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TITLE PAGE

Nicotine Pharmacokinetics in Rats is altered as a function of Age, impacting the Interpretation of Animal Model Data

Authors: Evelyn L. Craig, Bin Zhao, Jason Z. Cui, Maria Novalen, Sharon Miksys, Rachel F. Tyndale

Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health, Departments of Pharmacology & Toxicology, and Psychiatry, University of Toronto, Toronto, Canada (E.L.C., B.Z., M.N., S.M., R.F.T.)

Child & Family Research Institute, Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, Canada (J.Z.C.)

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RUNNING TITLE PAGE

Nicotine Pharmacokinetics, altered as a function of Rat Age

Corresponding Author:

Rachel F. Tyndale

Department of Pharmacology & Toxicology, University of Toronto
1 King's College Circle, Rm. 4326, Medical Sciences Building
Toronto, Ontario, M5S 1A8, Canada

Phone: +1 (416) 978-6374
Fax: +1 (416) 978-6395
E-mail address: r.tyndale@utoronto.ca

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Abbreviations:

AD, adult; AUC, area under the concentration-time curve; CL, plasma clearance; C_{max}, maximal metabolite concentration; EA, early adolescent; HPLC, high-performance liquid chromatography; K_P, brain to plasma partition coefficient; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PND, postnatal day; VD, volume of distribution.

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ABSTRACT

Several behavioral studies report that adolescent rats display a preference for nicotine compared to adults. However, age-related pharmacokinetic differences may confound the interpretation of these findings. Thus, differences in pharmacokinetic analyses of nicotine were investigated. Nicotine was administered via acute subcutaneous (1.0 mg base/kg) or intravenous (0.2 mg base/kg) injection to early adolescent (EA; PND25) and adult (AD; PND71) male Wistar rats. Nicotine and its primary metabolite cotinine, and additional metabolites nornicotine, nicotine-1'-N-oxide, *trans*-3'-hydroxycotinine and norcotinine were sampled from 10 minutes to 8 hours (plasma) and 2 to 8 hours (brain) post nicotine and analyzed by LC-MS/MS. Following subcutaneous nicotine, the EA cohort had *lower* levels of plasma nicotine, cotinine and nicotine-1'-N-oxide at multiple time points, resulting in a lower area under the plasma concentration-time curve (AUC) for nicotine ($p<0.001$), cotinine ($p<0.01$) and nicotine-1'-N-oxide ($p<0.001$). Brain levels were also lower for these compounds. In contrast, the EA cohort had *higher* plasma and brain AUCs ($p<0.001$) for the minor metabolite nornicotine. Brain to plasma ratios varied for nicotine and its metabolites, and by age. Following intravenous nicotine administration similar age-related differences were observed, and this route allowed detection of a 1.6-fold larger volume of distribution and 2-fold higher plasma clearance in EA cohort compared to the AD cohort respectively. Thus, unlike in humans, there are substantial age differences in nicotine pharmacokinetics such that for a given nicotine dose, adolescent rats will have lower plasma and brain nicotine compared to adults suggesting that this should be considered when interpreting animal model data.

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INTRODUCTION

Tobacco dependence is primarily mediated through the pharmacological actions of nicotine (Benowitz et al., 2009; Caille et al., 2012). The study of the pharmacokinetics of nicotine provides insight into variation in nicotine levels and how this may subsequently influence nicotine-mediated behaviors. Among the factors that influence nicotine pharmacokinetics is age (Benowitz et al., 2009). For example, in humans over the age of 65, nicotine clearance is reduced (Molander et al., 2001) and in neonates, the nicotine half-life is longer compared to adults. No differences in nicotine pharmacokinetics have been observed between human adolescents and adults (Gourlay and Benowitz, 1996; Al Koudsi et al., 2010). However, differences in nicotine plasma levels between adolescent and adult rats given the same nicotine dose in a behavioral study were noted, suggesting potential age-related variation in nicotine pharmacokinetics (Shram et al., 2008).

Nicotine is inactivated and eliminated from the body by various metabolic pathways, primarily mediated by hepatic enzymes (Nakajima and Yokoi, 2005). Nicotine's primary route of elimination in most mammalian species is via its conversion to cotinine by C-oxidation (Messina et al., 1997; Nakajima et al., 1998). Oxidative nicotine metabolism also produces minor nicotine metabolites including nornicotine (Green et al., 2001) and nicotine-1'-N-oxide (Ochiai et al., 2006). Measuring the pharmacokinetics of nicotine and its metabolites over time, preferably in both the plasma and brain, enhances the interpretation of nicotine's effects (Hukkanen et al., 2005).

The majority of current adult smokers began smoking in adolescence with 88% of first cigarette use occurring before age 18 (Liang et al., 2003; CDC, 2012). Human adolescents have a higher risk of developing nicotine dependence, relative to adults, even with limited tobacco exposure (Rose et al., 2012). Initiation of smoking during adolescence has been associated with a lower likelihood of quitting in adulthood, increased cigarette consumption and increased difficulty quitting (Khuder et al., 1999). Animal models can be used to investigate aspects of the nicotine dependence cycle that cannot be easily investigated in humans, such as the initiation of use (Casey and Jones, 2010). For example, using the conditioned place preference (CPP) paradigm, which assesses drug reward in rodents (Perna

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et al., 2011), acute low doses of subcutaneously delivered nicotine elicited a preference for the nicotine-paired chamber in adolescent but not in adult rats (Vastola et al., 2002; Belluzzi et al., 2004). When first exposed to nicotine in adolescence in an intravenous self-administration paradigm, both male (Levin et al., 2007) and female (Levin et al., 2003) rats displayed enhanced rates of responding compared to animals first exposed in adulthood. These behavioral differences between adolescents and adults may be attributed to a reduced sensitivity to nicotine's aversive effects (Vastola et al., 2002), differences in reward set points during development (Koob and Le Moal, 2000) and/or pharmacokinetic differences (Levin et al., 2003). Plasma nicotine and cotinine levels, measured at a single time point, were significantly lower in adolescent compared to adult rats (Shram et al., 2008). Adolescent rats also had enhanced up-regulation of brain nicotinic acetylcholine receptors in response to nicotine and significantly lower brain nicotine levels than those reported in adults (Doura et al., 2008). These studies suggest that pharmacokinetic differences may contribute to the behavioral differences observed between adolescent and adult rats.

This study was conducted to directly assess these potential age-specific pharmacokinetic differences in nicotine and its metabolites and to assist in understanding the potential confound of extrapolating the results of rat behavioral studies to the interpretation of human nicotine-mediated behaviors, given the apparent species differences in nicotine pharmacokinetics as a function of age. Acute dosing of subcutaneous and intravenous nicotine was used to assess the impact of age on the plasma and brain levels of nicotine and its metabolites in early adolescent and adult rats.

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MATERIALS AND METHODS

Animals. Male Wistar rats were purchased from Charles River Laboratories (St-Constant, Quebec, Canada). Rats were aged by postnatal days (PND); early adolescent (EA, PND25) and adult (AD; PND71) rats were used in each experiment. These age groups were selected according to the previously established developmental periods of EA (PND21 – 34) and AD (over PND60) (Spear, 2000; McCormick and Mathews, 2010). Male rats were chosen as they had been used in the behavioral studies, and to avoid the sex-specific influence of sexually dimorphic growth hormone release on drug metabolism which occurs in rats, and is not relevant in humans (Shapiro et al., 1995); please see Discussion. All EA (81 ± 8 g) and AD (349 ± 25 g) animals were housed in pairs or groups of three and maintained on a 12-hour light:12-hour dark, light and temperature-controlled cycle. Water and food were available ad libitum. All experimental procedures were conducted in accordance with the guidelines provided by the Canadian Council on Animal Care and approved by the Animal Care Committee of the University of Toronto.

