also used an illicit drug (cocaine, heroin, etc.) compared with 6.1 percent of adolescent who did not smoke cigarettes (SAMHSA 2011). Previously, Breslau and Peterson (1996) reported similar results, showing that individuals under the age of 15 who smoke cigarettes are eighty times more likely to use illegal drugs as compared to non-smokers. Indeed, early onset of drug abuse has been hypothesized to increase the risk of later drug addiction (Anthony and Petronis 1995; Clark et al. 1998; Palmer et al. 2009). The possibility that use of nicotine products during adolescence might increase the risk of opioid abuse in adulthood is particularly relevant to the current opioid crisis.

There is extensive literature regarding nicotine-opioid functional interactions. For instance, nicotine-stimulated release of endogenous opioids in various brain regions contributes to opiate reinforcement and reward (for review, see Berrendero et al. 2010). Furthermore, bilateral injections of mecamylamine, a non-selective nicotinic antagonist, into the hippocampal CA1 region significantly inhibited morphine condition place preference (CPP) in rats (Rezayof et al. 2006). The majority of these studies were reported with adult rodents; however, exposure to nicotine during adolescence might increase vulnerability to opioids in adulthood (for review, see Slotkin 2002). Indeed, some rodent studies but not others (Pomfrey et al., 2015; Kelley and Middaugh, 1999), suggest that nicotine exposure during adolescence enhanced reward responses to other drugs of abuse such as cocaine and amphetamines (Collins and Izenwasser, 2004; McMillen et al., 2005; McQuown et al., 2007, Dao et al., 2011, Dickson et al., 2014; Alajaji et al. 2016). Despite the above evidence and the high incidence of nicotine abuse among adolescents, relatively few studies have investigated the long-term effects of adolescent nicotine exposure on opioid use and subsequent dependence later in life.

Adolescence is a period where the brain is undergoing major developmental changes in addition to other various biological, hormonal and behavioral changes (for review, see Spear 2000 and Schramm-Sapota et al., 2009), and this may contribute to long lasting changes in brain function that underlie increased vulnerability to substance abuse. Moreover, Campbell et al. (2000) reported that for most drugs of abuse, rewarding properties are higher during adolescence while withdrawal symptoms are reduced. This shift in balance could contribute to increased experimentation during this stage of development thus increasing the likelihood of substance use disorders later in life. Various neuronal adaptations occur following repeated exposure to nicotine during early development that could influence diverse signaling pathways and circuits persisting into adulthood (Slotkin 2002). For example, the

level of nAChRs increases in the brain the day after the first exposure to nicotine (Abreu-Villaca et al. 2003), indicating a very rapid timeline for brain remodeling following drug exposure. Furthermore, nAChR upregulation persists at significant levels one month after treatment (Abreu-Villaca et al. 2003). Since nAChRs affect the release of virtually every major neurotransmitter, these overall neuroadaptations during adolescence can have broad and profound effects in adulthood (Dani and Bertrand 2007). This is in accord with a report that the opioid agonist morphine elicited enhanced locomotor activity in nicotine-experienced adult mice than in animals that were not exposed to nicotine (Biala and Weglinska 2004). The molecular basis for enhanced opioid effects is not clear. A primary suspect is the mu opioid receptor (MOR), which plays an essential role in opiates reward and dependence (for review see Contet et al., 2004). Marco et al. (2006) found sub-chronic nicotine treatment (0.4 mg/kg/day for 10 days) during adolescence induced a significant decrease in mu opioid receptor protein (MOR) in hippocampus and striatum of adult rats. However, they did not determine whether the decrease in expression was associated with changes in MOR function.

We previously reported that nicotine exposure in mice during early adolescence enhanced the rewarding response to nicotine (Kota et al., 2009), as well as cocaine and amphetamine (Alajaji et al, 2016), in adulthood. Given these results and those summarized above, the aim of the present study was to examine the effect of repeated low-dose exposure to nicotine in mice during adolescence on the subsequent behavioral responses to morphine in adulthood. In particular, we investigated the effects of nicotine exposure for seven days during early and late adolescence in mice on morphine reward in the conditioned place preference (CPP) test as well as morphine physical dependence once the animals reached adult age and evaluated MOR-mediated G-protein activity to assess possible changes in receptor function.

