ORIGINAL INVESTIGATION

Age-Related Differences in the Disposition of Nicotine and Metabolites in Rat Brain and Plasma

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ABSTRACT

Introduction: Studies have evaluated the behavioral and neurochemical impact of nicotine administration in rodents. However, the distribution of nicotine and metabolites in rat brain and plasma as a function of age has not been investigated. This is a significant issue because human adolescents have a greater risk for developing nicotine addiction than adults, and reasons underlying this observation have not been fully determined. Thus, in this present study, we evaluated the impact of the transition from adolescence (postnatal day [PND 40]) to adulthood (PND 90) on nicotine distribution in rats.

Methods: PND 40, 60, and 90 rats received a single injection of (–) nicotine (0.8 mg/kg, subcutaneously). Liquid chromatography tandem-mass spectrometry was used to measure concentration of nicotine and metabolites in selected biological matrices.

Results: Nicotine, cotinine, and nornicotine were detected in rat striata and frontal cortex 30 min, 1 hr, 2 hr, and 4 hr after a single administration. These and several additional metabolites (nicotine-1'-oxide, cotinine-N-oxide, nornicotine, and *trans*-3'-hydroxycotinine) were also detected in plasma at these same timepoints. The mean concentration of nicotine in brain and plasma was lower in PND 40 versus PND 90 rats. In contrast, the mean concentration of nornicotine was higher in the plasma and brain of PND 40 versus PND 90 rats.

Conclusions: Nicotine and metabolite distribution differs between adolescent and adult rats. These data suggest that adolescent rats metabolize nicotine to some metabolites faster than adult rats. Further studies are needed to investigate the potential correlation between age, drug distribution, and nicotine addiction.

INTRODUCTION

Nicotine (NIC) is a potent psychostimulant, and its use and abuse are important public health problems. Interestingly, most adult smokers who are addicted to cigarettes began smoking when they were adolescents (Centers for Disease Control and Prevention, 1994; Breslau & Peterson, 1996; Lynch & Bonnie, 1994). Furthermore, adolescents have a greater risk for developing NIC addiction than adults, although reasons underlying this finding have not been fully elucidated (Kandel & Chen, 2000). Previous clinical studies have evaluated NIC metabolism in smokers to determine its potential role in addiction and withdrawal (Rubinstein, Benowitz, Auerback, & Moscicki, 2008; Sofuoglu, Herman, Nadim, & Jatlow, 2012). Among these, it was demonstrated that individuals who metabolize NIC quickly have both an increased risk for developing NIC addiction and greater negative symptoms upon NIC withdrawal (Benowitz, 2010; Rubinstein et al.,

2008; Sofuoglu et al., 2012). In addition, studies with adolescents demonstrated that adolescents with an inactive allele in CYP2A6, the major enzyme that metabolizes NIC in humans (Hukkanen, Jacob, & Benowitz, 2005), progress to NIC dependence at a slower rate than those with normal NIC metabolism (Audrain-McGovern et al., 2007). These findings suggest that higher rates of NIC metabolism correlate with increased withdrawal symptoms and higher risk for developing NIC addiction (Audrain-McGovern et al., 2007; Moolchan et al., 2009). In contrast, another study reported that although adolescents with inactive alleles in CYP2A6 smoke fewer cigarettes, they are at higher risk for developing NIC addiction (O'Loughlin et al., 2004). Accordingly, differences in drug disposition into target tissue as a function of age and metabolism may influence the likelihood of developing NIC addiction.

Previous studies have shown that chronological age can affect biotransformation of drugs in humans and rodents

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(Klingler, 2005). However, the available data regarding differences in NIC metabolism in human and rodent adolescents and adults are ambiguous. For example, no age-related differences in CYP2A6 expression across a wide range of ages, 2–64 years, have been seen (Al Koudsi, Hoffmann, Assadzadeh, & Tyndale, 2010). In contrast, others have shown that CYP2A6 activity changes with age in a 25- to 65-year-old cohort of patients (Johnstone et al., 2006). In rodents, CYP2B1, the major NIC metabolizing enzyme in rats, is found at higher levels in the liver of adolescent rats (postnatal day [PND] 21) in comparison with adult rats (PND 84), although no differences in the activity of this enzyme were found between these two ages (Yun et al., 2010). In contrast, others have found higher concentrations of NIC metabolites in liver and kidneys of adolescent rats in comparison with adult rats, suggesting a higher rate of NIC metabolism in adolescents (Kyerematen, Owens, Chattopadhyay, deBethizy, & Vesell, 1988). In summary, the effects of age-related differences in NIC metabolism on NIC addiction are still unclear.

Several preclinical studies aimed to investigate the increased risk of NIC addiction in adolescents have demonstrated that younger rats find NIC more rewarding and less aversive than adult rats (Belluzzi, Lee, Oliff, & Leslie, 2004; Shram, Funk, Li, & Lê, 2006; Torres, Tejeda, Natividad, & O'Dell, 2008; Wilmouth & Spear, 2004). In these studies, periadolescent rats (PND 28–38) spent more time in an environment previously paired with moderate to high doses of NIC than adult rats (PND 63–73) in a conditioned place-preference test (Belluzzi et al., 2004; Shram et al., 2006; Torres et al., 2008) and adolescents consumed more NIC than adults in a conditioned taste-avoidance test, suggesting that adolescent rats are less sensitive to the aversive effects of NIC (Shram et al., 2006; Torres et al., 2008; Wilmouth & Spear, 2004). In contrast, NIC self-administration did not differ between adolescent and adult rats indicating that different models, that is, conditioned place preference and self-administration, can reveal distinct results (Shram et al., 2008). In addition to studies evaluating the rewarding properties of NIC, others have shown reduced withdrawal symptoms to NIC in adolescent rats when assessed by intracranial self-stimulation procedure and fewer somatic signs including eye blinks, body shakes, chews, cheek tremors, escape attempts, foot licks, and others in comparison with adult rats (O'Dell et al., 2006). Altogether, NIC reward and abuse studies have revealed behavioral differences between adolescent and adult rats, but few of them have investigated any neurochemical differences among these ages that could explain the behavioral data.