Reagents. (-) Nicotine hydrogen tartrate salt was purchased from Sigma (St. Louis, MO, USA). Nicotine-d_4, rac-cotinine, rac-cotinine-d_3, *trans*-3'-hydroxycotinine, *trans*-3'-hydroxycotinine-d_3, (R, S)-norcotinine, (R, S)-nornicotine, (1'S, 2'S)-nicotine-1'-oxide and rac-*trans* nicotine-1'-oxide-methyl-d_3 were obtained from Toronto Research Chemicals (North York, ON, Canada). The purity of all compounds used was over 98%.

In Vivo Nicotine Pharmacokinetics. Animals received either an acute subcutaneous (s.c. 1.0 mg free base / kg body weight, higher dose allowing better quantification of nicotine and metabolites) or intravenous (i.v. 0.2 mg free base / kg body weight, a lower dose required to avoid toxicity) administration of (-) nicotine hydrogen tartrate dissolved in sterile saline (0.9% NaCl). Fresh nicotine solutions were prepared and titrated to a pH of 7.4. Venous blood (saphenous vein, 100-200 μl) was

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collected at 10 minutes, 30 minutes, 1 hour, 2 hours and 4 hours after nicotine injection as well as trunk blood at sacrifice (2, 4 or 8 hours after nicotine injection). For each animal, two timed blood samples were obtained from the saphenous vein and the third sampled from trunk blood at time of sacrifice. The sample timing and animal numbers were based on previous nicotine studies performed in our laboratory in adult rats e.g. Micu et al. (2003), and pilot subcutaneous nicotine injection studies performed in adolescent rats. Thus, the total number of animals used was 30 for the EA and 14 for the AD groups for the subcutaneous study, and 25 for the EA and 23 for AD groups for the intravenous dosing study; as each animal provided 3 of the 6 blood sampling times, this resulted in an average of 10, 9, 6, and 7 samples per time point respectively. Blood samples were placed on ice and plasma was collected after centrifugation at 3500g for 10 minutes and stored at -30°C until analyzed. Following sacrifice brains were immediately dissected on ice, frozen on dry ice, and stored at -80°C.

Plasma Sample Preparation. Sample preparation and extraction was adapted from a previously described method (Vieira-Brock et al., 2011). Briefly, 100 µl of 20 ng/ml deuterium-labeled internal standard in 0.01 M HCl, 900 µl water and 100 µl 30% perchloric acid were added to 100 µl of plasma or 100 µl of standards, for a final volume of 1.2 ml. After vortex-mixing and centrifugation at 5400g for 5 minutes, the supernatant was added to Oasis® MCX solid phase extraction cartridges (3 cc, 60 mg; Waters, Milford, MA) which had been pre-conditioned with 2 ml HPLC-grade methanol followed by 2 ml 2% formic acid. The cartridges were washed with 1 ml 2% formic acid and 1 ml HPLC-grade methanol. Analytes were eluted with 1.5 ml 5% (v/v) ammonium hydroxide in methanol and 1.5 ml dichloromethane:isopropanol:ammonium hydroxide (v/v 78:20:2). The samples were fortified with 100 µl 10% (v/v) hydrochloric acid in methanol to enhance nicotine recovery and then dried under a stream of nitrogen at 37°C. Dry samples were then re-constituted in 100 µl 100 mM ammonium acetate in water:methanol:acetic acid (v/v 79:20:1) and 50 µl was injected onto an LC-MS/MS.

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Brain Sample Preparation. Brain samples (whole brain) were thawed on ice, weighed, homogenized in three volumes of ice-cold saline (0.9% NaCl) and centrifuged at 3000g for 10 minutes. The supernatant was collected and frozen at -80°C until time of analysis. The brain supernatant preparation was performed as described above using 1.0 ml of brain supernatant (without the addition of water), as previously described (Vieira-Brock et al., 2011). The mean recovery (N = 5) was over 80% for all compounds assessed, using labeled standards. The recovery of nicotine was 85.4% in EA (range: 82.6-87.3%) and 85.3% in AD (range: 83.2-88.4%) brain samples; the recovery of cotinine was 85.5% in EA (range: 81.2-90.1%) and 85.8% in AD (range: 81.6-89.9%); the recovery of nornicotine was 89.0% in EA (range: 86.3-93.3%) and 90.3% in AD (range: 88.8-93.3%); the recovery of nicotine-1'-N-oxide was 86.7% in EA (range: 83.8-90.2%) and 84.6% in AD (range: 82.9-87.3%); the recovery of trans-3'-hydroxycotinine was 85.3% in EA (range: 84.5-86.6%) and 84.3% in AD (range: 82.8-86.5%); and the recovery of norcotinine was 81.1% in EA (range: 77.2-86.1%) and 80.7% in AD (range: 76.4-85.7%).

Liquid Chromatography-Mass Spectrometry Conditions. The chromatographic conditions were adapted from an established method (Jacob et al., 2011) and were performed using an Agilent 1260 HPLC system equipped with a 4.6 mm x 150 mm Phenomenex Synergi Polar RP column (4 µm). The mobile phase consisted of a 10 mM ammonium acetate/0.1% acetic acid in water (solvent A) and 10 mM ammonium acetate/0.1% acetic acid in methanol (solvent B). The flow rate was 0.7 ml/min and the following gradient was used: the initial composition was 80% solvent A, changing to 100% solvent B over 6.5 minutes. From 6.5 to 8 minutes, 100% solvent B was maintained and was then changed to 80% solvent A at 8.1 minutes and maintained at this composition until the end of the run; total run time was 13 minutes. The spectrometric analysis was performed on an Agilent 6430 triple-quadruple mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) ion source operated in the positive ion mode. The vaporizer temperature was optimized to 450°C, the gas temperature 350°C, and the corona discharge current was set at 5 µA. The limit of quantification was 1 ng/ml for all

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analytes. Intra-day coefficients of variation (CV) were assessed for all metabolites to determine intra-run assay precision. The CV values were 2.3% for nicotine, 2.5% for cotinine, 1.9% for nornicotine, 1.6% for nicotine-1'-N-oxide, 1.1% for *trans*-3'-hydroxycotinine and 3.0% for norcotinine.

Pharmacokinetic Analysis. In vivo pharmacokinetic parameters were determined using non-compartmental analysis and were calculated using PK Functions for Microsoft Excel (J.I. Usansky, A. Desia, D. Tan-Liu, Department of Pharmacokinetics and Drug Metabolism, Allergon, Irvine, CA). The area under the concentration-time curves from 10 minutes to 4 hours ($AUC_{(10min\text{-}4hr)}$) or 2 to 8 hours ($AUC_{(2\text{-}8hr)}$) in plasma and 2 to 8 hours ($AUC_{(2\text{-}8hr)}$) in brain were determined using the log-linear trapezoidal rule. Half-life ($t_{1/2}$) was estimated using the equation: $t_{1/2} = \ln(2) / k_{el}$, where k_{el} is the terminal elimination rate constant. The terminal elimination rate constant was estimated by linear regression of the terminal phase of the semi-logarithmic concentration-time curve. The brain-to-plasma partition coefficient (K_P) was calculated as a ratio of brain $AUC_{(2\text{-}8hr)}$ to plasma $AUC_{(2\text{-}8hr)}$. Following acute i.v. nicotine administration, the initial volume of distribution (VD) was calculated as Dose/C_0 where, C_0 is the initial plasma nicotine concentration, extrapolated to time zero of the concentration-time curve. The plasma clearance (CL) was calculated as $\ln(2) \times V_C / t_{1/2}$ (Toutain and Bousquet-Mélou, 2004c).