2. Materials and Methods

2.1 Animals

Experimentally naïve male ICR mice were obtained from Harlan Laboratories (Indianapolis, IN). Adolescent mice arrived on postnatal day (PND) 21 and weighed approximately 18–23 grams at the start of the experiment. Adult mice arrived on PND 65 and weighed approximately 30–35 grams. Mice were obtained from different litters and housed four per cage. They were allowed to acclimate for seven days prior to experiments. The mice were handled for three days prior to the experiment with unlimited access to food and water. All mice were housed in humidity and temperature controlled (22 °C) vivarium on a 12-hr light/dark cycle (lights on at 6 a.m., off at 6 p.m.). Animals were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care. Experiments were conducted during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and as stated in the National Institutes of Health guide for the care and use of Laboratory animals.

2.2 Drugs and Reagents

(–)-nicotine hydrogen tartrate salt [(–)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA); morphine, [d-Ala2,

(NMe)Phe⁴, Gly⁵-OH] enkephalin (DAMGO), and naloxone were supplied by the National Institute on Drug Abuse (Rockville, MD). All drugs were dissolved in sterile saline (0.9% sodium chloride) and prepared fresh before each experiment. Nicotine, morphine and saline were injected subcutaneously (s.c.) and naloxone was injected intraperitoneally (i.p.). Control groups received saline injections at the same volume and by the same route of administration. All doses are expressed as the free base of the drug. [³⁵S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Guanosine diphosphate (GDP) was purchased from Sigma-Aldrich (St. Louis, MO). Econosafe scintillation fluid was purchased from Research Products International. All other reagent grade chemicals were obtained from Sigma Chemical Co. (St. Louis MO) or Fisher Scientific (Pittsburgh PA).

2.3 Drug Exposure Protocol

Mice received nicotine during early adolescence (PND 28–34), late adolescence (PND 50–57) or adulthood (PND 70–77) (n=7–8/group treatment). Nicotine (0.1 or 0.5 mg/kg) or saline was administered to mice by s.c. injections twice daily approximately seven hours apart (0900 and 1600) for seven days. Mice were then housed in their home cages where each age group matured for 35 days (PND 70) at which point they were evaluated in the CPP paradigm as described below. The doses of nicotine and exposure periods were chosen based on our recent reports (Kota et al., 2009; Kota et al., 2011; Alajaji et al., 2016) that showed that these experimental conditions were optimal for nicotine behavioral enhancement in mice.

For the morphine dose-response curve study, nicotine (0.5 mg/kg, s.c.) or saline was administered to separate groups of early adolescent (PND 28) mice and at PND 70, they were tested for morphine CPP at various doses (vehicle control, 2.5, 5 and 10 mg/kg, s.c.).

Control studies to examine nicotine treatment in late adolescent and adults were performed using a separate group of mice. Nicotine (0.1 and 0.5 mg/kg) or saline was administered to late adolescent (PND50) and adult (PND 70) mice s.c. twice daily (09:00 and 16:00) for 7 days. Mice were then housed in their home cages for 35 days and evaluated for morphine reward at a dose of 10 mg/kg as described below in the CPP test. The dose of 10 mg/kg of morphine was based on the results of the CPP dose-response curve as outlined above.

Conditioned Place Preference Test—Mice were tested for morphine preference using the CPP test after a 35-day maturation period. A five-day unbiased CPP paradigm was utilized in this study as described in Kota et al. (2007). Briefly, place conditioning boxes consisted of two distinct sides (20 cm X 20 cm X 20 cm) with a smaller center gray compartment that separated the two sides. Openings from the center compartment allowed access to either side of the chamber. Mice were handled for three days prior to the start of the CPP procedure. On day 1, animals were placed in the boxes and allowed to move freely from side to side for 15 min. Time spent in each side of the chamber was recorded. The times spent in the white and black chambers were used to establish baseline chamber preferences, if any. Mice were separated into vehicle and drug groups such that initial chamber biases were approximately balanced. On days 2–4 (conditioning days), twice per

