Chronic Nicotine Exposure Attenuates Methamphetamine-Induced Dopaminergic Deficits

Paula L. Vieira-Brock, Lisa M. McFadden, Shannon M. Nielsen, Jonathan D. Ellis, Elliot T. Walters, Kristen A. Stout, J. Michael McIntosh, Diana G. Wilkins, Glen R. Hanson, and Annette E. Fleckenstein

Departments of Pharmacology and Toxicology (P.V.-B., L.M.M., S.M.N., J.D.E., E.T.W., K.A.S., G.R.H.), Psychiatry and Biology (J.M.M.), and Pathology (D.G.W.), School of Dentistry (G.R.H., A.E.F.), University of Utah, Salt Lake City, Utah; and George E. Wahlen Veterans Affairs Medical Center, Salt Lake City, Utah (J.M.M.)

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ABSTRACT

Repeated methamphetamine (METH) administrations cause persistent dopaminergic deficits resembling aspects of Parkinson's disease. Many METH abusers smoke cigarettes and thus self-administer nicotine; yet few studies have investigated the effects of nicotine on METH-induced dopaminergic deficits. This interaction is of interest because preclinical studies demonstrate that nicotine can be neuroprotective, perhaps owing to effects involving $\alpha 4\beta 2$ and $\alpha 6\beta 2$ nicotinic acetylcholine receptors (nAChRs). This study revealed that oral nicotine exposure beginning in adolescence [postnatal day (PND) 40] through adulthood [PND 96] attenuated METH-induced striatal dopaminergic deficits when METH was administered at PND 89. This protection did not appear to be due to nicotine-induced alterations in METH pharmacokinetics. Short-term (i.e., 21-day) high-dose nicotine

exposure also protected when administered from PND 40 to PND 61 (with METH at PND 54), but this protective effect did not persist. Short-term (i.e., 21-day) high-dose nicotine exposure did not protect when administered postadolescence (i.e., beginning at PND 61, with METH at PND 75). However, protection was engendered if the duration of nicotine exposure was extended to 39 days (with METH at PND 93). Autoradiographic analysis revealed that nicotine increased striatal $\alpha 4\beta 2$ expression, as assessed using [125]epibatidine. Both METH and nicotine decreased striatal $\alpha 6\beta 2$ expression, as assessed using [125] α -conotoxin MII. These findings indicate that nicotine protects against METH-induced striatal dopaminergic deficits, perhaps by affecting $\alpha 4\beta 2$ and/or $\alpha 6\beta 2$ expression, and that both age of onset and duration of nicotine exposure affect this protection.

Introduction

Methamphetamine (METH) is a potent psychostimulant abused among adolescents and young adults (Grant et al., 2007; Johnston et al., 2014). Repeated METH administrations to humans (Sekine et al., 2001; Volkow et al., 2001; McCann et al., 2008) and rodents (McFadden et al., 2012; Kousik et al., 2014) cause long-term striatal dopaminergic deficits resembling some aspects of Parkinson's disease (PD) (McCann et al., 1998; Lotharius and Brundin, 2002; Kish et al., 2008). In fact, individuals with a history of amphetamine (AMPH)/METH abuse have an increased risk for developing PD (Callaghan et al., 2010, 2012; Curtin et al., 2015). Although the majority of patients with PD have never abused METH, overlapping neuropathologies may underlie the degenerative processes

involving these two conditions (for review, see Granado et al., 2013; Kousik et al., 2014). Preclinical studies indicate that aberrant dopamine (DA) sequestration and release leading to oxidative stress might be one of the mechanisms that likely contribute to this dopaminergic damage (Fleckenstein et al., 1997; Lotharius and Brundin, 2002; for review, see Riddle et al., 2006).

Clinical evidence suggests that PD is less likely to occur among cigarette smokers (Hernán et al., 2001, 2002; Chen et al., 2010) and preclinical research indicates that nicotine is neuroprotective against nigrostriatal dopaminergic deficits (Huang et al., 2009; García-Montes et al., 2012; for review, see Quik et al., 2012). However—and despite the fact that the majority of METH abusers smoke cigarettes (approximately 80%; McCann et al., 2008) and thus self-administer nicotine—few studies have specifically assessed the effect of nicotine on METH-induced dopaminergic deficits. Of these studies, results reveal that acute nicotine injections protect against METH-induced striatal dopaminergic deficits (Maggio et al., 1998; Ryan et al., 2001). The effect of chronic nicotine exposure has not been explored.