Statistical Analysis. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA). Variables were described by their mean ± standard deviation (SD) and were compared between the EA and AD groups using a one-tailed, unpaired Student's *t* test with Welch's correction. Due to the study design, whereby individual animals were sampled across varying time points, only group means are reported for $t_{1/2}$, VD, and CL.

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RESULTS

Plasma Nicotine and Metabolite Levels differ between Early Adolescent and Adult Rats

Plasma nicotine levels were lower in the $EA_{(SC)}$ group compared to the $AD_{(SC)}$ group at 1 hour (p<0.05), 2 hours (*p*<0.001) and 4 hours (*p*<0.01) following acute s.c. nicotine administration (Figure 1A); pharmacokinetic parameters are reported in Table 1.The mean plasma nicotine $AUC_{(10min\text{-}4hr)}$ was also lower in the $EA_{(SC)}$ compared to the $AD_{(SC)}$ group (*p*<0.001), indicative of lower total nicotine exposure in the $EA_{(SC)}$ animals (Figure 1B). If the lower plasma nicotine levels in the $EA_{(SC)}$ group were due to an increased rate of conversion to cotinine, the major metabolic route for nicotine, higher plasma cotinine levels might be expected in the younger animals. However, plasma cotinine levels were lower in the $EA_{(SC)}$ group at 2 hours (*p*<0.05), 4 hours (*p*<0.05) and 8 hours (*p*<0.01) compared to those in the $AD_{(SC)}$ group (Figure 2A). The mean plasma cotinine $AUC_{(10min\text{-}4hr)}$ was also lower in the $EA_{(SC)}$ compared to the $AD_{(SC)}$ group (*p*<0.01) (Figure 2D). We also investigated whether the formation of minor metabolites, nornicotine and nicotine-1'-N-oxide, contributed to the observed age differences in nicotine levels. Plasma nornicotine levels were low, as expected, and higher at 30 minutes (p<0.05), 1 hour (*p*<0.001) and 2 hours (*p*<0.01) in the $EA_{(SC)}$ compared to the $AD_{(SC)}$ group (Figure 2B) resulting in a higher $AUC_{(10min\text{-}4hr)}$ (*p*<0.001) (Figure 2D). The mean nornicotine C_{max} was also higher in the $EA_{(SC)}$ versus the $AD_{(SC)}$ group (*p*<0.001) (Table 1). The plasma levels of nicotine-1'-N-oxide were lower in the $EA_{(SC)}$ group at 10 minutes (*p*<0.05), 1hour (*p*<0.01), 2 hours (*p*<0.001), 4 hours (*p*<0.001) and 8 hours (*p*<0.015) following nicotine administration compared to the $AD_{(SC)}$ group (Figure 2C) resulting in a lower mean nicotine-1'-N-oxide $AUC_{(10min\text{-}4hr)}$ (*p*<0.001) (Figure 2D). The mean plasma C_{max} for nicotine-1'-N-oxide was also lower in the $EA_{(SC)}$ compared to the $AD_{(SC)}$ group (*p*<0.01) (Table 1). We also assessed the plasma levels of two additional minor nicotine metabolites. The mean plasma levels of *trans*-3'-hydroxycotinine and norcotinine were very low, near the limit of quantification (1 ng/ml), in both the $EA_{(SC)}$ and $AD_{(SC)}$ groups (Table 2).

Brain Levels of Nicotine and its Metabolites differ between Early Adolescent and Adult Rats

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Brain nicotine levels were lower in the $EA_{(SC)}$ compared to the $AD_{(SC)}$ group at 2 hours ($p<0.01$) following acute s.c. nicotine administration (Figure 3A, Table 3), consistent with a modestly lower $AUC_{(2\text{-}8hr)}$ ($p=0.06$) (Figure 3B). The brain nicotine K_P ratio from 2 to 8 hours was 3.9 in the $EA_{(SC)}$ group and 2.2 in the $AD_{(SC)}$ group, indicating higher partitioning of nicotine in the brain versus the plasma (Figure 3C). Brain cotinine levels were lower in the $EA_{(SC)}$ group at 2 hours ($p<0.05$), 4 hours ($p<0.05$), and 8 hours ($p<0.05$) compared to the $AD_{(SC)}$ group (Figure 4A) as was the brain cotinine $AUC_{(2\text{-}8hr)}$ ($p<0.01$) (Figure 4B). The brain cotinine K_P ratio from 2 to 8 hours was below 1 for both the $EA_{(SC)}$ and $AD_{(SC)}$ groups, indicating that there was lower partitioning of cotinine into the brain (Figure 4C). Brain nornicotine levels were higher in the $EA_{(SC)}$ group at 2 hours ($p<0.05$) and 8 hours ($p<0.05$) compared to the $AD_{(SC)}$ group (Figure 4A) as was the brain nornicotine $AUC_{(2\text{-}8hr)}$ ($p < 0.05$) (Figure 4B). The brain nornicotine K_P ratio from 2 to 8 hours was 3.2 in the $EA_{(SC)}$ group and 2.3 in the $AD_{(SC)}$ group, indicative of higher partitioning of nornicotine in the brain, as seen for nicotine (Figure 4C). The brain nicotine-1'-N-oxide levels were relatively low and did not differ between the $EA_{(SC)}$ and $AD_{(SC)}$ animals (Figure 4A); the K_P ratio from 2 to 8 hours was also below one, as seen for cotinine (Figure 4C). Mean brain levels of *trans*-3-hydroxycotinine and norcotinine were low, near the limit of quantification (1 ng/g), in both the $EA_{(SC)}$ and $AD_{(SC)}$ groups (Table 4).

Plasma and Brain Nicotine Levels also differed by Age Following Intravenous Nicotine Administration

Plasma and brain nicotine levels were compared between the EA and AD age groups following an acute i.v. nicotine administration. The use of the i.v. route of administration, with 100% bioavailability, permits investigation of whether the differences in plasma and brain nicotine levels between the EA and AD age groups following acute s.c. nicotine resulted from differences in absorption, due perhaps to differences in body composition between these ages. Intravenous injection can also be used to examine the volume of distribution and clearance.

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Plasma nicotine levels were lower in the $EA_{(IV)}$ compared to the $AD_{(IV)}$ group at 30 minutes ($p<0.05$), 1 hour ($p<0.05$), 2 hours ($p<0.001$) and 4 hours ($p<0.05$) (Figure 5A) resulting in a lower plasma nicotine $AUC_{(10min\text{-}4hr)}$ ($p<0.001$) (Figure 5B). Brain nicotine levels were not significantly different between the $EA_{(IV)}$ and $AD_{(IV)}$ groups at any time point (Figure 6A); the mean brain nicotine $AUC_{(2\text{-}8hr)}$ - trended towards being lower in the $EA_{(IV)}$ group compared to the $AD_{(IV)}$ group ($p = 0.09$) (Figure 6B). The brain nicotine K_P ratio from 2 to 8 hours was 6.1 in the $EA_{(IV)}$ group compared to 3.2 the $AD_{(IV)}$ group (Figure 6C), indicating increased nicotine partitioning into the brain of EA rats, as observed following subcutaneous nicotine administration. Pharmacokinetic parameters for nicotine and metabolites following acute i.v. administration were similar to those seen following subcutaneous administration, with comparable age-related differences, and are reported in Supplementary Table 1. Due to the relatively flat elimination curve for nicotine from the brain (Fig 6A) we examined the later time points of 18 and 24 hours. The only metabolite detected in the plasma beyond 8 hours was cotinine, detected in both age groups above the limit of quantification at 18 and 24 hours after nicotine administration. Nicotine and cotinine were detectable in the brain at 18 and 24 hours while other metabolites were not, suggesting a long residence time for these two compounds.