Few studies have investigated the concentrations of NIC and cotinine (COT), the major metabolite of NIC, in biological matrices of adolescent and adult rats. One study reported that plasma levels of NIC in adolescent rats (PND 36–45) were lower in comparison with adult rats (after PND 70) without differences in COT levels among these ages (O'Dell et al., 2006). In these studies, investigators increased the dose of NIC in the adolescent group in order to achieve comparable drug levels with the adult group and correlate the outcome measure (withdrawal signs). Results indicated a dose–response relationship in adults but not in adolescents, that is, increased NIC and COT levels in plasma increased withdrawal signs in adults but not in adolescents (O'Dell et al., 2006). Similarly, another study investigated the concentrations of several NIC metabolites in different strains of rats after *in vitro* administration of NIC to liver and kidney tissues and found higher levels of total

metabolite formation in Sprague-Dawley adolescent rats (PND 40) in comparison with PND 100 (Kyerematen et al., 1988). In another study aimed to demonstrate that NIC-induced changes in the expression of nicotinic acetylcholine receptors differ between periadolescent and adult rats, plasma and brain levels of NIC and COT were reported. In both biological matrices, NIC and COT concentrations were lower in adolescents compared with adults suggesting that pharmacokinetic differences could have contributed to the distinct pattern of nicotinic acetylcholine receptor expression (Doura, Gold, Keller, & Perry, 2008). In both cases, NIC administration occurred via osmotic minipumps, and it was delivered for several days (7–14 days).

As noted above, the impact of NIC metabolism during development has important translational relevance. However, few preclinical studies have evaluated whether disposition of NIC and metabolites is different between adolescent and adult rats. Thus, the purpose of this study was to investigate the impact of the transition from adolescence (defined herein as PND 40) to adulthood (defined herein as PND 90) on NIC disposition in two selected brain regions and plasma of rats. Since some of the NIC metabolites such as COT and nornicotine (NNIC) are also known to potentially have pharmacological activity in the brain (Crooks & Dwoskin, 1997), we also investigated the disposition of several NIC metabolites.

Striatum and frontal cortex tissues were particularly selected for study because of their role in addiction (as reviewed, for example, by Koob & Volkow, 2010). As defined by Koob and Volkow, the dorsal striatum (caudate and putamen) is involved in the anticipation stage of addiction and transition to habitual compulsive use. The frontal cortical region is implicated in the executive function and in the craving stage of addiction. This region is also known to be undeveloped until young adulthood in humans and rodents (Spear, 2000). Further studies evaluating NIC disposition in other brain regions also involved in drug addiction such as the ventral tegmental area and nucleus accumbens would be useful to the this field. Therefore, this current study explored whether age influences the distribution of NIC and metabolites in plasma and in brain regions that are affected by drug addiction.

METHODS

Animals

A total of 90 male Sprague-Dawley rats (Charles River Laboratories), age PND 40 (adolescents: 130–170 g, based on Charles River Laboratories, PND 38–42 lie within this weight range), PND 60 (young adults: 230–270 g, based on Charles River Laboratories, PND 55–62 lie within this weight range), or PND 90 (adults: 390–430 g), were treated with a single administration of (–) NIC (0.8 mg free base/kg, subcutaneously) or saline vehicle (1 ml/kg, subcutaneously). The age of the animals were selected based upon reviews by Spear (2000), Tirelli et al. (2003), and McCormick and Matthews (2010), in which early adolescence period is defined to span from PND 21 to 34, a mid-adolescence period from PND 34 to 46, and a late adolescence period from PND 46 to 59. Rats were maintained under controlled lighting and temperature with food and water provided *ad libitum*. The NIC-treated animals were sacrificed by decapitation 30 min, 1 hr, 2 hr, or 4 hr after treatment, and saline-treated animals

were sacrificed 2 hr after injection. Striata and frontal cortex tissue were dissected on ice and immediately frozen on dry ice. Trunk blood was collected in sodium heparin-containing tubes and centrifuged at $3,000 \times g$ for 15 min to obtain plasma. Brains utilized for the preparation of calibration curve samples and quality control samples were obtained from nontreated rats and tissues immediately frozen on dry ice. Brain tissue and plasma samples were stored at -80°C until day of analysis. All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Utah Institutional of Animal Care and Use Committee.

Chemicals and Reagents

Calibrators, quality control working solutions, and deuterated internal standards were prepared in high-performance liquid chromatography (HPLC)-grade methanol as described in Miller, Norris, Rollins, Tiffany, and Wilkins (2010) and Vieira-Brock, Miller, Nielsen, Fleckenstein, and Wilkins (2011). (–) NIC hydrogen tartrate salt ($\geq 98\%$) was obtained from Sigma and used for preparation of liquid chromatography tandem-mass spectrometry (LC-MS/MS) calibrators and quality control samples, as well as for animal administration studies. ($\pm$) Cotinine- d_3 (COT- d_3) was obtained from Cerilliant. The following reference standards and deuterated internal standards were obtained from Toronto Research Chemicals: *trans*-3'-hydroxycotinine (3-HC) and *trans*-3'-hydroxycotinine- d_3 (3-HC- d_3); (S)-Cotinine-N-oxide (CNO) and (R,S)-Cotinine-N-oxide-methyl- d_3 (CNO- d_3); (–) COT; cotinine N- β -D-glucuronide (COT GLUC) and cotinine- d_3 N- β -D-glucuronide (COT GLUC- d_3); (R,S)-Norcotinine (NCOT) and (R,S)-Norcotinine pyridyl- d_4 (NCOT- d_4); ($\pm$) nicotine- d_3 (NIC- d_3); nicotine-N-(4-deoxy-4,5-didehydro)- β -D-glucuronide (NIC GLUC) and nicotine-N-(4-deoxy-4,5-didehydro)- β -D-glucuronide-methyl- d_3 (NIC GLUC- d_3); (R,S)-Nornicotine (NNIC) and (R,S)-Nornicotine- d_4 (NNIC- d_4); (1'S, 2'S)-Nicotine-1'-oxide and (1'R, 2'S)-Nicotine-1'-oxide mixture (NNO), and ($\pm$)-*trans*-nicotine-1'-oxide-methyl- d_3 (NNO- d_3). Solid-phase extraction cartridges (Oasis® MCX and HLB [60 mg, 3 ml]) were obtained from Waters. HPLC-grade methanol, dichloromethane (DCM), and isopropyl alcohol (IPA) were obtained from Honeywell Burdick & Jackson. Ammonium acetate and glacial acetic acid were obtained from Spectrum. Concentrated hydrochloric acid (HCl), concentrated formic acid, and concentrated ammonium hydroxide were obtained from Fisher Scientific. All chemicals and reagents were HPLC grade ($\geq 99\%$ purity).