day (8:00 am and 1:00 pm), mice were injected with vehicle or drug and subsequently paired with either the white or black chamber, where they were allowed to roam for 15 min. Vehicle-treated animals were paired with saline in both chambers and drug-treated animals received saline in one chamber and nicotine in the opposite chamber. Pairing of the drug with either the black or white chamber was randomized within the drug-treated group of mice. On day 5 (test day), mice did not receive an injection. They were placed into the center chamber for 5 min, the partitions were lifted, and they were allowed to roam freely for 15 min. Data are expressed as preference score (time spent on drug-paired side minus time spent on saline-paired side). A positive number indicates a preference for the drug-paired side, whereas a negative number indicates an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

2.4 Induction of Morphine Dependence and Naloxone-Precipitated Withdrawal

The changes in morphine CPP prompt us to investigate if nicotine pretreatment in early adolescence would alter morphine withdrawal, an important aspect of dependence. Another group of early-adolescent (PND 28) mice were treated with saline or nicotine (0.5 mg/kg; s.c. twice daily for 7 days) (n=8/group treatment). When mice reached adulthood (PND 70), they were tested for morphine physical dependence using a modified method of our previously reported procedure (Muldoon et al., 2014). Mice were randomly divided into four groups: saline-saline-saline, saline-morphine-naloxone, nicotine-saline-saline and nicotine-morphine-naloxone, which indicate the adolescent pretreatment, adulthood treatment and challenge treatment respectively. Depending on the treatment groups, mice were injected with saline or morphine s.c. three times daily at 0900, 1200 and 1500 pm for 3 consecutive days according for the following schedule. On day 1 morphine was given at 50 mg/kg, on day 2 morphine was given at 75 mg/kg and on day 3 morphine was given at 100 mg/kg. On the morning of day 4, 100 mg/kg of morphine was injected at 0900 only. Two hours later, mice received an i.p. injection of either naloxone (2.0 mg/kg) or saline. Immediately following the injection, animals were placed individually in a glass beaker and observed for the following morphine withdrawal signs: head shakes, paw tremors, body tremors, and backing, ptosis, curls, and jumps. Results were reported as the average (mean $\pm$ S.E.M.) of the total signs per group during the 30 min observation period. All testing was conducted in a blind manner.

2.5 Agonist-stimulated [35 S]GTP γ S Binding

Separate groups of mice were treated with nicotine during adolescence [Early adolescent (PND 28) with either saline or nicotine (0.5 mg/kg, s.c.) twice for seven days] and euthanized by decapitation on day PND70 (adulthood) (n=7/group treatment). Three brain regions were chosen on the basis of their role in morphine reward and CPP as well as the presence of a good ratio of background/signal for [35 S]GTP γ S signal from previous observations (Selley et al., 1997). Striatum, hippocampus and thalamus were dissected on ice, and samples were stored at -80°C until use. [35 S]GTP γ S binding assays were conducted as previously published with minor modification (Selley et al., 1997). Tissue samples were thawed and homogenized in 5 ml of cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl_2 , 1 mM EGTA, pH 7.4). The homogenate was centrifuged at $50,000 \times g$ at 4°C for 10 min and the pellet was resuspended in assay buffer (50 mM Tris-HCl, 3 mM MgCl_2 , 1

mM EGTA, 100 mM NaCl, pH 7.7). Protein was determined (PMID:942051), and membranes were then incubated with 4 mU/ml adenosine deaminase for 10 min at 30°C. Concentration-effect curves were generated by incubating membranes from each region (5–6 µg) in assay buffer with 30 µM GDP, 0.1 nM [³⁵S]GTPγS, and varying concentrations of the MOR-selective full agonist DAMGO. Basal binding was determined in the absence of agonist and non-specific binding was measured using 20 µM GTPγS. Samples were incubated for 2 hr at 30°C with agitation. The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold 50 mM Tris-HCl, pH 7.2. Bound radioactivity was measured using liquid scintillation spectrophotometry at 95% efficiency after overnight extraction in Econo-Safe scintillation fluid. Data are reported as mean ± SEM of at least 4 experiments, each performed in triplicate. Non-specific binding was subtracted from each sample. Net stimulated [³⁵S]GTPγS binding was calculated as agonist-stimulated minus basal [³⁵S]GTPγS binding, and percent stimulation was calculated as (net-stimulated/basal [³⁵S]GTPγS binding) × 100%.