Volume of Distribution and Clearance differ between Early Adolescent and Adult Rats

The $EA_{(IV)}$ group had a mean volume of distribution that was approximately 1.6-fold larger than the $AD_{(IV)}$ group (Figure 7A). Furthermore, the estimated mean clearance rate over 2-fold faster in the $EA_{(IV)}$ group compared to that of the $AD_{(IV)}$ group (Figure 7B).

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DISCUSSION

There are nicotine-mediated behavioral differences between adolescent and adult rats (Belluzzi et al., 2004; Levin et al., 2007). We have shown that rat age (adolescent versus adult) substantially impacts nicotine pharmacokinetics, and consequently, the plasma and brain levels of nicotine and its metabolites, which may contribute to these observed behavioral differences.

Pharmacokinetic parameters including absorption (Mangoni and Jackson, 2003), drug distribution (Turnheim, 1998; Molander et al., 2001), excretion (McLean and Le Couteur, 2004) and metabolism (Klinger, 2005) can be altered by age. Characterization of nicotine pharmacokinetics using subcutaneous nicotine can be influenced by absorption (Hukkanen et al., 2005). However, the pharmacokinetic differences between early adolescent and adult rats are not due to differences in nicotine absorption as we obtained similar differences following intravenous nicotine administration, where bioavailability is 100% (Toutain and Bousquet-Mélou, 2004a) . Throughout development, body mass composition is altered in most mammalian species, with younger animals having a higher ratio of lean to fatty mass than older animals (Wolden-Hanson, 2010). As nicotine primarily distributes into lean mass, a change in this body mass composition could potentially impact nicotine's volume of distribution (Molander et al., 2001). As adolescent animals tend to have primarily lean body mass they would be expected to have a larger volume of distribution, relative to their body weight, for nicotine resulting in lower plasma nicotine levels (Tutka et al., 2005). Of note, the substantial change in volume of distribution (approximately 1.6 times larger in EA) suggests that there are likely additional factors contributing to this difference.

Nicotine is excreted by glomerular filtration and tubular secretion with variable absorption, depending on urinary pH. Renal clearance accounts for approximately 5% of total nicotine clearance in humans (Benowitz et al., 2009). In vitro expression of rat organic cation transporter 1 and 2 (OCT 1 and 2) indicated that they are basolaterally-located transporters contributing to tubular secretion of cationic drugs, removing them from the plasma to be secreted in the urine (Urakami et al., 1998). In rats, renal transporter mRNA expression is increased in early adolescents (de Zwart et al., 2008). With

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developmental changes in transporter protein expression, it is predicted that the distribution and elimination of substrates would also change with age (de Zwart et al., 2008). The removal of a drug from the body is reflected by the plasma clearance, impacting plasma concentration (Toutain and Bousquet-Mélou, 2004b). The early adolescent animals have increased nicotine clearance, consistent with the lower plasma nicotine levels observed in this age group, relative to the adult cohort.

In rats nicotine is metabolized to cotinine by the hepatic enzyme CYP2B1 as the rat CYP2A family, chiefly responsible for nicotine metabolism in humans (Benowitz et al., 2006), is essentially inactive towards nicotine (Hammond et al., 1991; Nakayama et al., 1993). CYP2B expression changes through development; early adolescent rats had higher levels of hepatic CYP2B protein compared to adults (Yun et al., 2010). However, our data do not support a faster conversion of nicotine to cotinine in the early adolescent cohort as both nicotine and cotinine levels are lower. Moreover, as nicotine is a high extraction ratio drug its clearance is less impacted by the expression and activity of hepatic enzymes (Benowitz et al., 2009). This, and the faster nicotine clearance in younger animals may be attributed, in part, to increased hepatic blood flow (Woodhouse and Wynne, 1992). Furthermore, most of the other metabolites were present at relatively lower levels in the adolescent compared to the adult cohort, suggesting that an increased rate of metabolism to these metabolites is not responsible, at least in full, for the lower nicotine levels in the adolescent cohort. Together, this suggests that the lower levels of nicotine in the adolescent cohort are consistent with an effect of volume of distribution and clearance, rather than with a change in metabolic pathways.

Sex-dependent differences in drug metabolism and pharmacokinetics have been demonstrated in several species, including rats (Czerniak, 2001). In general, female rats have rates of hepatic drug metabolism 3-to 5-fold lower than those of males (Kyerematen et al., 1988a); the longer nicotine half-life observed in female versus male rats, and the lower plasma cotinine levels, are consistent with lower recovery of urinary nicotine metabolites (Kyerematen et al., 1988a). Non-metabolic sex differences in nicotine pharmacokinetics include altered clearance and volume of distribution (Czerniak, 2001); female rats have a larger volume of distribution than males (Kyerematen et al., 1988a). However, despite the

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larger volume of distribution, and consistent with slower metabolism, female rats have higher plasma and brain nicotine levels compared to male rats, likely due to sex differences in nicotine distribution and metabolism (Rosecrans, 1972; Harrod et al., 2007). These sex differences in rat nicotine pharmacokinetics may also contribute to sex differences in nicotine-mediated behaviors, such as those seen for nicotine in the CPP paradigm (Torres et al., 2009), which may be related to the higher plasma and brain nicotine levels in females relative to males. Our findings of a potential role for differences in nicotine volume of distribution and clearance, and the known rodent sex differences in these parameters, suggest that differences between males and females of different ages may occur and should perhaps be considered when comparisons of nicotine-evoked behaviors are made.

Following acute nicotine administration, lower levels of nicotine were also observed in the brains of the adolescent animals. The lower brain nicotine levels may be important to the interpretation of differences in nicotine-mediated behaviors between adolescent and adult rats. Adolescent rats display increased rates of nicotine self-administration relative to adults (Levin et al., 2003; Levin et al., 2007), often attributed to an enhanced preference for nicotine. However, due to the lower brain nicotine levels observed in adolescent rats, it is possible that adolescent animals are self-administering more nicotine to attain rewarding brain levels comparable to those achieved by adults (Koob and Le Moal, 2000), effectively compensating for lower brain nicotine levels acquired per injection. We also observed a higher brain to plasma $AUC_{(2\text{-}8hr)}$ ratio in the early adolescents compared to the adults. Understanding the differing brain levels of nicotine measured directly, or by age-specific extrapolations from plasma levels, may be useful when interpreting animal behavioral data.

In contrast to the lower plasma and brain levels of nicotine, plasma nornicotine levels were higher in early adolescent compared to adult rats. In both humans and rats, nicotine is metabolized to nornicotine by N-demethylation (Cundy and Crooks, 1984; Yamanaka et al., 2005), accounting for a urinary recovery of 2-3% of a given nicotine dose in humans and approximately 8% in rats (Yamanaka et al., 2005). In this, and former studies, nornicotine represents a more substantial proportion of the nicotine dose in the brain (Plowchalk et al., 1992; Crooks et al., 1997). Nornicotine has psychoactive

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properties and is self-administered by rats (Bardo et al., 1999); this metabolite also has a high affinity for nicotinic receptors and can evoke dopamine release in the striatum (Green et al., 2001). Nornicotine desensitizes nicotinic receptor subtypes with a lower potency than nicotine, although cross-sensitization was observed between nicotine and nornicotine, indicating common receptor subtypes (Dwoskin et al., 2001). We detected a significantly higher brain nornicotine $AUC_{(2\text{-}8hr)}$ in the early adolescent cohort, indicating higher total brain nornicotine exposure. In this age group, where for a given dose of nicotine, brain nicotine levels are lower and brain nornicotine levels are higher than those in adults, the pharmacological actions of this metabolite may be contributing to observed age differences in nicotine-mediated behaviors.