Previous studies have suggested that $\alpha 4\beta 2$ and $\alpha 6\beta 2$ subtypes of nicotinic acetylcholine receptors (nAChRs)

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ABBREVIATIONS: [125]RTI-55, [125]3 β -(4'-iodophenyl)tropan-2 β -carboxylic acid methyl ester; α CtxMII, α -conotoxin MII; AMPH, amphetamine; DA, dopamine; DAT, dopamine transporter; METH, methamphetamine; nAChR, nicotinic acetylcholine receptor; PD, Parkinson's disease; PND, postnatal day.

contribute to the neuroprotective effects of the stimulant, although other nicotinic subunits also likely contribute (Ryan et al., 2001; Khwaja et al., 2007; Takeuchi et al., 2009; Quik et al., 2011). For example, $\alpha 4\beta 2$ antagonist administration inhibits the protection afforded by nicotine in rotenone-treated mice (Takeuchi et al., 2009). Furthermore, the protective effect of chronic nicotine against 6-hydroxy-DA was lost in $\alpha 4$ -knockout mice (Ryan et al., 2001). Of note, however, are other studies demonstrating that $\alpha 6\beta 2$ nAChR binding is increased in $\alpha 4$ -knockout mice, leading to the suggestion that the loss of protection in $\alpha 4$ -knockout mice was due to the increase in $\alpha 6\beta 2$ expression (Perez et al., 2008). Similarly, others have suggested that nicotine-induced reductions in $\alpha 6\beta 2$ nAChRs expression mediate neuroprotection against paraquat-induced dopaminergic damage (Khwaja et al., 2007). Overall, these and other studies suggest that $\alpha 4\beta 2$ and/or $\alpha 6\beta 2$ nAChRs contribute to the neuroprotective effects of nicotine. Given that these receptor subtypes modulate DA release (Meyer et al., 2008) and aberrant DA release contributes to METH-induced dopaminergic deficit (Di Chiara and Imperato, 1988; Howard et al., 2011), the potential role of these receptor subtypes merits attention.

It is important to note that the majority of humans addicted to cigarettes initiate smoking during adolescence (Kandel and Logan, 1984; Chen and Kandel, 1995; Breslau and Peterson, 1996; Centers for Disease Control and Prevention, 2002). Furthermore, epidemiologic studies indicate that those who did not develop PD were more likely to have smoked before the age of 20 years (Chen et al., 2010). These data suggest that cigarette smoking (and thus nicotine exposure) starting at a young age may contribute to neuroprotection. However, whether age of nicotine initiation is a factor in neuroprotection is unknown.

This series of studies aimed to investigate any potential age-related effect of nicotine neuroprotection in the METH model of striatal dopaminergic dysfunction. To more closely mimic the intermittent and chronic nature of nicotine exposure in smoking, nicotine was given long-term via drinking water. The data described herein demonstrate that prolonged oral nicotine exposure protects against METH-induced striatal dopaminergic deficits, perhaps by affecting $\alpha 4\beta 2$ and/or $\alpha 6\beta 2$ expression, and that both age of onset and duration of nicotine exposure affect this protection.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) initially weighing 125–150 g [corresponding to postnatal day (PND) 40] or 245–270 g [corresponding to PND 60] (for reviews, see Spear, 2000; Tirelli et al., 2003) were housed two to three rats per cage and maintained under a controlled light/dark cycle (14:10 hours) and in an ambient environment of 20°C (with the exception of the 6-hour period during which METH or saline vehicle was administered, during which the ambient environment was maintained at 24°C). Food and water were available ad libitum. During METH or saline administrations, core body (rectal) temperatures were measured using a digital thermometer (Physitemp Instruments, Clifton, NJ) every 1 hour beginning 30 minutes before the first saline or METH administration and continuing until 30 minutes after the final saline or METH administration. Rats were placed in a cooler environment during METH exposure if their body temperature exceeded 40.5°C and were returned to their home cage once their body temperature dropped to 40°C. All experiments were approved by

the University of Utah Institutional Animal Care and Use Committee, in accordance with the 2011 National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, Eighth Edition.

Drug Treatments. METH hydrochloride was provided by the National Institutes of Health National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC) and was administered at 4×7.5 mg/kg s.c., at 2-hour intervals calculated as free base. (–)-Nicotine (1.010 g/ml; Sigma-Aldrich, St. Louis, MO) was administered ad libitum p.o. at concentrations of 10, 20, 50, or 75 μ g/ml via the water bottles, as delineated in Fig. 1. To increase palatability, 1% saccharin (Sweet & Low; Cumberland Packing Corp., Brooklyn, NY) was added to the animals' drinking water in experiments in which nicotine concentration started at the highest concentration (i.e., 75 μ g/ml; experiments in Fig. 1, B–D) or during the highest escalating rate (Fig. 1E). In these studies, nicotine water consumption was approximately 30 ml/rat per day, tap water consumption was approximately 45 ml/rat per day, and saccharin water consumption was approximately 60 ml/rat per day, similar to previous reports (Bordia et al., 2008). These nicotine doses in rats yield plasma concentrations similar to plasma nicotine and cotinine concentrations typically found in human smokers (10–50 ng/ml for nicotine and 300 ng/ml for cotinine) (Benowitz, 1994; Matta et al., 2007).