2.6. Data analysis

For all behavioral data, graphs and statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software; San Diego, CA). All CPP results were expressed as mean preference scores ± standard error of the mean. Preference scores were measured in seconds, and indicate time spent in the drug-paired side during post conditioning - time spent in the drug paired side pre-conditioning (baseline). Statistical analyses of CPP and withdrawal studies were performed with mixed-factor (two-way) ANOVA and Bonferroni post hoc analyses were used to determine significant differences between groups ($p < 0.05$). Non-linear iterative regression analyses of DAMGO concentration-effect curves were performed with Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) to determine $E_{\max}$ and EC_{50} values. Significant differences in the DAMGO concentration-effect curves between groups were determined in each region by two-way ANOVA. $E_{\max}$ and EC_{50} values were compared between groups in each region using the two-tailed Student's t -test.

3. Results

3.1 Early-adolescent nicotine exposure enhances morphine-induced CPP in adulthood

The first study assessed the effect of early-adolescent (PND 28–34) exposure to 0.1 or 0.5 mg/kg nicotine on morphine-mediated reward-like effects in adulthood because we have previously identified this as a vulnerable period for cocaine and nicotine reward (Kota et al., 2011; Alajaji et al., 2016). Mice were evaluated in the CPP test and as shown in Figure 1A, morphine (10mg/kg) elicited a significant preference in mice that received saline or nicotine during early adolescence [$F_{\text{pretreatment}}(1, 42) = 204.3, p < 0.0001$]. Exposure to nicotine during early adolescence significantly induced a greater preference for morphine in adulthood [$F_{\text{treatment}}(2, 42) = 8.09, p < 0.0001$ $p < 0.001$] in a nicotine treatment dose-related manner as compared to mice that received saline during adolescence. Adolescent pretreatment x adult treatment interaction was significant [$F_{\text{interaction}}(2, 42) = 3.59, p < 0.03$].

A second group of early-adolescent mice (PND 28) received 0.5 mg/kg nicotine (twice daily for 7 days) and animals were tested at PND 70 with various doses of morphine (0, 2.5, 5, and 10 mg/kg) in the CPP procedure (Figure 1B). Pretreatment with 0.5 mg/kg nicotine during early adolescence produced an apparent upward shift in the morphine dose-response curve, where doses of 5 and 10 mg/kg morphine evoked a significant CPP response in adulthood as compared to saline control mice [$F_{\text{pretreatment}}(2, 56) = 42.16, p < 0.0001$] and [$F_{\text{treatment}}(2, 56) = 75.18, p < 0.0001$]. In addition, dose x treatment interaction was significant [$F_{\text{interaction}}(3, 56) = 6.44, p < 0.0008$].

3.2 The early adolescent period plays a critical role in the effects of nicotine on morphine preference in adulthood

To determine whether enhancement of morphine preference is specific to nicotine treatment during early adolescence, late adolescent (PND 50) and adult (PND 70) mice were administered nicotine (0.1 and 0.5 mg/kg) for 7 days, and then tested for morphine-induced CPP after the same drug-free period (35 days) as early adolescents (Figures 2A & B). All mice exhibited a significant preference for the morphine-paired side, as shown by one-way ANOVA for late adolescent [$F_{\text{treatment}}(1,36) = 330.6; p < 0.0001$] (Figure 2A) and adult exposure (Figure 2B) [$F_{\text{treatment}}(1,36) = 414.1; p < 0.0001$]. In contrast to data from mice that received nicotine during early adolescence (Figure 1A), mice treated with nicotine during late adolescence (Figure 2A) or adulthood (Figure 2B) did not demonstrate any significant differences from mice pretreated with saline when assessed for morphine CPP in adulthood [$F_{\text{pretreatment}}(2,36) = 1.59; p < 0.21$]. Similarly, mice that received either nicotine or saline during adulthood displayed similar preference for morphine (Figure 2B) later in life [$F_{\text{pretreatment}}(2,36) = 0.65; p < 0.52$].