Thus, given the same dose of nicotine, early adolescent rats have lower plasma and brain levels of nicotine, higher nicotine brain to plasma ratios, and differing levels of various nicotine metabolites in both plasma and brain compared to adult rats. Differences in volume of distribution and clearance are likely primarily responsible for these differences in nicotine levels. Additional contributing factors include differences in renal and brain transport, hepatic blood flow, and possibly, minor contributions from differences in metabolic pathways. Adolescence is a key period of development where, in humans, the majority of initiation of nicotine use occurs (Breslau and Peterson, 1996). Animal models are useful for examining the underlying neurobiological vulnerability to dependence in adolescence in ways that cannot be easily investigated in humans (Caille et al., 2012). However, the rat may not be a suitable animal model of human nicotine pharmacokinetics given species-specific age differences in nicotine pharmacokinetics (Gourlay and Benowitz, 1996; Benowitz et al., 2009), differences in the enzyme primarily responsible for nicotine metabolism (Nakayama et al., 1993; Benowitz et al., 2006) and differences in the relative importance of various metabolic pathways of nicotine (Kyerematen et al., 1988b; Hukkanen et al., 2005). Overall, the results presented here highlight both the importance of age as a factor contributing to age differences in nicotine pharmacokinetics in rats and the importance of taking these differences, as well as potential pharmacodynamic differences, into account when

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interpreting the results of animal model data and extrapolating them to interpretations of human smoking behaviors.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Craig, Cui, Miksys, Tyndale

Conducted experiments: Craig, Cui, Miksys, Zhao, Novalen

Performed data analyses: Craig, Cui, Zhao

Wrote or contributed to the writing of the manuscript: Craig, Tyndale

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FOOTNOTES

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Reprint Requests:

Rachel F. Tyndale

Department of Pharmacology & Toxicology, University of Toronto
1 King's College Circle, Rm. 4326, Medical Sciences Building
Toronto, Ontario M5S 1A8, Canada

E-mail address: r.tyndale@utoronto.ca

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LEGENDS FOR FIGURES

Fig. 1. Plasma nicotine levels and $AUC_{(10min\text{-}4hr)}$ were lower in the $EA_{(SC)}$ group following subcutaneous nicotine administration. (A) Plasma nicotine levels measured over time. (B) Plasma nicotine $AUC_{(10min\text{-}4hr)}$. *****$p<0.05$, **$p<0.01$, *******$p<0.001$.

Fig. 2. Plasma cotinine and nicotine-1'-N-oxide levels and $AUC_{(10min\text{-}4hr)}$ were lower, and plasma nornicotine levels and $AUC_{(10min\text{-}4hr)}$ were higher, in the early adolescent ($EA_{SC)}$) group following subcutaneous nicotine administration. (A-C) Plasma levels of cotinine, nornicotine and nicotine-1'-N-oxide measured over time. (D) Plasma cotinine (COT), nicotine-1'-N-oxide (NNO) and nornicotine (NNIC) $AUC_{(10min\text{-}4hr)}$. *****$p<0.05$, **$p<0.01$, *******$p<0.001$**.**

Fig. 3. Brain nicotine levels were lower in the $EA_{(SC)}$ group following subcutaneous nicotine administration. (A) Brain nicotine levels measured over time. (B) There was no significant difference in mean brain nicotine $AUC_{(2\text{-}8hr)}$, though a trend was noted ($p=0.06$). (C) The brain to plasma partition coefficient (K_P) was larger than 1 in both the $EA_{(SC)}$ and $AD_{(SC)}$ groups.******$p<0.01$**.**

Fig. 4. Brain cotinine levels and $AUC_{(2\text{-}8hr)}$ were lower while brain nornicotine levels and $AUC_{(2\text{-}8hr)}$ were higher in the $EA_{(SC)}$ group following subcutaneous nicotine administration. (A) Brain cotinine, nornicotine and nicotine-1'-N-oxide levels measured over time. (B) Brain cotinine (COT), nornicotine (NNIC) and nicotine-1'-N-oxide (NNO) $AUC_{(2\text{-}8hr)}$. (C) The brain to plasma partition coefficient (K_P) was below 1 for cotinine and nicotine-1'-N-oxide (NNO) in both age groups. In contrast, the K_P for nornicotine was above 1 in both age groups. *****$p<0.05$, ******$p<0.01$.

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Fig. 5. Plasma nicotine levels and $AUC_{(10min-4hr)}$ were lower in the $EA_{(IV)}$ group following intravenous nicotine administration. (A) Plasma nicotine levels measured over time. (B) Plasma nicotine $AUC_{(10min-4hr)}$. *****$p<0.05$, *******$p<0.001$.

Fig. 6. Brain nicotine levels were not significantly different in the $EA_{(IV)}$ compared to AD $_{(IV)}$ group following intravenous nicotine administration. (A) Brain nicotine levels measured over time. (B) A trend towards a lower brain nicotine $AUC_{(2\text{-}8\ hr)}$ was noted in the $EA_{(IV)}$ group (p=0.09). (C) The K_P for brain nicotine was larger than 1 in both age groups.

Fig. 7. Age-related differences in volume of distribution and nicotine clearance may alter pharmacokinetics between the two age groups. (A) The initial volume of distribution was approximately 1.6-fold larger in the $EA_{(IV)}$ versus the $AD_{(IV)}$ group. (B) Nicotine clearance was approximately 2-fold faster in the $EA_{(IV)}$ group compared to the $AD_{(IV)}$ group.

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TABLES

Table 1.

Pharmacokinetic Parameters of Plasma Nicotine and Metabolites following Subcutaneous Nicotine

	NIC		COT		NNIC		NNO	
Parameter[a]	**EA**	**AD**	**EA**	**AD**	**EA**	**AD**	**EA**	**AD**
C_{max} (ng/ml)	217 (37)	253 (65)	206 (73)	361 (112)	13** (2.5)	9 (1.8)	66** (23)	105 (8.4)
$t_{1/2}$ (min)	62	74	446	543	123	181	66	89
$AUC_{(10min\text{-}4hr)}$ (ng.hr/ml)	278*** (29)	479 (56)	658** (52)	960 (55)	39*** (1.7)	26 (1.7)	144*** (9.4)	255 (12)
$AUC_{(2\text{-}8hr)}$ (ng.hr/ml)	87* (29)	249 (47)	988* (193)	1893 (272)	37 (4.8)	34 (6.8)	66** (16)	148 (20)

[a] Mean (SD); NIC = Nicotine; COT = Cotinine; NNIC = Nornicotine; NNO = Nicotine-1'-N-oxide; EA = Early adolescent; AD = Adult . C_{max} = Maximal metabolite concentration, $t_{1/2}$ = Terminal half-life, AUC = Area under the plasma concentration-time curve from 10 min to 4 hr and 2 to 8 hr. **p*<0.05, ***p*<0.01, ****p*<0.001, significantly different from $AD_{(SC)}$ group.

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Table 2.

Plasma *Trans*-3'-Hydroxycotinine and Norcotinine Levels following Subcutaneous Nicotine

	Mean Plasma 3HC Level ng/ml plasma (SD)		**Mean Plasma NCOT Level** ng/ml plasma (SD)	
Timepoint	EA	AD	EA	AD
10 min	< LOQ	< LOQ	< LOQ	< LOQ
30 min	< LOQ	1.4 (3.7)	2.4 (0.8)	2.7 (3.8)
1 hr	1.4 (0.8)	1.4 (0.1)	3.5 (1.3)	3.3 (0.3)
2 hr	2.9 (1.6)	2.8 (0.9)	7.7 (3.1)	5.2 (1.8)
4 hr	2.9 (1.2)	4.0 (1.9)	7.6 (2.6)	7.8 (3.1)
8 hr	1.7 (0.4)	4.0 (0.5)	3.8 (0.7)	6.3 (1.0)

LOQ = Limit of quantification. 3HC = *Trans*-3'-hydroxycotinine; NCOT = Norcotinine.