Tissue Preparation. Rats were decapitated 7 days after METH treatment. Brains were hemisected, and the left striatum was dissected out on ice, placed in cold sucrose buffer (0.32 M sucrose, 3.8 mM NaH_2PO_4 , and 12.7 mM Na_2HPO_4), and used for [^3H]DA uptake and Western blotting as described below. The contralateral brains were rapidly removed and frozen in isopentane on dry ice and stored at -80°C . Frozen right hemisected brains were sliced at 12- μ m thick at the level of the anterior striatum (1.5 mm from bregma; Paxinos and Watson, 2006) using a cryostat. Eight slices (four per rat) were mounted on each Superfrost Plus glass microslide (VWR International, Radnor, PA) and stored at -80°C for subsequent use in autoradiography assays. Hippocampal and perirhinal cortex tissues were also analyzed and data were reported in a separate article (Vieira-Brock et al., 2015).

[^3H]DA Uptake Assay. Striatal synaptosomes were prepared as previously described (Hanson et al., 2009). After decapitation, the striatum was quickly dissected out and homogenized in ice-cold sucrose buffer (0.32 M sucrose, 3.8 mM NaH_2PO_4 , and 12.7 mM Na_2HPO_4). [^3H]DA uptake assays were conducted according to Hanson et al. (2009). For plasmalemmal uptake of [^3H]DA, striatal synaptosomes were prepared accordingly and resuspended in ice-cold Krebs' buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl_2 , 16 mM sodium phosphate, 1.4 mM MgSO_4 , 11 mM dextrose, and 1 mM ascorbic acid, pH 7.4). Assay tubes containing 1.5 mg striatal tissue and 1 μ M pargyline were incubated (3 minutes, 37°C ; Sigma-Aldrich) with [7,8- ^3H]DA (0.5 nM final concentration; Perkin Elmer, Boston, MA). Nonspecific values were ascertained in the presence of 10 μ M cocaine. Samples were filtered using a filtering manifold (Brandel Inc., Gaithersburg, MD) through Whatman GF/B filters (Whatman International LTD, Maidstone, UK) soaked previously in 0.05% polyethylenimine and washed three times with 3 ml ice-cold 0.32 M sucrose. Protein concentration was used for normalization and determined by the Bradford Protein Assay.

Dopamine Transporter Western Blotting. Western blotting was conducted according to our previous method (Hadlock et al., 2009). Equal quantities of protein (8 μ g) were loaded into each well of a 4%–12% NuPAGE Novex Bis-Tris Midi gradient gel (Invitrogen, Carlsbad, CA) and electrophoresed by using a XCell4 SureLock Midi-Cell (Invitrogen). Membranes were blocked for 30 minutes with Starting-Block Blocking Buffer (Thermo Fisher Scientific, Waltham, MA) and incubated for 1 hour at room temperature with a rabbit polyclonal N-terminal dopamine transporter (DAT) antibody at 1:5000 dilution (a generous gift from Dr. Roxanne Vaughan, University of North Dakota, Grand Forks, ND; Freed et al., 1995). The polyvinylidene difluoride membrane was then washed five times in Tris-buffered saline

Fig. 1. Experimental designs. (A) In paradigm A, rats received tap water or nicotine water (10–75 $\mu\text{g/ml}$) from PND 40 to PND 96 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 89. (B) In paradigm B, rats received saccharin water or nicotine plus saccharin water (at 75 $\mu\text{g/ml}$) from PND 40 to PND 61 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 54. (C) In paradigm C, rats received saccharin water or nicotine plus saccharin water (at 75 $\mu\text{g/ml}$) from PND 40 to PND 61 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 82. (D) In paradigm D, rats received saccharin water or nicotine plus saccharin water (at 75 $\mu\text{g/ml}$) from PND 61 to PND 82 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 75. (E) In paradigm E, rats received saccharin water or nicotine plus saccharin water (10–75 $\mu\text{g/ml}$) from PND 61 to PND 100 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 93.

with Tween (250 mM NaCl, 50 mM Tris, pH 7.4, and 0.05% Tween 20). The membranes were then incubated for 1 hour with a horseradish peroxidase-conjugated secondary antibody (BioSource International, Camarillo, CA). After five washes in Tris-buffered saline with Tween, the bands were visualized by using Western Lightning Plus chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA) and quantified by densitometry using a FluorChem SP imaging system (Alpha Innotech, San Leandro, CA). Protein concentrations were determined by using the Bradford Protein Assay.

Brain METH and AMPH Concentrations. Brain concentrations of METH and its metabolite, AMPH, were measured by liquid chromatography–tandem mass spectrometry as described previously (Truong et al., 2005). The whole brains (except for the striatum) were weighed and homogenized separately in 10 ml water. A VibraCell homogenizer (Sonics, Newton, CT) was used for the homogenization. A 0.5-ml volume of the homogenate was used for the analysis. An Agilent liquid chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a ThermoQuest Finnigan TSQ 7000 tandem mass spectrometer (Thermo Fisher Scientific) was used for the analysis. Electrospray ionization was used. The lower limit of quantification was 1 ng/ml in the homogenates.