Early adolescent nicotine exposure does not affect morphine withdrawal—

Mice were pretreated with saline or nicotine (0.5 mg/kg s.c.; two injections each day for 7 days) during early adolescence (PND 28) and then allowed to mature to adulthood (PND 70). Repeated morphine or saline was then administered for 3 days as described in methods and on day 4 mice received saline or naloxone injection to assess withdrawal signs. A two-way ANOVA (adolescent pretreatment $\times$ morphine-naloxone) indicated that expression of somatic withdrawal signs was significantly increased in morphine-naloxone treated mice [$F_{\text{treatment}}(1,20) = 63.11, p < 0.0001$]. However, there was no significant effect of adolescent nicotine treatment on expression of withdrawal signs [$F_{\text{pretreatment}}(1,20) = 0.0006, p = 0.9806$] (Figure 3). In addition, the separate analysis of individual signs revealed no significant effect of adolescent pretreatment on any sign evaluated (data not shown).

3.3 Effect of Adolescent nicotine treatment on MOR-stimulated [^{35}S]GTP γ S binding

To determine whether repeated nicotine treatment during adolescence directly affected MOR signaling in adult mouse brain, DAMGO-stimulated [^{35}S]GTP γ S binding was conducted in thalamus, striatum and hippocampus. Results in thalamus (Fig. 4) showed DAMGO produced concentration-dependent stimulation of G-protein activity, but there was no difference in the DAMGO concentration-effect curves between vehicle- and nicotine-treated mice, as indicated by two-way ANOVA (main effect of DAMGO concentration [$F(6,56) = 66.38, p < 0.0001$], no main effect of nicotine [$F(1,56) = 0.6446, p = 0.4255$] and no

interaction between nicotine and DAMGO concentration [$F_{6,56} = 0.0313$, $p = 0.9999$]. Likewise, DAMGO significantly stimulated G-protein activation in striatum [$F_{6,55} = 41.26$, $p < 0.0001$], but there was no effect of nicotine treatment [$F(1,55) = 0.0178$, $p = 0.8941$] nor was there an interaction between nicotine and DAMGO [$F(1,55) = 0.29$, $p = 0.9391$]. DAMGO also stimulated G-protein activity in the hippocampus [$F(6,39) = 56.92$, $p < 0.0001$], and in this region there was a small but significant effect of nicotine treatment to increase DAMGO-stimulated activity [$F(1,39) = 12.19$, $p = 0.0012$], but no interaction [$F(6,39) = 0.2361$, $p = 0.962$]. Basal [^{35}S]GTP γ S binding ranged from 115.9 ± 7.9 fmol/mg in thalamus to 200.0 ± 6.5 fmol/mg in striatum of vehicle treated mice, but there were no differences in basal activity between vehicle- and nicotine-treated mice in any region (Data not shown). Non-linear regression analysis was performed to determine the E_{max} and EC_{50} values of DAMGO in all three regions (Table 1 Supplement), but there were no significant differences between vehicle- and nicotine-treated mice in either parameter in any region examined, including hippocampus. Thus, there was only a slight increase MOR-mediated G-protein activation in hippocampus of nicotine-treated mice, which could not be definitively attributed to a change in either the potency or maximal stimulation by DAMGO.

4. Discussion

The present study investigated the effect of repeated nicotine administration during early adolescence on behavioral effects of morphine in adult mice. We report that a 7-day exposure to nicotine during early adolescence significantly enhanced morphine preference in the CPP test in adult mice. In contrast, the same treatment with nicotine had no effect on expression of somatic withdrawal signs in morphine-dependent adult mice. MOR-mediated G-protein activity in thalamus and striatum of adult mice was not significantly altered by adolescent nicotine treatment, although there was a very slight increase in hippocampus, suggesting that this is not the primary mechanism underlying enhanced reward-like behavior.