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Table 3.

Pharmacokinetic Parameters of Brain Nicotine and Metabolites following Subcutaneous Nicotine

	NIC		COT		NNIC		NNO	
Parameter[a]	**EA**	**AD**	**EA**	**AD**	**EA**	**AD**	**EA**	**AD**
$t_{1/2}$ **(min)**	159	97	449	347	139	137	172	112
AUC$_{(2\text{–}8hr)}$ (ng.hr/g)	343 (105)	534 (59)	706** (58)	972 (46)	120* (20)	78 (13)	13 (3.4)	19 (3.0)

[a] Mean (SD); NIC = Nicotine; COT = Cotinine; NNIC = Nornicotine; NNO = Nicotine-1'-N-oxide; EA = Early adolescent; AD = Adult. $t_{1/2}$ = Terminal half-life, AUC = Area under the brain concentration-time curve from 2 to 8 hr and 2 hr to infinity. *$p<0.05$, **$p<0.01$, significantly different from $AD_{(SC)}$ group.

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Table 4.

Brain *Trans*-3'-Hydroxycotinine and Norcotinine Levels following Subcutaneous Nicotine

	Mean Brain 3HC Level ng/g brain tissue (SD)		**Mean Brain NCOT Level** ng/g brain tissue (SD)	
Timepoint	**EA**	**AD**	**EA**	**AD**
2 hr	< LOQ	< LOQ	4.3 (1.1)	3.1 (0.9)
4 hr	1.0 (0.7)	1.4 (0.2)	4.9 (1.9)	5.1 (0.4)
8 hr	< LOQ	1.2 (0.3)	2.7 (0.1)	3.5 (0.6)

LOQ = Limit of quantification. 3HC = *Trans*-3'-hydroxycotinine; NCOT = Norcotinine.

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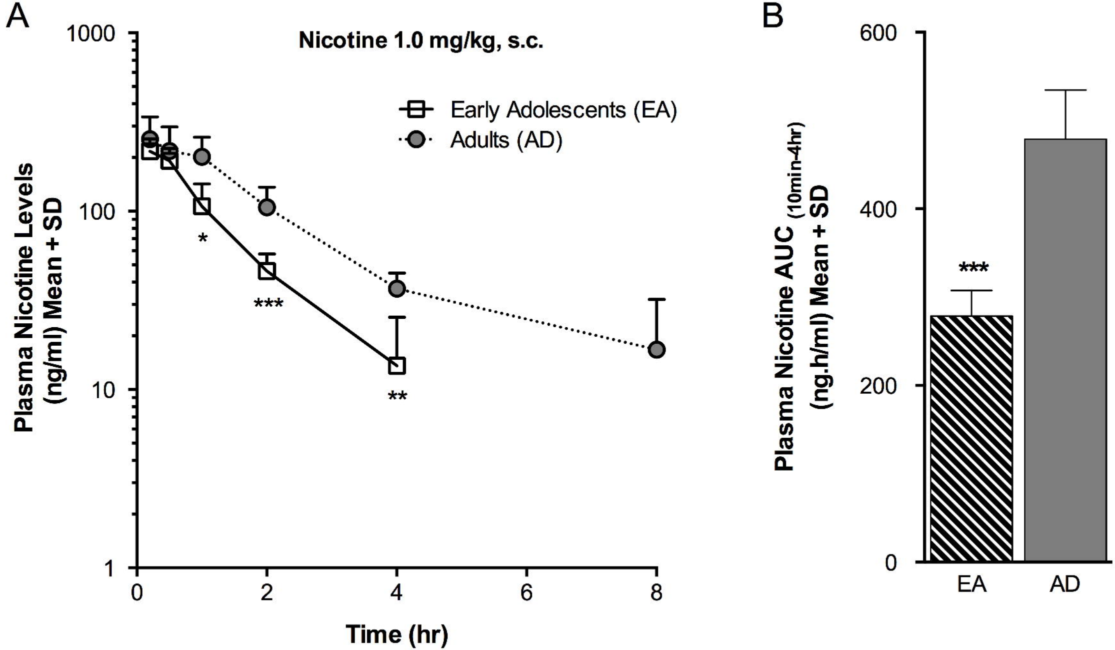
A
Nicotine 1.0 mg/kg, s.c.
Early Adolescents (EA)
Adults (AD)
Plasma Nicotine Levels
(ng/ml) Mean + SD
1000
100
10
1
*
***
**
0
2
4
6
8
Time (hr)
B
Plasma Nicotine AUC (10min-4hr)
(ng.h/ml) Mean + SD
600
400
200
0
***
EA
AD

Figure 1

DMD Fast Forward. Published on June 30, 2014 as DOI: 10.1124/dmd.114.058719
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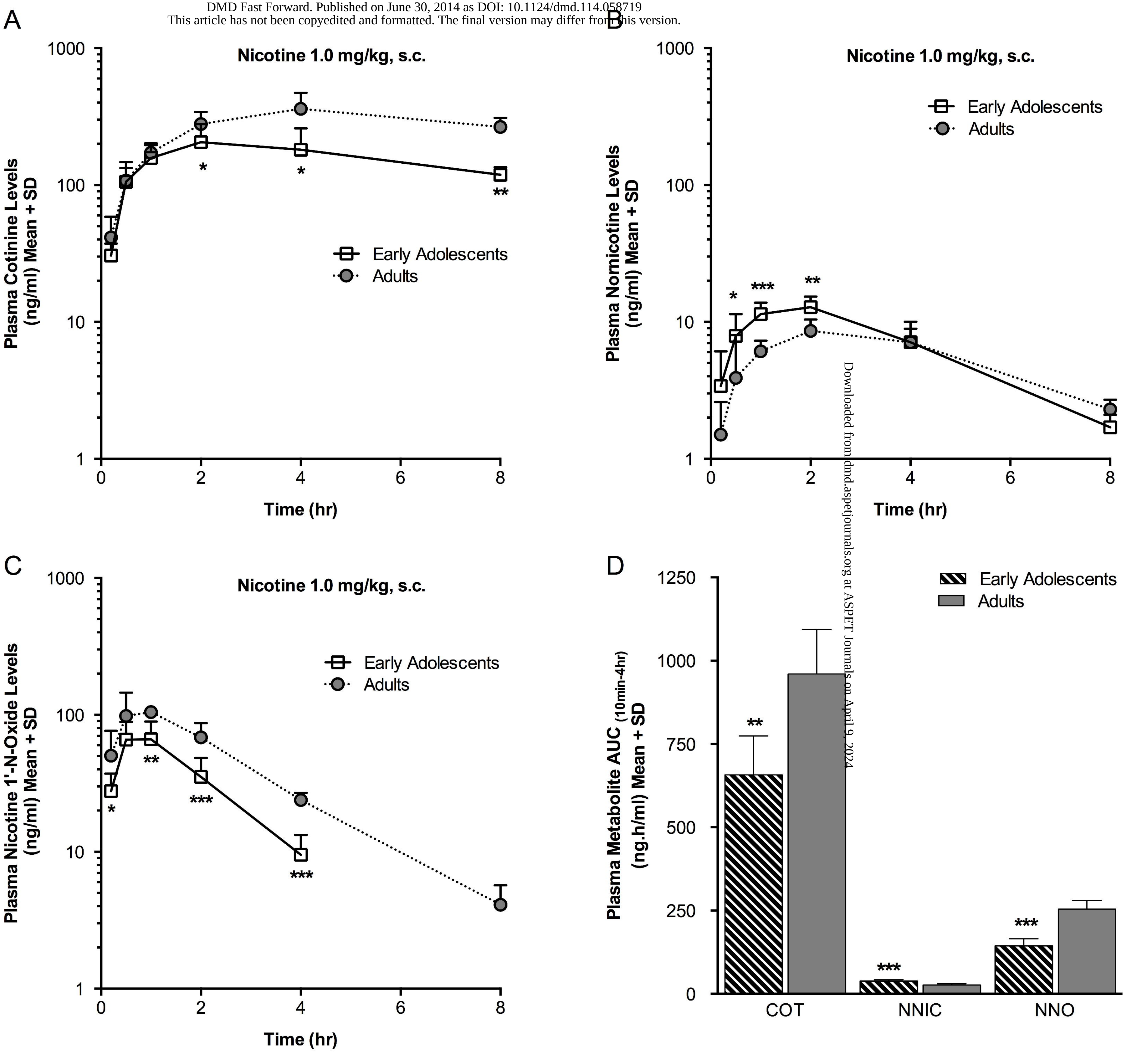