[^{125}I]RTI-55 Autoradiography. DAT density was used as a marker of dopaminergic integrity and assessed via [^{125}I]3 β -(4'-iodophenyl)tropan-2 β -carboxylic acid methyl ester ([^{125}I]RTI-55) binding to striatal slices as previously described (O'Dell et al., 2012). Briefly, slides were thawed on a slide warmer (5–10 minutes) and preincubated in sucrose buffer (10 mM sodium phosphate, 120 mM sodium chloride, and 320 mM sucrose, pH 7.4) containing 100 nM

fluoxetine at room temperature for 5 minutes, followed by a 2-hour incubation in sucrose buffer containing 25 pM [^{125}I]RTI-55 (2200 Ci/mmol; PerkinElmer Life and Analytical Sciences). Nonspecific binding was determined by slides incubated in sucrose buffer containing 25 pM [^{125}I]RTI-55 and 100 nM fluoxetine plus 100 μM nomifensine (Sigma-Aldrich). Slides were rinsed twice in ice-cold buffer and distilled water for 2 minutes and air dried. Sample slides and standard [^{125}I] microscale slides (American Radiolabeled Chemicals, St. Louis, MO) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co., Rochester, NY) for 24 hours to keep variables constant.

[^{125}I]Epibatidine Autoradiography. $\alpha 4\beta 2$ nAChR density was assessed via [^{125}I]epibatidine binding to striatal slices as previously described (Lai et al., 2005; Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 minutes) and preincubated in binding buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , and 1.0 mM MgCl_2 , pH 7.5) plus 100 nM α -conotoxin MII (αCtxMII) (synthesized as previously described by Whiteaker et al., 2000) at room temperature for 30 minutes. The nonradiolabeled αCtxMII was used to inhibit epibatidine binding to $\alpha 6\beta 2$ nAChR, followed by a 40-minute incubation in binding buffer containing 0.015 nM [^{125}I]epibatidine (2200 Ci/mmol; PerkinElmer Life and Analytical Sciences) in the presence of 100 nM αCtxMII . Nonspecific binding was determined by slides incubated in binding buffer containing 0.015 nM [^{125}I]epibatidine plus 0.1 mM nicotine. Slides were rinsed twice in ice-cold buffer for 5 minutes, followed by a 10-second rinse in distilled water. Slides were air dried. Sample slides and standard [^{125}I] microscale slides (American Radiolabeled Chemicals) were placed on one cassette and

exposed to the same Kodak MR film (Eastman Kodak Co.) for 24 hour to keep variables constant.

[¹²⁵I]αCtXMII Autoradiography. α6β2 nAChR density was assessed via [¹²⁵I]αCtXMII binding to striatal slices as previously described (Lai et al., 2005; Huang et al., 2009). Briefly, slides were thawed on a slide warmer (5–10 minutes) and preincubated in buffer A (pH 7.5, 20 nM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 0.1% bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride) at room temperature for 2 × 15 minutes, followed by a 1-hour incubation in buffer B (pH 7.5, 20 nM HEPES, 144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 0.2% bovine serum albumin, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A; Sigma-Aldrich) containing 0.5 nM [¹²⁵I]αCtXMII (approximately 2200 Ci/mmol, which was synthesized as previously described; Whiteaker et al., 2000). Nonspecific binding was determined by slides incubated in 0.5 nM [¹²⁵I]αCtXMII buffer B also containing 0.1 mM nicotine (Sigma-Aldrich). Slides were rinsed in room temperature buffer A for 10 minutes, then in ice-cold buffer A for another 10 minutes, followed by 2 × 10 minutes in 0.1 × ice-cold buffer A, and finally in 4°C distilled water for 2 × 10 seconds. Slides were air dried. Sample slides and standard [¹²⁵I] microscale slides (American Radiolabeled Chemicals) were placed on one cassette and exposed to the same Kodak MR film (Eastman Kodak Co.) for 4 days to keep variables constant.

Statistical Analyses. Statistical analyses were conducted using GraphPad Prism 5.01 software (GraphPad Software Inc., La Jolla, CA). For autoradiography, optical densities from four replicate slices per rat were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) by an analyst blinded to the experimental groups. Specific binding was obtained by subtracting film background

from mean density values and was converted to femtomoles per milligram using the standard curve generated from ¹²⁵I standards. The optical densities of the samples were within the linear range of the standards. Data were analyzed using two-way analysis of variance, except for temperature data, for which one-way analysis of variance was used followed by the Newman–Keuls post hoc test. Bonferroni adjustments were applied as appropriate. For comparisons between two groups, data were analyzed using the *t* test. Differences among groups were considered significant if the probability of error was less than 5%.