Calibrators and Quality Control Samples

Calibrators and quality control working solutions were prepared in methanol at concentrations of 10 $\mu\text{g}/\text{ml}$, 1 $\mu\text{g}/\text{ml}$, 0.1 $\mu\text{g}/\text{ml}$, and 0.01 $\mu\text{g}/\text{ml}$ for 3-HC, CNO, COT, COT GLUC, NCOT, NIC hydrogen tartrate salt (weight corrected for NIC free base), NIC GLUC, NNIC, and NNO. The NIC hydrogen tartrate salt was used as it was determined to be more stable in solution than NIC free base. The analytical balance used was XS3DU Mettler Toledo microbalance. All working solutions were stored in the freezer at -20°C and equilibrated to room temperature before each analytical run. Finally, a single combined methanolic deuterated internal standard working solution

was prepared at 1 $\mu\text{g}/\text{ml}$ and contained 3-HC- d_3 , CNO- d_3 , COT- d_3 , COT GLUC- d_3 , NCOT- d_4 , NIC- d_3 , NIC GLUC- d_3 , NNIC- d_4 , and NNO- d_3 . The working deuterated internal standard solution was stored in the freezer at -20°C as well. For information regarding the final concentration of calibrators and quality control samples, as well as lower limit of quantification and detection for each analyte, see studies by Miller et al. (2010) and Vieira-Brock et al. (2011).

Sample Preparation and Drug Extraction

Brain and plasma samples were extracted and analyzed as per our previously published analytical procedures (Miller et al., 2010; Vieira-Brock et al., 2011). Briefly, rat brain tissues from drug administration studies were weighed and homogenized in cold Milli-Q water utilizing a 2 ml-Wheaton Safe-Grind tissue grinder. For frontal cortex (weight approximately 45 mg), the right amount of water volume added was to achieve a final concentration of 20 mg tissue/ml. For striatal tissue, the whole left striatum (weight approximately 25 mg) was homogenized in 1 ml water volume. Thawed plasma samples were diluted by adding 750 μl of analyte-free rat plasma (BioChemed) to 250 μl of study sample and mixed. Calibrators and quality control samples were prepared in analyte-free brain homogenates obtained from homogenization of whole brains of nontreated rats in sufficient volume of Milli-Q water to achieve a final concentration of 20 mg tissue/ml. Calibrators were prepared by fortification of 1 ml analyte-free rat plasma or 1 ml analyte-free brain homogenate with increasing concentrations of NIC and metabolites (see references for detailed concentrations). Quality control samples were prepared by fortifying 1 ml of analyte-free brain homogenate or plasma with NIC and metabolites at low, medium, and high concentrations (see references for detailed concentrations). All brain homogenate samples, including study samples, were fortified with 25 ng/ml deuterated internal standards prior to extraction. All plasma samples were fortified with 50 ng/ml deuterated internal standards. Samples (calibrators, quality control, and study samples) were acidified to precipitate proteins and to ensure extraction on MCX cartridges as described by the manufacturer (Waters®). Samples were centrifuged to aid with clean-up, brain supernatant was loaded onto conditioned MCX cartridges, and plasma supernatant was transferred onto conditioned MCX and HLB combination cartridges. Analytes were eluted with 5% (v/v) ammoniated methanol and DCM:IPA:aqueous ammonium hydroxide (for brain only) and dried until completion at 40°C (Zymark Turbovap®). The samples were reconstituted with initial mobile phase condition (10 mM ammonium acetate [pH 5.0] + 0.001% formic acid: HPLC-grade methanol [85:15 v/v]).

LC-MS/MS Conditions and Criteria for Identification of Analytes

The LC-MS/MS method was performed as described in Vieira-Brock et al. (2011). Analytes were resolved on a Discovery® HS F5 HPLC column (10 cm $\times$ 4 mm $\times$ 3 μm , Supelco®) and Acquity UPLC® system (Waters®). The mobile phase consisted of a gradient elution of 10 mM ammonium acetate with 0.001% formic acid at pH 5.0 (aqueous) and methanol (organic). Initial conditions were as follows: 85% aqueous and 15% organic phase, increased linearly to 76% organic phase at 11.6 min, and held for 3.4 min to re-equilibrate the HPLC

column. The flow rate was set at 0.6 ml/min. Mass spectrometric control and analysis were performed on a Quattro Premier XE™ triple quadrupole mass spectrometer (Waters®) equipped with MassLynx™ v4.1 software. The mass spectrometry used positive-ion electrospray ionization and multiple reaction monitoring (MRM) transition data acquisition.

The limits of quantification (LOQ) for each analyte and matrix were previously identified by our laboratory (Miller et al., 2010; Vieira-Brock et al., 2011). In plasma, the LOQ for NIC and each metabolite was 1 ng/ml. Of note, the selected amount of sample used for this current study was 250 µl of plasma in order to be able to identify and quantify all of the metabolites, given that NIC and COT concentrations are much higher than some of the minor metabolites. Thus, the data reported herein are adjusted from measured concentrations (in 250 µl sample) to the final reported concentration in 1 ml. The LOQ for brain samples are as follows 25 pg/mg for COT, NNIC, 3-HC, CNO, NNO, NCOT and NIC GLUC, and 100 pg/mg for NIC and COT GLUC.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 5.01 software. Mean concentrations $\pm$ standard deviations (SD) were analyzed using one-way analysis of variance followed by Newman–Keuls post-hoc test. A Kruskal–Wallis nonparametric test followed by Dunn's post-hoc test was used whenever homogeneity of variance was not achieved. Differences among groups were considered significant if the probability of error was less than 5% ($p < .05$).