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Figure 2

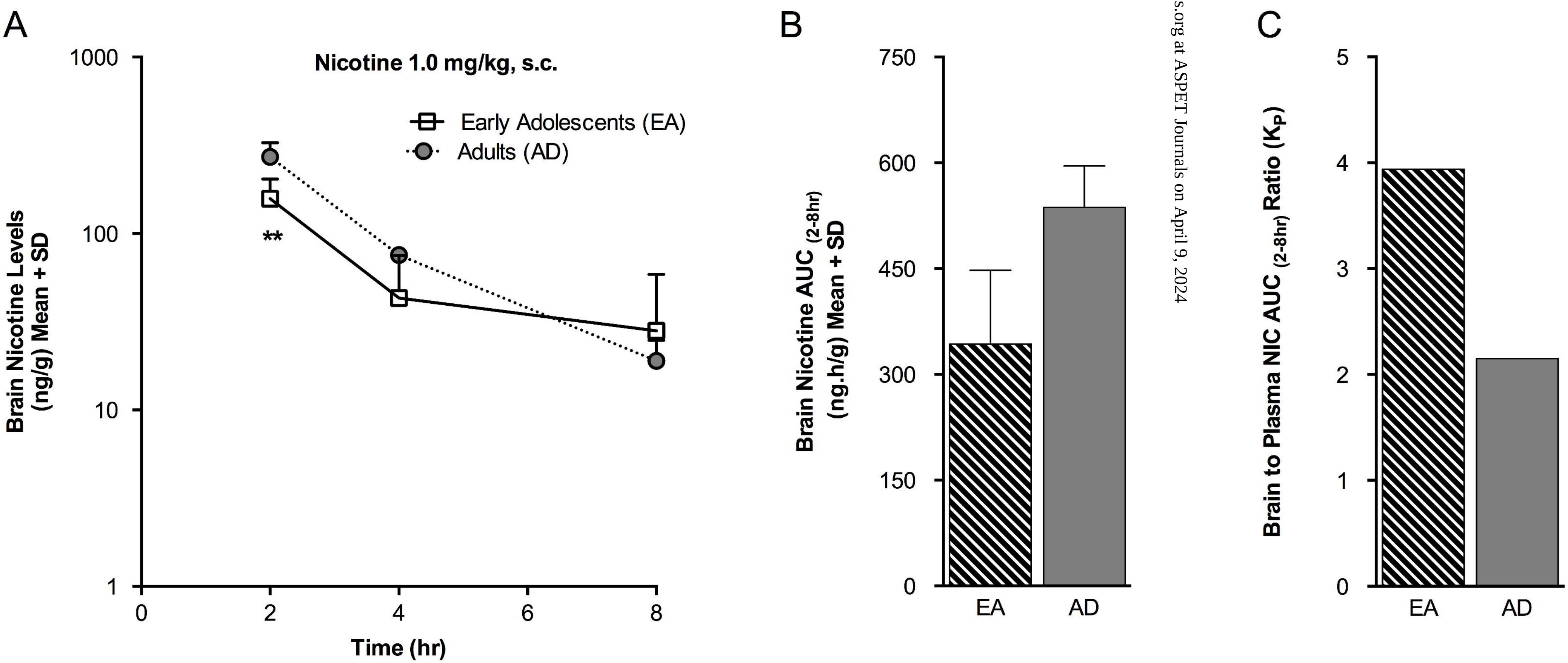

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Figure 3

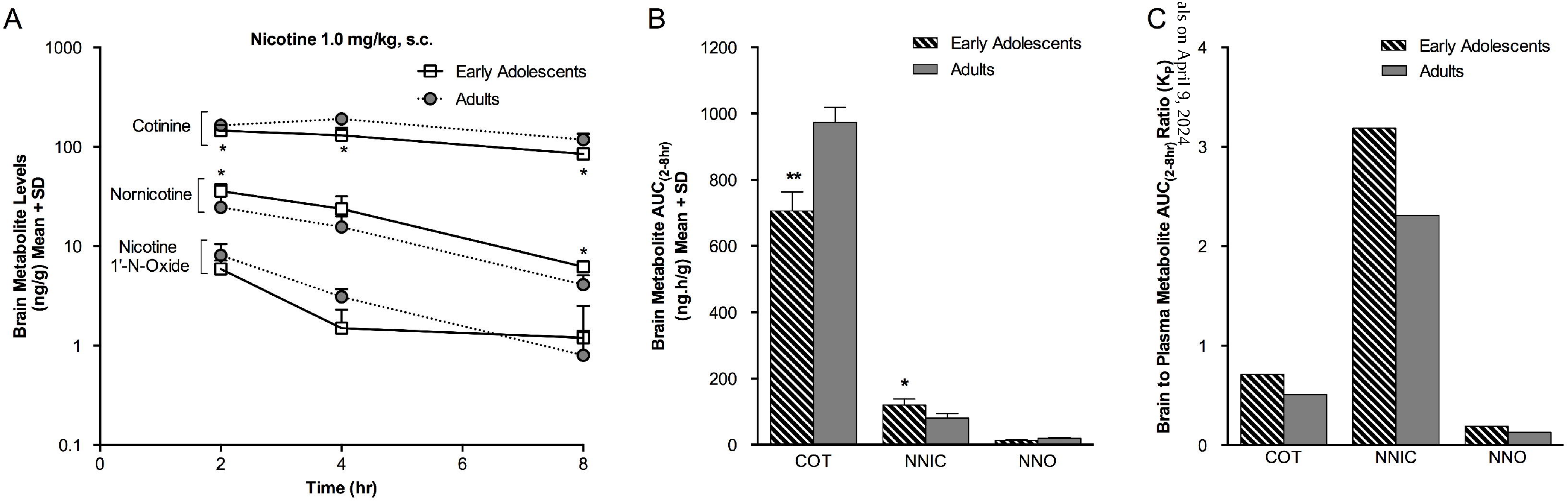

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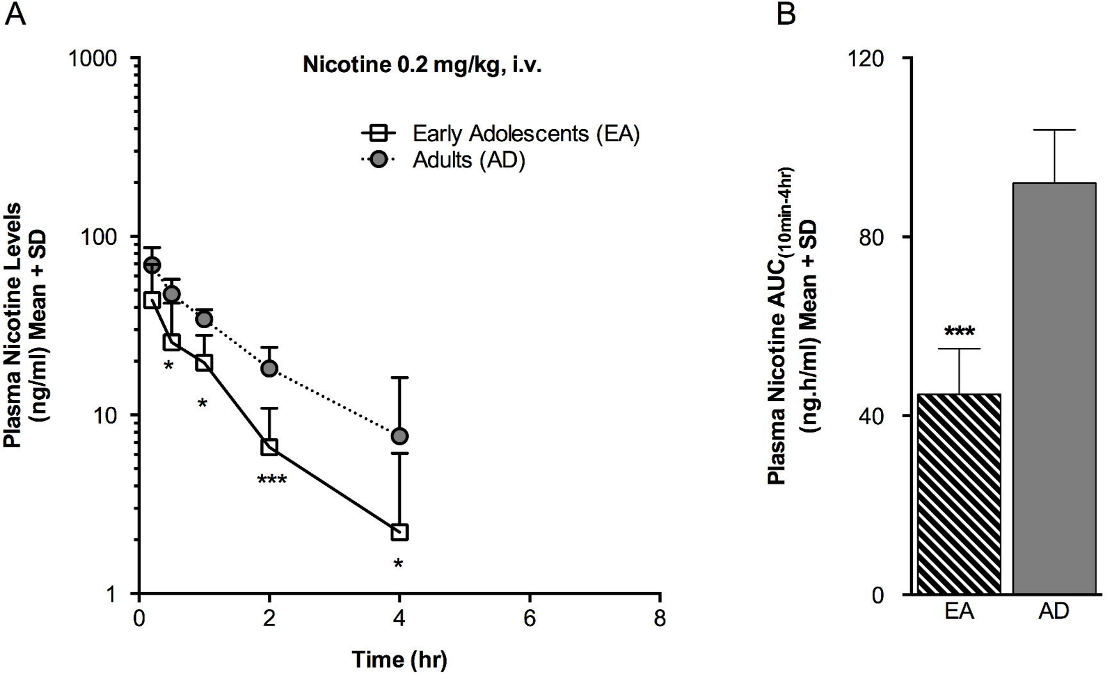
Figure 4



A
Nicotine 0.2 mg/kg, i.v.