Results

Figure 1 depicts the experimental design of the studies presented herein, with additional details provided in the *Materials and Methods*. As shown in Fig. 1A, rats were exposed to an escalating-dose regimen of nicotine for a total of 56 days beginning in adolescence (PND 40) until young adulthood (PND 96), with METH administered on PND 89. As shown in Fig. 1B, the focus then shifted to assessing the effects of high-dose (75 μg/ml) nicotine exposure for 21 days beginning at PND 40 through PND 61, with METH administered on PND 54. As presented in Fig. 1C, rats were similarly exposed to high-dose nicotine for 21 days beginning at PND 40 through PND 61, with METH administered 21 days later on PND 82. In Fig. 1D, rats received high-dose nicotine for 21 days beginning at PND 61 through PND 82, with METH administered on PND 75. Finally, as shown in Fig. 1E, rats received an

Fig. 2. Chronic nicotine administration attenuates METH-induced deficits in striatal DAT function (A), immunoreactivity (B), and expression (C), with no change in METH-induced hyperthermia (D). These data are derived from the paradigm described in Fig. 1A. Data are expressed as mean values ± S.E.M. of *n* = 6–10 subjects. **P* < 0.05 (significantly different from saline control); [#]*P* < 0.05 (significantly different from SM); ^{##}*P* < 0.01 (significantly different from SM). NM, nicotine water/METH injections; NS, nicotine water/saline injections; SM, tap water/METH injections; SS, tap water/saline injections.

escalating-dose regimen beginning at PND 61 through PND 100, with METH administered at PND 93.

Results presented in Fig. 2 demonstrate that ad libitum exposure to an escalating-dose regimen of nicotine (10–75 $\mu\text{g}/\text{ml}$; see Fig. 1, paradigm A for details) from PND 40 to PND 96 attenuated the persistent (e.g., 7-day) METH-induced decrease in striatal [^3H]DAT uptake, DAT immunoreactivity, and [^{125}I]RTI-55 binding. For data presented in Fig. 2A, there was no interaction effect of METH and nicotine ($P = 0.169$), and there were main effects of nicotine ($P = 0.029$) and METH ($P < 0.0001$) per se. A post hoc comparison revealed significant differences between the saline/METH and nicotine/METH groups ($P < 0.05$). As shown in Fig. 2B, there was an interaction effect of METH and nicotine ($P = 0.038$) and main effects of nicotine ($P = 0.020$) and METH ($P < 0.0001$) per se, and a post hoc comparison revealed differences between the saline/METH and nicotine/METH groups ($P < 0.01$). In Fig. 2C, there was no interaction effect of METH and nicotine ($P = 0.052$), no main effect of nicotine ($P = 0.249$), and a main effect of METH ($P < 0.0001$). A post hoc comparison revealed differences between the saline/METH and nicotine/METH groups ($P < 0.05$). This nicotine regimen generally did not attenuate METH-induced hyperthermia (Fig. 2D).

Results presented in Fig. 3A demonstrate that ad libitum exposure to nicotine (75 $\mu\text{g}/\text{ml}$; see the *Materials and Methods* and Fig. 1, paradigm B for details) from PND 40 to PND 61 attenuated the persistent (e.g., 7-day) METH-induced decrease in striatal [^3H]DA uptake when METH was administered on

PND 54. In particular, there was an interaction effect of METH and nicotine ($P = 0.019$), and a post hoc comparison tests revealed differences between the saline/METH and nicotine/METH groups ($P < 0.01$). Similarly, for striatal [^{125}I]RTI-55 autoradiography (mean $\pm$ S.E.M. tap water/saline injections, 3.32 ± 0.06 fmol/mg; tap water/METH injections, 1.26 ± 0.24 fmol/mg; nicotine water/saline injections, 3.28 ± 0.03 fmol/mg; and nicotine water/METH injections, 2.44 ± 0.15 fmol/mg), there was an interaction effect of METH and nicotine ($P = 0.001$), and a post hoc comparison revealed differences between the saline/METH and nicotine/METH groups ($P < 0.001$). In other words, 21 days of nicotine exposure afforded protection when (as was accomplished as shown in Fig. 2) exposure was initiated on PND 40. This nicotine regimen did not attenuate METH-induced hyperthermia (data not shown).

To note, the protection afforded by 21-day nicotine exposure (75 $\mu\text{g}/\text{ml}$; Fig. 3B) does not persist when nicotine exposure is initiated on PND 40 but terminated on PND 61 (see Fig. 1, paradigm C for details). In particular, there was no interaction effect of METH and nicotine ($P = 0.691$) and no main effect of nicotine ($P = 0.304$), although there was a main effect of METH ($P < 0.0001$). A post hoc comparison revealed no differences between the saline/METH and nicotine/METH groups ($P > 0.05$). This nicotine regimen did not attenuate METH-induced hyperthermia (data not shown).