The area under the brain and plasma concentration–time curves (AUC) were calculated using GraphPad Prism 5.01 software from 30 min to 4 hr timepoints. Half-life ($t_{1/2}$) and elimination constant (K_{el}) calculations followed equations $t_{1/2} = \ln(2)/K_{el}$ and $K_{el} = \ln(C_1) - \ln(C_2)/t_2 - t_1$.

Figure 1. Nicotine (NIC) distribution in plasma differs among age. PND 40, 60, and 90 rats ($n = 4-6$) were treated with a single injection of NIC (0.8 mg/kg, subcutaneously) and sacrificed 30 min, 1 hr, 2 hr, and 4 hr after. Data shown as mean NIC concentration $\pm$ SD in ng per ml of plasma. Statistical analysis performed by one-way analysis of variance with significance achieved by $p < .05$. #, Mean concentration significantly different from PND 40. +, Mean concentration significantly different from PND 60. ^, Mean concentration significantly different from PND 90.

RESULTS

NIC, COT, NNIC, and NNO were detected and quantified in plasma of PND 40, 60, and 90 rats 30 min, 1 hr, 2 hr, and 4 h after a single NIC administration (Figures 1–3 and Table 1). Additional metabolites (CNO, NCOT, and 3-HC) were detected in some plasma samples and only found at concentrations above the LOQ at specific age and timepoints (Table 1). In brain, NIC, COT, and NNIC were quantified in striata and frontal cortex at the same four timepoints and all age groups (Table 2). NIC GLUC and COT GLUC were not detected in either brain or plasma. NIC and metabolites were not detected in samples from saline-treated animals.

Figure 2. Nornicotine (NNIC) distribution in plasma differs among age. PND 40, 60, and 90 rats ($n = 4-6$) were treated with a single injection of NIC (0.8 mg/kg, subcutaneously) and sacrificed 30 min, 1 hr, 2 hr, and 4 hr after. Data shown as mean NNIC concentration $\pm$ SD in ng per ml of plasma. Statistical analysis performed by one-way analysis of variance with significance achieved by $p < .05$. #, Mean concentration significantly different from PND 40. +, Mean concentration significantly different from PND 60. ^, Mean concentration significantly different from PND 90.

Figure 3. Cotinine (COT) distribution in plasma as a function of age. PND 40, 60, and 90 rats ($n = 4-6$) were treated with a single injection of NIC (0.8 mg/kg, subcutaneously) and sacrificed 30 min, 1 hr, 2 hr, and 4 hr after. Data shown as mean NNIC concentration $\pm$ SD in ng per ml of plasma. Statistical analysis performed by one-way analysis of variance with significance achieved by $p < .05$. #, Mean concentration significantly different from PND 40. +, Mean concentration significantly different from PND 60.

Table 1. Nicotine-1'-Oxide (NNO), Norcotinine (NCOT), Cotinine-N-Oxide (CNO), and *Trans*-3'-Hydroxycotinine (3-HC) Mean Concentrations $\pm$ SD in Plasma of PND 40, 60, and 90 Rats That Were Treated With a Single NIC Administration (0.8 mg/kg, Subcutaneously) and Sacrificed at 30 min, 1 hr, 2 hr, and 4 hr Later ($N = 4-6$)

Analyte, timepoint	Mean concentration (SD) (ng/ml plasma)			Analyte, timepoint	Mean concentration (SD) (ng/ml plasma)		
	PND 40	PND 60	PND 90		PND 40	PND 60	PND 90
NNO, 30 min	106.6 (13.0) ^{a,b}	81.0 (13.0) ^c	73.5 (9.8) ^c	NCOT, 30 min	4.1 (1.0)	<LOQ	<LOQ
NNO, 1 hr	81.3 (8.5)	74.0 (11.9)	73.5 (6.4)	NCOT, 1 hr	5.4 (0.7) ^{a,b}	3.9 (0.7) ^c	3.7 (0.5) ^c
NNO, 2 hr	40.1 (7.5)	44.7 (8.4)	40.7 (10.1)	NCOT, 2 hr	7.1 (1.3)	6.1 (1.2)	5.5 (1.3)
NNO, 4 hr	7.6 (2.2)	9.9 (5.7)	9.6 (1.8)	NCOT, 4 hr	8.5 (1.9)	6.6 (2.1)	6.6 (0.9)
CNO, 30 min	3.7 (0.2)	<LOQ	<LOQ	3-HC, 30 min	<LOQ	<LOQ	<LOQ
CNO, 1 hr	5.5 (0.7) ^b	4.4 (0.3)	3.8 (0.2) ^c	3-HC, 1 hr	<LOQ	<LOQ	<LOQ
CNO, 2 hr	6.2 (1.5) ^b	5.2 (0.6)	4.5 (0.4) ^c	3-HC, 2 hr	<LOQ	<LOQ	<LOQ
CNO, 4 hr	8.9 (1.3) ^{a,b}	6.9 (1.3) ^c	5.9 (0.7) ^c	3-HC, 4 hr	4.0 (0.4)	<LOQ	<LOQ

Note. LOQ = limit of quantification; PND = postnatal day.

NNO, NCOT, CNO, and 3-HC concentrations were measured by liquid chromatography tandem-mass spectrometry as described in the Methods section. Either one-way analysis of variance or Kruskal–Wallis statistic test was used to assess differences in analyte concentration among PND 40, 60, and 90 rats at each timepoint.

^aSignificantly different from PND 60.

^bSignificantly different from PND 90 ($p < .05$).

^cSignificantly different from PND 40.