Low doses of nicotine (0.1 and 0.5 mg/kg) given daily during early adolescence appear to selectively influence processes related to morphine-induced preference in the CPP test in adult mice. Nicotine pretreatment enhanced morphine preference and shifted upward the dose-response curve. Interestingly, the priming effects of nicotine on morphine depended on the age of exposure to the drug. An increased behavioral response to morphine was not observed when nicotine exposure occurred during late adolescence (PND 50–57) or adulthood (PND 70–77), emphasizing early adolescence as a critical period to increase vulnerability to later opioid use.

Our results are consistent with previous behavioral studies that showed that exposure to nicotine during this developmental stage can have long-term consequences on later drug-taking behavior. Indeed, rats and mice pre-exposed to nicotine during adolescence demonstrated enhanced rewarding and reinforcing effects of nicotine (Adriani et al., 2003; Kota et al., 2009), alcohol (Spear et al., 2000) methamphetamine (Dao et al., 2011) and cocaine (Collins and Izenwasser, 2004; Dao et al., 2011, Alajaji et al. 2016).

The enhanced rewarding effects of morphine by nicotine, two drugs of abuse with different primary targets, suggests that nicotine induces long-term molecular changes in brain circuits implicated in the rewarding and reinforcing effects of drugs of abuse. For example, functional and structural alterations have been identified in the nucleus accumbens, amygdala, hippocampus and prefrontal cortex following adolescent nicotine exposure (see Smith et al., 2015 for review). Additionally, the mesocorticolimbic dopamine pathway, which is still developing during the adolescent period (Spear, 2000), shows lasting alterations in receptors and protein expression (Kobb and LeMoal, 2001; Nestler, 2001). A study by Doura et al. (2010) showed that adolescent rats that were subjected to chronic nicotine exhibited age-specific persistent gene expression changes in the ventral tegmental area. Moreover, nicotine exposure during adolescence induced a long-lasting increase in FosB in the nucleus accumbens and hippocampus of rats (Soderstrom et al., 2007). Our studies showed that nicotine treatment during early adolescence induced FosB, a stable splice variant of the transcription factor FosB that promotes reward and drug sensitization in the nucleus accumbens (Alajaji 2016). In fact, there was a (four-fold) greater induction of FosB in the NAc of nicotine-treated adolescent compared to adult mice. These adaptations in the mesolimbic system are likely to contribute to lasting enhanced drug reward seen in adulthood.

Another potential mechanism for nicotine priming effects on morphine-induced preference behavior in mice is through receptor adaption. Neuronal nicotinic receptors continue to develop during adolescence and higher levels of mRNAs for $\beta 2$ nicotinic subunits and $\alpha 4\beta 2^*$ high-affinity binding sites have been measured in adolescent compared to adult rodents (Azam et al., 2007; Levine et al., 2007). Moreover, exposure to nicotine in mice and rats during adolescence induces a long-lasting increase in brain $\alpha 4\beta 2^*$ nAChRs levels and functions upon reaching adulthood (Slotkin, 2002; Adriana et al., 2003; Kota et al., 2007). It is unlikely that these variations are attributable to the pharmacokinetic difference in nicotine metabolism because our results showed that nicotine blood levels in early adolescent and adult ICR were similar after repeated exposure to the nicotine (Alajaji et al., 2016). Previous studies reported reduced MOR expression in striatum and hippocampus following adolescent nicotine exposure in rats (Marco et al., 2006), whereas we found a slight increase in MOR-mediated G-protein activity only in the hippocampus. This might be explained by differences in species and treatment parameters or *in vitro* assessment (e.g. western blot versus DAMGO-stimulated [35 S]GTP γ S binding). Finally, adolescent pretreatment with nicotine enhanced morphine rewarding-like properties, it has no effect on the expression of physical dependence signs in morphine-dependent adult mice later in life. This suggest some region-specific neuronal regulatory mechanisms induced by nicotine during adolescence exposure. Finally, nicotine is not unique in terms of its long-term behavioral effects on morphine after adolescence exposure. Molet et al., (2013) showed that, in DBA/2J mice, early adolescent exposure to ethanol enhanced morphine CPP later in adulthood. Overall, the results from the current study support previous reports suggesting that adolescence is a unique developmental stage during which nicotine has long-term effects on future drug-taking behavior.