In contrast with the results presented in Fig. 3A, 21 days of nicotine (75 $\mu\text{g}/\text{ml}$) exposure was not sufficient to attenuate the persistent (7-day) METH-induced decrease in striatal

Fig. 3. (A) Short-term (i.e., 21-day) nicotine administration starting in adolescence attenuates METH-induced deficits in striatal DAT function. These data are derived from paradigm B described in Fig. 1B. (B) Nicotine neuroprotective effects on METH-induced deficits in striatal DAT function do not persist for 4 weeks. These data are derived from paradigm C described in Fig. 1C. (C) Short-term (i.e., 21-day) NIC administration starting in adulthood does not attenuate METH-induced deficits in striatal DAT function. These data are derived from paradigm D described in Fig. 1D. (D) Long-term (i.e., 39-day) nicotine administration starting in adulthood attenuates METH-induced deficits in striatal DAT function. These data are derived from paradigm E described in Fig. 1E. Data are expressed as mean values $\pm$ S.E.M. of $n = 8$ to 10 (A), $n = 8$ to 11 (B), $n = 6$ to 7 (C), or 9 to 10 (D) subjects. ### $P < 0.01$ (significantly different from SM). NM, nicotine water/METH injections; NS, nicotine water/saline injections; SM, tap water/METH injections; SS, tap water/saline injections.

[³H]DA uptake when nicotine exposure was initiated on PND 61 (Fig. 3C; see Fig. 1, paradigm D for details). In particular, there was no interaction effect of METH and nicotine ($P = 0.122$) and no main effect of nicotine ($P = 0.456$), although there was a main effect of METH ($P < 0.0001$). A post hoc comparison revealed no differences between the saline/METH and nicotine/METH groups ($P > 0.05$). This nicotine regimen did not attenuate METH-induced hyperthermia (data not shown).

Results presented in Fig. 3D demonstrate that ad libitum exposure to an escalating-dose regimen of nicotine (10–75 μ g/ml; see the *Materials and Methods* and Fig. 1, paradigm E for details) from PND 61 to PND 100 attenuated the persistent (e.g., 7-day) METH-induced decrease in striatal [³H]DA uptake. In particular, there was an interaction effect of METH and nicotine ($P = 0.004$), and a post hoc comparison revealed differences between the saline/METH and nicotine/METH groups ($P < 0.05$). For striatal [¹²⁵I]RTI-55 autoradiography (mean $\pm$ S.E.M. tap water/saline injections, 3.44 ± 0.05 fmol/mg; tap water/METH injections, 1.57 ± 0.25 fmol/mg; nicotine water/saline injections, 3.31 ± 0.06 fmol/mg; and nicotine water/METH injections, 2.16 ± 0.26 fmol/mg), there was a trend for an interaction effect of METH and nicotine ($P = 0.063$), and there was a main effect of METH ($P < 0.0001$) and no main effect of nicotine ($P > 0.05$). A post hoc comparison also revealed differences between the saline/METH and nicotine/METH groups ($P < 0.05$). This NIC regimen did not attenuate METH-induced hyperthermia (data not shown).

The concentrations of METH and its metabolite, AMPH, were evaluated in rats exposed to tap or nicotine water to investigate whether NIC alters METH pharmacokinetics. From PND 40, rats received increasing concentrations of NIC via drinking water (10–75 μ g/ml) for 49 days as described for PND 40 to PND 89 in Fig. 1 (paradigm A). METH (4×7.5 mg/kg per injection) or saline (4×1 ml/kg per injection) was

administered on PND 89 and rats were euthanized 1 hour later. Results revealed that neither METH nor AMPH concentrations differed between METH-treated rats preexposed to tap water or nicotine water [for METH, 8.08 ± 0.52 and 6.73 ± 0.66 ng/mg tissue for saline and nicotine pretreatment, respectively; $t(10) = 1.61$, $P = 0.14$; for AMPH, 1.62 ± 0.15 and 1.64 ± 0.21 ng/mg tissue for saline and nicotine pretreatment, respectively; $t(10) = 0.08$, $P = 0.93$]. Neither METH nor AMPH was detected in the saline-treated rats preexposed to tap or nicotine water (below the lower limit of quantification).

Results presented in Fig. 4 indicate that chronic nicotine treatment increased striatal [¹²⁵I]epibatidine binding density, as assessed by autoradiography in the striatum of both saline- and METH-treated rats. For data presented in Fig. 4A (i.e., rats treated as described in paradigm A), there was no interaction effect of METH and nicotine ($P = 0.124$). There were main effects of nicotine ($P < 0.0001$) and METH ($P = 0.007$). A post hoc comparison revealed differences between the saline/saline and nicotine/saline groups ($P < 0.05$) and the saline/METH and nicotine/METH groups ($P < 0.001$). As shown in Fig. 4B (i.e., rats treated as described in paradigm B), there was no interaction effect of METH and nicotine ($P = 0.960$), and there was a main effect of nicotine ($P < 0.0001$) and METH ($P < 0.007$) per se. A post hoc comparison revealed differences between the saline/saline and nicotine/saline groups ($P < 0.001$) and the saline/METH and nicotine/METH groups ($P < 0.001$). As presented in Fig. 4C (i.e., rats treated as described in paradigm E), there was no interaction effect of METH and nicotine ($P = 0.249$) and there was a main effect of nicotine ($P < 0.0001$). A post hoc comparison revealed differences between the saline/saline and nicotine/saline groups ($P < 0.001$) and the saline/METH and nicotine/METH groups ($P < 0.001$).