Table 2. Nicotine (NIC), Cotinine (COT), and Norcotinine (NNIC) Mean Concentrations $\pm$ SD in Striatal and Frontal Cortical Tissues of PND 40, 60, and 90 Rats That Were Treated With a Single NIC Administration (0.8 mg/kg, Subcutaneously) and Sacrificed at 30 min, 1 hr, 2 hr, and 4 hr Later ($N = 4-6$)

Analyte, timepoint	Mean concentration (SD) (pg/mg striatum wet weight)			Mean concentration (SD) (pg/mg frontal cortex wet weight)		
	PND 40	PND 60	PND 90	PND 40	PND 60	PND 90
NIC, 30 min	600.0 (136.4) ^a	571.7 (92.0) ^a	868.0 (72.2) ^{b,c}	492.5 (64.2) ^{a,b}	780.0 (167.8) ^c	741.7 (98.3) ^c
NIC, 1 hr	315.0 (74.2)	388.3 (86.8)	411.7 (29.3)	322.0 (67.1) ^{a,b}	498.3 (29.9) ^c	565.0 (98.3) ^c
NIC, 2 hr	126.7 (21.6) ^a	165.0 (32.1)	255.0 (69.8) ^c	191.7 (59.4) ^{a,b}	275.0 (47.6) ^c	302.0 (39.0) ^c
NIC, 4 hr	<LOQ	<LOQ	80.0 (23.7)	<LOQ	<LOQ	120.0 (37.4)
COT, 30 min	90.2 (28.5)	65.0 (12.2)	76.0 (15.2)	70.0 (21.6)	73.3 (5.2)	58.3 (7.5)
COT, 1 hr	115.0 (29.5)	111.7 (27.9)	91.7 (7.5)	131.7 (9.8) ^{a,b}	113.3 (16.3) ^c	106.7 (10.3) ^c
COT, 2 hr	128.3 (31.9)	118.3 (11.7)	136.7 (28.7)	153.3 (8.2)	141.7 (9.8)	145.0 (16.4)
COT, 4 hr	148.3 (21.4)	123.3 (28.0)	156.7 (19.7)	161.7 (14.7)	115.0 (33.9) ^a	180.0 (39.0)
NNIC, 30 min	30.0 (21.9)	21.7 (4.1)	20.0 (0.0)	45.0 (12.9)	40.0 (6.3)	40.0 (6.3)
NNIC, 1 hr	43.3 (10.3) ^{a,b}	30.0 (8.9) ^c	25.0 (8.4) ^c	66.7 (5.2) ^{a,b}	47.1 (8.2) ^c	50.0 (6.3) ^c
NNIC, 2 hr	43.3 (10.3) ^{a,b}	28.3 (4.1) ^c	25.0 (5.5) ^c	70.0 (8.9) ^{a,b}	50.0 (8.9) ^c	56.0 (8.9) ^c
NNIC, 4 hr	33.3 (5.2) ^{a,b}	20.0 (6.3) ^c	23.3 (5.2) ^c	51.7 (4.1) ^b	33.3 (12.1) ^c	41.7 (9.8)

Note. PND = postnatal day.

NIC, NNIC, and COT concentrations were measured by liquid chromatography tandem-mass spectrometry as described in the Methods section. Either one-way analysis of variance or Kruskal–Wallis statistical test was used to assess differences in analyte concentration among PND 40, 60 and 90 rats at each timepoint.

^aSignificantly different from PND 90 ($p < .05$).

^bSignificantly different from PND 60.

^cSignificantly different from PND 40.

Results presented in Figure 1 demonstrate that the mean concentration of NIC in plasma was significantly lower in PND 40 versus PND 90 ($q = 6.067$, $p < .01$) and PND 60 versus PND 90 ($q = 3.139$, $p < .05$) rats as assessed 30 min after a single NIC injection ($F(2,12) = 9.29$, $p = .004$). After 1 h of NIC administration, the mean concentration of NIC in plasma was significantly lower in PND 40 versus PND 60 ($q = 5.560$, $p < .01$) and PND 40 versus PND 90 ($q = 8.286$, $p < .001$)

($F(2,15) = 17.83$, $p < .001$). At the 2 hr timepoint, the mean concentration of NIC in plasma was significantly lower in PND 40 versus PND 60 ($q = 8.273$, $p < .001$), PND 40 versus PND 90 ($q = 13.39$, $p < .001$), and PND 60 versus PND 90 ($q = 5.120$, $p < .01$) ($F(2,15) = 45.67$, $p < .001$). Finally, at the 4 hr timepoint, the mean concentration of NIC in plasma was significantly lower in PND 40 versus PND 90 rats (Kruskal–Wallis statistic = 15.17, $p < .001$). The K_{el} of NIC in PND 40

Table 3. Pharmacokinetics Parameters in Order of Age—PND 40, PND 60, and PND 90 Rats

	AUC from 30 min to 4 hr (SD) (pg·h/mg for brain or ng·h/ml for plasma)									K _{el} (h ⁻¹) (SD)			t _{1/2} (min) (SD)		
	NIC			COT			NNIC			NIC			NIC		
	PND 40	PND 60	PND 90	PND 40	PND 60	PND 90	PND 40	PND 60	PND 90	PND 40	PND 60	PND 90	PND 40	PND 60	PND 90
Striatum	792 (122) ^a	853 (50) ^a	1201 (125) ^{b,c}	470 (55)	417 (45)	465 (52)	146 (18) ^{a,b}	96 (8) ^c	90 (10) ^c	0.89 (0.1) ^{a,b}	0.73 (0.1) ^c	0.69 (0.1) ^c	47 (5.7)	58 (8.3)	61 (10)
Frontal cortex	840 (28) ^{a,b}	1,258 (78) ^c	1,334 (113) ^c	521 (14)	449 (47) ^{a,c}	507 (38)	221 (4.3) ^{a,b}	163 (26) ^c	185 (14) ^c	0.81 (0.1) ^a	0.81 (0.2) ^a	0.53 (0.1) ^{b,c}	52 (5.5) ^a	54 (11) ^a	80 (9.4) ^{b,c}
Plasma	193 (11) ^{a,b}	284 (30) ^{a,c}	345 (13) ^{b,c}	950 (76)	927 (82)	942 (126)	41 (3.4) ^{a,b}	27 (2.2)	24 (2.4)	1.19 (1.2) ^a	0.89 (0.2)	0.66 (0.1) ^c	35 (1.2) ^a	48 (7.0)	63 (6.0) ^c

Note. AUC = area under the brain and plasma concentration–time curves; COT = cotinine; NIC = nicotine; NNIC = normicotine; PND = postnatal day.