These findings have interesting implications in the ongoing opioid crisis. One approach to mitigate risk of opioid abuse is to identify vulnerable populations when prescribing opioid

medications (Volkow and McLellan, 2016). Our findings suggest that patients who smoked during adolescence might have an increased susceptibility to opioid use disorder as adults. Moreover, identification of molecular targets that underlie this vulnerability could provide novel treatment strategies.

5. Conclusions

We report here for the first time that exposure to nicotine in male mice for seven days during early adolescence enhances the preference to morphine in CPP following a five-week drug free period, indicating enhanced rewarding effects. In contrast, adolescent pretreatment with nicotine has no effect on expression of somatic withdrawal signs in morphine-dependent adult mice. These findings were not associated with an alteration in MOR-mediated G-protein activity in adulthood. While our studies involved male mice only, these results support previous reports suggesting that adolescence is a unique developmental stage during which nicotine has long-term effects on future drug-taking behavior. Further studies are needed to explore possible sex differences to these effects and to identify the neurotransmitters and mechanisms involved in increased vulnerability to drug abuse.

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Abbreviations:

nAChR	Nicotinic acetylcholine receptor
CPP	conditioned place preference
s.c.	subcutaneous injection

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Highlights

- Early adolescent nicotine exposure in mice enhances subsequent morphine reward.
- Early adolescent exposure in mice did not enhance subsequent morphine physical dependence
- Nicotine is a risk factor underlying adolescent susceptibility to morphine in mice

Figure 1. The effect of early-adolescent nicotine exposure on morphine-induced CPP in adult mice.

(A) Early adolescent (PND 28) male ICR mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) twice for seven days and were assessed for morphine preference in the CPP test on PND 70. X axis label represents mice conditioned with either saline or morphine (10 mg/kg, s.c.) * $p < 0.05$ from respective adolescent treatment (saline, nicotine 0.1 or nicotine 0.5)-saline control; ^ $p < 0.05$ from saline-morphine (B) Dose-response relationship for morphine-induced CPP in mice that were exposed to 0.5 mg/kg nicotine in adolescent (7-day protocol). * $p < 0.05$ from respective adolescent saline treatment-saline control; # $p < 0.05$ from respective saline-morphine control. Results are expressed as mean $\pm$ SEM of $n=8$ /group. Nic = nicotine.

Figure 2. The influence of adolescent period of nicotine exposure on cocaine condition preference in adulthood.

(A) Late adolescent (PND 50) mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) twice a day for 7 days and were assessed for morphine CPP test on PND 92. (B) Adult (PND 70) mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) two times per day for 7 days and were assessed for the morphine CPP test on PND 112. The legend represents the pretreatment group during early adolescence. X axis label represents mice conditioned with either saline or morphine (10 mg/kg, s.c.). Results are expressed as mean $\pm$ SEM of $n = 8$ /group. * $p < 0.05$ from respective saline control. Nic = nicotine.

Figure 3. The effect of early-adolescent nicotine exposure on morphine physical dependence in adult mice.

Early adolescent (PND 28) male ICR mice were injected s.c. with either saline or nicotine (0.5 mg/kg) twice for seven days and were assessed for morphine physical dependence on PND 70. All groups received naloxone (2 mg/kg, s.c.). Results are expressed as mean $\pm$ SEM of $n=8$ /group. * $p<0.05$ from respective saline control.

Figure 4. The effect of early-adolescent nicotine exposure on morphine-stimulated [35S]GTP γ S binding in adult mice.

Early adolescent (PND 28) male ICR mice were injected s.c. with either saline or nicotine (0.5 mg/kg) twice for seven days and were assessed for morphine-stimulated [35S]GTP γ S binding in the thalamus, striatum and hippocampus of adult mice on PND 70. Results are expressed as mean $\pm$ SEM of % stimulation above basal for n=7/group.