Results presented in Fig. 5 indicate that both nicotine and METH treatment decreased striatal [¹²⁵I] α CtxMII binding

Fig. 4. Long-term nicotine administration increases striatal $\alpha 4 \beta 2$ nAChR binding in saline-treated and METH-treated rats. (A) Rats received tap water or nicotine water (10–75 μ g/ml) from PND 40 to PND 96 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 89, as delineated in paradigm A in Fig. 1A. (B) Rats received saccharin water or nicotine plus saccharin water (75 μ g/ml) from PND 40 to PND 61 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 54, as delineated in paradigm B in Fig. 1B. (C) Rats received saccharin water or nicotine plus saccharin water (10–75 μ g/ml) from PND 61 to PND 100 and METH (4×7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 93, as delineated in paradigm E in Fig. 1E. Brains were harvested 7 days after METH and $\alpha 4 \beta 2$ density was assessed via [¹²⁵I]epibatidine autoradiography. Data are expressed as mean values $\pm$ S.E.M. of $n = 8$ –12 subjects (A), $n = 8$ –10 subjects (B), and $n = 10$ subjects (C). ^ $P < 0.05$ (significantly different from SS); ^^ $P < 0.001$ (significantly different from SS); ### $P < 0.001$ (significantly different from SM). NM, nicotine water/METH injections; NS, nicotine water/saline injections; SM, tap water/METH injections; SS, tap water/saline injections.

density, as assessed by autoradiography. For data presented in Fig. 5A (i.e., rats treated as described in Fig. 1, paradigm A), there was no interaction effect of METH and nicotine ($P = 0.275$). There was a main effect of METH ($P < 0.0001$) and nicotine ($P < 0.003$) per se. A post hoc comparison revealed differences between the saline/saline and nicotine/saline ($P < 0.05$), the saline/saline and saline/METH ($P < 0.001$) and the nicotine/saline and nicotine/METH groups ($P < 0.05$), but not the saline/METH and nicotine/METH groups ($P > 0.05$). As shown in Fig. 5B (i.e., rats treated as described in paradigm B), there was no interaction effect of METH and nicotine ($P = 0.179$), and there was a main effect of nicotine ($P = 0.003$) and METH ($P < 0.0001$) per se. A post hoc comparison revealed differences between the saline/saline and nicotine/saline ($P < 0.01$), the saline/saline and saline/METH ($P < 0.001$), and the nicotine/saline and nicotine/METH groups ($P < 0.01$), but not the saline/METH and nicotine/METH groups ($P > 0.05$). As shown in Fig. 5C (i.e., rats treated as described in paradigm E), there was no interaction effect of METH and nicotine ($P = 0.066$), and there was a main effect of nicotine ($P = 0.0002$) and METH ($P < 0.0001$) per se. A post hoc comparison revealed differences between the saline/saline and nicotine/saline ($P < 0.001$), the saline/saline and saline/METH ($P < 0.001$), and the nicotine/saline and nicotine/METH groups ($P < 0.05$), but not the saline/METH and nicotine/METH groups ($P > 0.05$).

Representative autoradiograms of the [125 I]RTI-55, [125 I]epibatidine, and [125 I] α CtxMII studies are presented in Fig. 6.

Discussion

Previous studies have demonstrated dopaminergic neuroprotection afforded by chronic oral nicotine exposure. For example, rats exposed for 7 weeks to escalating doses (12.5–50 μ g/ml) of nicotine in drinking water beginning in adolescence are partially protected against 6-hydroxy-DA-induced loss of striatal DAT (Huang et al., 2009). In addition, 6 weeks of nicotine exposure to mice via drinking water attenuated paraquat-induced deficits in striatal DAT density deficits when nicotine was initiated during adulthood (Khwaja et al., 2007). Given the clinical relevance of evaluating chronic nicotine exposure as described in the *Introduction*, our studies extended this work to determine the effects of chronic nicotine exposure on METH-induced dopaminergic deficits.

The first of our studies demonstrated that long-term (i.e., 56 days), escalating-dose (i.e., 10–75 μ g/ml) oral nicotine exposure, initiated during a period corresponding to human adolescence (i.e., paradigm A), attenuates the persistent (7-day) striatal dopaminergic deficits in rats treated with METH during young adulthood. In these studies, nicotine was administered before and during the 7-day period after METH exposure. This effect does not appear to be attributable to nicotine-mediated alterations in METH pharmacokinetics.