AUC, elimination constant (K_{el}), and half-life (t_{1/2}) ± SD for NIC, COT, and NNIC in brain and plasma. (N = 4–6). NIC, NNIC, and COT AUC, K_{el}, and t_{1/2} were measured as described in the Methods section. Either one-way analysis of variance or Kruskal–Wallis statistical test was utilized to assess differences in analyte concentration among PND 40, 60, and 90 rats each timepoint.

^aSignificantly different from PND90 (p < .05).

^bSignificantly different from PND60.

^cSignificantly different from PND40.

rats' plasma was higher than in PND 90 rats, and the $t_{1/2}$ of NIC in plasma of PND 40 rats was lower than in PND 90 rats (Kruskal–Wallis statistic = 11.95, $p = .0025$) (Table 3).

In contrast, as shown in Figure 2, plasma NNIC mean concentration was higher in PND 40 versus PND 60 ($q = 5.23$, $p < .01$), in PND 40 versus PND 90 ($q = 8.43$, $p < .001$) and in PND 60 versus PND 90 ($q = 3.19$, $p < .05$) rats at the 30 min timepoint ($F(2,12) = 17.81$, $p < .001$). At the 1 hr timepoint, NNIC mean concentration was higher in PND 40 versus PND 60 ($q = 6.867$, $p < .05$), PND 40 versus PND 90 ($q = 11.10$, $p < .001$), and in PND 60 versus PND 90 ($q = 4.229$, $p < .05$) ($F(2,15) = 31.36$, $p < .001$). After 2 h of NIC administration, the mean concentration of NNIC in plasma was higher in PND 40 versus PND 60 ($q = 4.88$, $p < .01$) and in PND 40 versus PND 90 ($q = 6.36$, $p < .01$) ($F(2,15) = 11.08$, $p = .001$). Finally, at the 4 hr timepoint, plasma NNIC mean concentration was higher in PND 40 versus PND 60 ($q = 5.83$, $p < .01$) and in PND 40 versus PND 90 ($q = 4.0$, $p < .05$) ($F(2,14) = 8.50$, $p = .003$). COT concentrations in plasma were mostly similar among age groups (Figure 3). The mean concentration of COT only differed at the 1 hr timepoint with measured values lower in PND 90 versus PND 40 ($q = 4.56$, $p < .05$) and in PND 90 versus PND 60 ($q = 3.29$, $p < .05$) rats ($F(2,15) = 5.53$, $p = .016$).

Several additional metabolites were also detected in plasma and demonstrated similar patterns (Table 1). The mean concentration of NNO was significantly higher in PND 40 versus PND 60 ($q = 4.59$, $p < .01$) and in PND 40 versus PND 90 rats ($q = 6.16$, $p < .01$) at the 30 min timepoint ($F(2,12) = 9.90$, $p = .003$), although no differences among age groups were observed at 1 hr, 2 hr, and 4 hr timepoints. Similarly, the mean concentration of NCOT was significantly higher in PND 40 versus PND 60 ($q = 5.38$, $p < .01$) and in PND 40 versus PND 90 rats ($q = 7.06$, $p < .001$) at the 1 hr timepoint ($F(2,15) = 13.59$, $p < .001$). Moreover, CNO mean concentration was higher in PND 40 versus PND 90 rats at 1 hr (Kruskal–Wallis statistic = 14.56, $p < .001$), 2 hr (Kruskal–Wallis statistic = 7.14, $p = .028$), and 4 hr timepoints ($q = 6.46$, $p < .01$ for PND 40 versus 90 comparison and $q = 4.16$, $p < .05$ for PND 40 versus 60 comparison; $F(2,15) = 10.71$, $p = .001$). Finally, although 3-HC mean concentrations were detected in PND 40, 60, and 90 rats at 1 hr, 2 hr, and 4 hr timepoints, they were only quantifiable at 4 hr timepoint in PND 40 rat plasma indicating higher concentrations in PND 40 rats (Table 1).

The mean concentrations of NIC in striatal tissues also differed among the age groups. Following 30 min of a single NIC injection, the mean concentration of NIC was lower in PND 40 and PND 60 versus PND 90 rats ($q = 5.68$, $p < .01$ and $q = 6.95$, $p < .001$, respectively) ($F(2,12) = 13.75$, $p < .001$) (Table 2). After 2 hr of treatment, NIC mean concentration was lower in PND 40 versus PND 90 rats (Kruskal–Wallis statistic = 11.06, $p = .004$) (Table 2). At the 4 hr timepoint, NIC mean concentration was lower than the LOQ in PND 40 and PND 60 but quantified in PND 90 rats (Table 2). Similar to results found in plasma, the K_{el} of NIC in striatal tissue of PND 40 rats was higher than both, PND 60 ($q = 3.38$, $p < .05$) and PND 90 rats ($q = 3.93$, $p < .05$) ($F(2,12) = 4.33$, $p = .0384$) (Table 3). The $t_{1/2}$ of NIC in adolescent striata did not achieve statistical significance ($F(2,12) = 3.21$, $p = .077$) (Table 3).