Early Adolescents (EA)
Adults (AD)
Plasma Nicotine Levels (ng/ml) Mean + SD
1000
100
10
1
0
2
4
6
8
Time (hr)
*
*
***
*
B
Plasma Nicotine AUC(10min-4hr) (ng.h/ml) Mean + SD
120
80
40
0
***
EA
AD

Figure 5

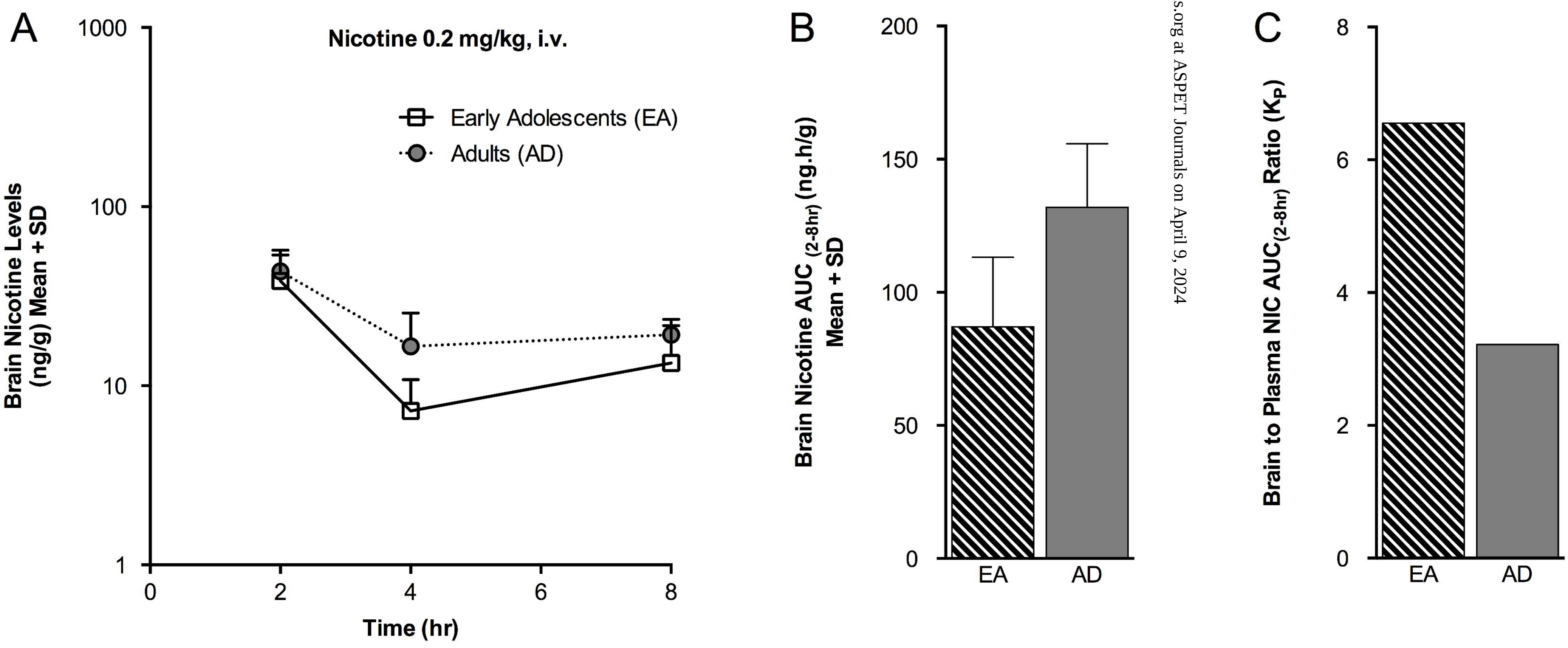


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Figure 6

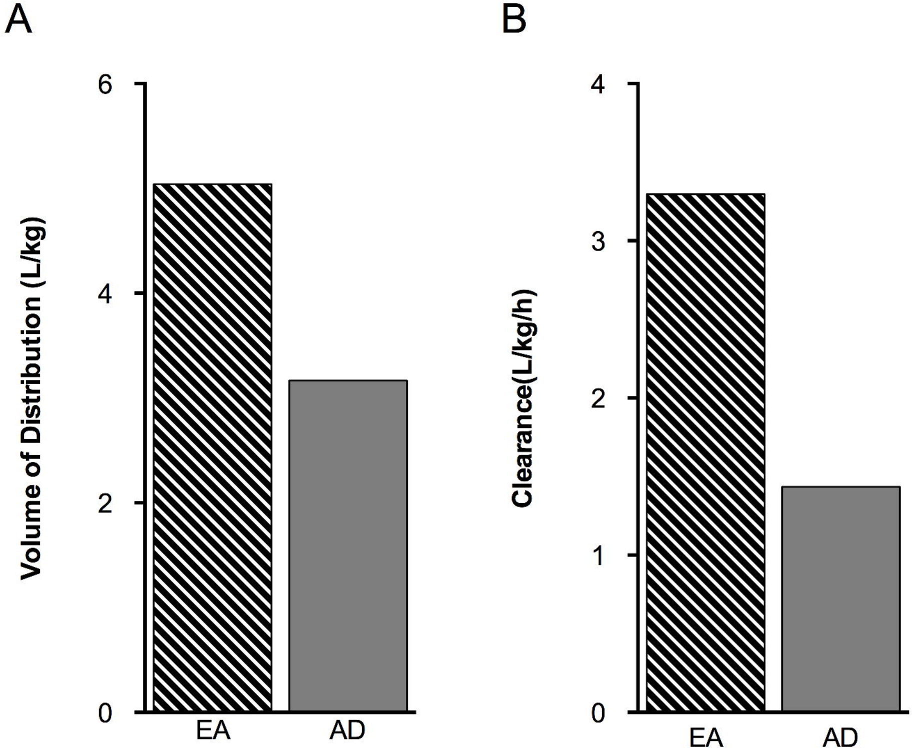

Figure 7