Similarly, the mean concentration of NIC in frontal cortex was significantly lower in adolescent versus adult rats at 30 min (PND 40 vs. PND 60: $q = 5.06$, $p < .01$; PND 40 vs. PND 90:

$q = 4.39$, $p < .01$; $F(2,13) = 7.11$, $p = .008$), 1 hr (PND 40 vs. PND 60: $q = 6.09$, $p < .001$; PND 40 vs. PND 90: $q = 8.40$, $p < .001$; $F(2,15) = 18.83$, $p < .001$) and 2 hr timepoints (PND 40 vs. PND 60: $q = 4.08$, $p < .05$; PND 40 vs. PND 90: $q = 5.15$, $p < .01$; $F(2,14) = 7.48$, $p = .006$). At the 4 hr timepoint, NIC mean concentration was lower than the LOQ in PND 40 and PND 60 but not in PND 90 rats (Table 2). The K_{el} of NIC in frontal cortex of PND 40 and PND 60 rats was higher than the K_{el} of NIC in frontal cortex of PND 90 rats ($q = 4.22$, $p < .05$ and $q = 4.51$, $p < .05$, respectively; $F(2,12) = 6.69$, $p = .011$). The $t_{1/2}$ of NIC in PND 40 versus PND 90 rats ($p < .01$) and PND 60 versus PND 90 rats ($p < .05$) were also lower in frontal cortical tissues (Kruskal–Wallis statistic = 10.49, $p = .0053$) (Table 3).

As demonstrated in Table 2, COT concentrations in the striatum were similar among PND 40, 60, and 90 rats as assessed 30 min ($F(2,14) = 2.06$, $p = .164$), 1 hr (Kruskal–Wallis statistic = 3.29, $p = .193$), 2 hr ($F(2,15) = 0.76$, $p = .482$) and 4 hr ($F(2,15) = 3.32$, $p = .064$) after a single NIC injection. In contrast, the mean concentration of NNIC in the striatum was higher in PND 40 versus PND 60 ($q = 3.53$, $p < .05$) and in PND 40 versus PND 90 rats ($q = 4.86$, $p < .01$) as assessed at 1 hr after a single NIC administration ($F(2,15) = 6.30$, $p = .010$). NNIC mean concentration in the striatum was also higher in PND 40 versus PND 60 ($q = 5.14$, $p < .01$) and in PND 40 versus PND 90 rats ($q = 6.28$, $p < .01$) at the 2 hr timepoint ($F(2,15) = 11.20$, $p = .001$) and at the 4 hr timepoint (PND 40 vs. PND 60: $q = 4.72$, $p < .05$; PND 40 vs. PND 90: $q = 4.05$, $p < .05$; $F(2,15) = 9.29$, $p = .002$) (Table 2). In frontal cortical tissues, COT mean concentrations were similar among PND 40, 60, and 90 rats when assessed 30 min ($F(2,13) = 2.60$, $p = .112$) or 2 hr ($F(2,15) = 1.50$, $p = .255$) after single NIC administration (Table 2). However, COT mean concentration in the frontal cortex at 1 hr timepoint was higher in PND 40 versus PND 60 ($q = 3.59$, $p < .05$) and in PND 40 versus PND 90 rats ($q = 4.89$, $p < .001$; $F(2,15) = 6.42$, $p = .009$) (Table 2). The mean concentration of NNIC in the frontal cortex of PND 40 rats was higher than that in both PND 60 ($q = 7.35$, $p < .001$) and PND 90 rats ($q = 6.12$, $p < .001$) at 1 hr after NIC administration ($F(2,15) = 15.50$, $p < .001$). The mean concentration of NNIC in the frontal cortex was also higher at the 2 hr timepoint in PND 40 versus PND 60 rats ($q = 5.48$, $p < .01$) and in PND 40 versus PND 90 rats ($q = 3.66$, $p < .05$; $F(2,15) = 5.63$, $p = .015$) and at the 4 hr timepoint (PND 40 vs. PND 60: $q = 4.82$, $p < .05$; $F(2,15) = 5.83$, $p = .013$) (Table 2).

The AUC for NIC in PND 40 and PND 60 striata were lower than in PND 90 ($q = 8.39$, $p < .001$ and $q = 7.49$, $p < .001$, respectively; $F(2,12) = 21.32$, $p < .001$) (Table 3). In contrast, the AUC for NNIC in PND 40 striata was higher than in PND 60 and 90 rats ($q = 9.54$, $p < .001$ and $q = 10.73$, $p < .001$, respectively; $F(2,15) = 34.61$, $p < .001$) (Table 3). In the frontal cortex, the AUC for NIC in PND 40 was lower than in PND 60 ($q = 10.50$, $p < .001$) and PND 90 rats ($q = 12.39$, $p < .001$; $F(2,11) = 43.10$, $p < .001$) (Table 3). In contrast, the AUC for NNIC in PND 40 frontal cortex was higher than in PND 60 and 90 rats ($q = 4.29$, $p < .05$ and $q = 6.92$, $p < .001$, respectively; $F(2,12) = 12.01$, $p = .0014$) (Table 3). In plasma, the AUC for NIC for lower in PND 40 versus PND 60 ($q = 9.61$, $p < .001$), in PND 40 versus PND 90 ($q = 16.69$, $p < .001$), and in PND 60 versus PND 90 rats ($q = 7.14$, $p < .001$; $F(2,12) = 69.64$, $p < .001$) (Table 3). In contrast, the AUC for NNIC in plasma was higher in PND 40 versus PND 60 ($q = 11.13$, $p < .001$) and 90

rats ($q = 14.14$, $p < .001$; $F(2,12) = 53.32$, $p < .001$). However, the AUC for COT was not statistically different among the age groups in plasma and striata, but in frontal cortex the AUC for COT was lower in PND 60 versus PND 90 ($q = 3.68$, $p < .05$) and in PND 60 versus PND 40 ($q = 4.09$, $p < .05$; $F(2,13) = 5.26$, $p = .021$) (Table 3).

DISCUSSION

This current study demonstrates that the distribution of NIC and metabolites in plasma, striata and frontal cortex changes as a function of postnatal age in rats. In particular, adolescent rats have lower concentrations of NIC in plasma, striata, and frontal cortex compared with adult rats. Further, adolescent rats have greater NNIC concentrations in these matrices. NNO, NCOT, CNO, and 3-HC concentrations quantified in plasma were also detected at higher concentrations at some timepoints in PND 40 versus PND 90 rats. These data suggest that adolescent rats may metabolize NIC faster to some metabolites compared with adult rats.

A previous study reported that PND 21 rats have a higher expression of the NIC hepatic metabolizing enzyme, CYP2B1, than PND 84 rats (Yun et al., 2010). In contrast, the activity of CYP2B1 was not different between PND 21 and PND 84 (Yun et al., 2010). In this current study, plasma COT concentrations were only different among age groups at 1 hr after a single NIC administration with higher levels found in PND 40 and PND 60 rats in comparison with PND 90 rats. Since CYP2B1 metabolizes NIC to COT in rats, our study is consistent with previous findings on the lack of age-related differences of CYP2B1 activity. However, because the ages of the rats used between the current and previous studies are different and because COT levels differed at the 1 hr timepoint in the present study, other factors besides enzymatic activity such as age-related differences in heart rate, blood flow, and other pharmacokinetics parameters (i.e., volume of distribution, absorption, and excretion) potentially contribute to these findings.

Age-related differences in heart rate and cerebral blood flow in rats have been reported. Previous studies have found that increases in heart rate are greater in adolescent versus adult rats during ethanol consumption, and adolescents consumed a higher volume of ethanol than adults, suggesting a correlation between heart rate and the greater reward value of ethanol among adolescent rats (Ristuccia & Spear, 2008). Others have shown that cerebral blood oxygenation increased in adult rats but decreased in adolescent rats after acute administration of methylphenidate (Canese et al., 2009). To our knowledge, age-related differences in heart rate and blood flow among rats that administered NIC have not yet been reported.

As in plasma, brain COT concentrations were only different at 1 hr timepoint in the frontal cortex. This lack of difference is predictable since the source of COT in the brain is primarily from distribution of plasma COT rather than metabolism of NIC to COT in the brain, thus levels in the plasma likely dictated the levels found in the brain (Crooks, Li, & Dwoskin, 1997; Lockman et al., 2005). Additionally, consistent with the literature indicating that the ability of COT to cross the blood–brain barrier is half that of NIC (Lockman et al., 2005), we found that the concentration of COT in the brain never reached the same levels than the concentration of NIC in the brain.

Besides COT, NNIC was the other metabolite found in plasma and brain after systemic single NIC administration. NNIC concentrations in plasma and brain were significantly higher in adolescent rats in comparison with adult rats. NIC is converted to NNIC in the periphery via *N*-demethylation in humans and rodents (Cundy & Crooks, 1984; Yamanaka et al., 2005). It has been suggested that in rats, NNIC source in the brain is primarily from local demethylation of NIC (Crooks et al., 1997). To our knowledge, no studies have yet indicated age-related differences in NIC demethylation in rats. Therefore, age-related differences in brain NNIC reported herein is likely due to differences in NIC demethylation between adolescents and adults; however, further studies are necessary to rule out the possibility of NNIC differences being due to distribution from plasma.

Higher concentrations of NNIC in adolescents might contribute to increased risk of NIC addiction in adolescents. NNIC is a pharmacologically active metabolite of NIC that might contribute to the addictive properties of NIC (Dwoskin, Buxton, Jewell, & Crooks, 1993; Goldberg, Risner, Stolerman, Reavill, & Garcha, 1989; Papke, Dwoskin, & Crooks, 2007). In operant behavioral studies using food reinforcement in rats, NNIC was found to be active and was suggested to contribute to the effect of NIC (Goldberg et al., 1989). In rat striatal slices, NNIC induced dopamine release to higher concentrations and for a longer period of time than NIC (Dwoskin et al., 1993). More recently, it has been demonstrated that NNIC has similar affinities than NIC to several subtypes of nicotinic acetylcholine receptors including those subtypes involved in dopamine release and NIC addiction such as $\alpha 5$ - and $\alpha 6$ -containing nicotinic acetylcholine receptors (Papke et al., 2007).

Of note, NIC, COT, and NNIC concentrations and $t_{1/2}$ in adult rats' whole brains have been previously reported (Ghosheh, Dwoskin, Li, & Crooks, 1999). The $t_{1/2}$ reported is comparable with this current study, whereas the concentrations reported in that study were lower than those reported here, perhaps due to differences in methodology. For example, the current and previous studies used different sample preparation techniques. In the previous study, the brain homogenates were centrifuged twice and incubated for 1 hr at 34°C before injecting directly to the HPLC column. In this current study, the brain homogenates were centrifuged only once without incubation, and analytes were extracted from the supernatant by solid-phase extraction that provides a very clean sample before being injected into the HPLC column. Also of note, the HPLC columns used are different. In the previous study, a C8 column was used, and in our current study, a HS F5 column was used. Generally, HS F5 columns have a greater retention than C8 columns allowing for higher recovery of analytes. Finally, the detectors used by the previous and the current studies are different. In the previous study, the detector used was UV fixed at 254 nm wavelength. Our study used MS/MS with MRM that minimizes interference from metabolites. Thus, differences in analyte recovery between the two extraction, chromatographic, and detection techniques may account for the differences observed.

The significance of these findings is represented by clinical and preclinical studies with NIC administration in adolescents. Cigarette smoking often starts during adolescence and in this case is usually reported to be more difficult to quit (Rubinstein et al., 2008). Individuals with faster NIC metabolism have increased smoking behavior relative to light or occasional smokers (Benowitz, 2010). NIC self-administration is more

rewarding to adolescent than adult rats as demonstrated by studies using a conditioned place-preference procedure (Shram & Lê, 2010). This latter observation might be explained by the reduced drug concentration in the brain of the younger animals that chose NIC over vehicle more frequently than adults in order to maintain the rewarding effect. The lower NIC concentrations found in rat brain of adolescents in this study may suggest another reason for the augmented smoking behavior seen in adolescents. Therefore, the findings presented in this current study revealed differential disposition of NIC and metabolites in adolescents that may contribute to their higher susceptibility to NIC addiction.

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DECLARATION OF INTERESTS

None declared.

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