A second series of experiments was conducted to address the question as to whether shorter-term nicotine administration also affords protection. Results revealed that exposure to nicotine (75 μ g/ml), initiated during adolescence and administered for 21 days, likewise attenuates METH-induced persistent

Fig. 5. Nicotine or METH administration reduces striatal α 6 β 2 nAChR binding. (A) Rats received tap water or NIC water (10–75 μ g/ml) from PND 40 to 96 and METH (4 $\times$ 7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 89, as delineated in paradigm A in Fig. 1A. (B) Rats received saccharin water or nicotine plus saccharin water (75 μ g/ml) from PND 40 to PND 61 and METH (4 $\times$ 7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 54, as delineated in paradigm B in Fig. 1B. (C) Rats received saccharin water or nicotine plus saccharin water (10–75 μ g/ml) from PND 61 to PND 100 and METH (4 $\times$ 7.5 mg/kg per injection s.c., 2 hours apart) or saline (1 ml/kg per injection) at PND 93, as delineated in paradigm E in Fig. 1E. Brains were harvested 7 days after METH and α 6 β 2 density was assessed via [125 I] α CtxMII autoradiography. Data are expressed as mean values $\pm$ S.E.M. of $n = 8$ –12 subjects (A), $n = 8$ –10 subjects (B), and $n = 10$ subjects (C). $^{\wedge}P < 0.05$ (significantly different from SS); $^{\wedge\wedge}P < 0.01$ (significantly different from SS); $^{\wedge\wedge\wedge}P < 0.001$ (significantly different from SS); $^{*}P < 0.05$ (significantly different from NS); $^{++}P < 0.01$ (significantly different from NS). NM, nicotine water/METH injections; NS, nicotine water/saline injections; SM, tap water/METH injections; SS, tap water/saline injections.

Fig. 6. Representative autoradiographs depicting the effects of nicotine and METH treatments. (A) DAT ($[^{125}\text{I}]\text{RTI-55}$ binding). (B) $\alpha 4\beta 2$ nAChR ($[^{125}\text{I}]\text{epibatidine}$ binding). (C) $\alpha 6\beta 2$ nAChR ($[^{125}\text{I}]\alpha\text{CtxMII}$) densities. Blank indicates nonspecific binding. NM, nicotine water/METH injections; NS, nicotine water/saline injections; SM, tap water/METH injections; SS, tap water/saline injections.

(7-day) striatal dopaminergic deficits. In these studies, nicotine was administered before and during the 7-day period after METH exposure (paradigm B). However, this protective effect does not persist. In particular, if nicotine exposure occurred during adolescence and METH was administered as rats approach young adulthood, protection was lost (paradigm C). These data are consistent with clinical findings indicating that the reduced risk for PD diminishes as time since quitting cigarette smoking increases (Chen et al., 2010). One possible explanation for these data is that nicotine must be present during the period during and after METH exposure to afford protection. However, our data that long-term nicotine exposure affords neuroprotection even when nicotine exposure is halted 2 or 24 hours before METH treatment research (Vieira-Brock et al., unpublished observations), suggesting that the protective effects afforded by nicotine are not a direct effect of having the drug “on board” during and after METH treatment.

To investigate the effect of age of onset of nicotine exposure, a third series of studies was conducted wherein nicotine exposure occurred postadolescence (i.e., for 21 days during the period approaching young adulthood). Results revealed that in this scenario, nicotine no longer affords protection against striatal METH-induced dopaminergic deficits (paradigm D). However, postadolescent exposure to an escalating-dose paradigm with longer-term (i.e., 39-day) nicotine exposure afforded protection (paradigm E). These data demonstrate that protection can be engendered postadolescence but requires an escalating-dose paradigm and/or (more likely) longer-term exposures.

Our studies also demonstrate that oral nicotine administration per se did not alter striatal DAT function and/or expression when assessed during adulthood. These data are consistent with previous findings demonstrating that chronic nicotine administration via drinking water beginning in adolescence did not affect striatal DAT expression when assessed in adulthood (Huang et al., 2009). Similarly, 7 days of nicotine administration via osmotic minipumps had no effect on striatal DAT function and expression in adult rats (Izenwasser and Cox, 1992; Collins et al., 2004).

It is well established that attenuation of METH-induced hyperthermia protects the persistent dopaminergic deficits

caused by the stimulant. For example, exposure of animals to a low ambient temperature attenuates both METH-induced hyperthermia and neurotoxicity (Bowyer et al., 1994; Ali et al., 1995). Prevention of METH-induced hyperthermia attenuates reactive species formation as well (Fleckenstein et al., 1997). Furthermore, selective inhibition of dopaminergic receptors by various agents also attenuates METH-induced hyperthermia and affords dopaminergic neuroprotection (Sonsalla et al., 1986). However, our results reveal that chronic nicotine exposure had little effect on METH-induced hyperthermia, thus indicating that mechanisms beyond alterations in body temperature contribute to its protection.

The $\alpha 4\beta 2$ and $\alpha 6\beta 2$ nAChRs are highly expressed on dopaminergic projections and regulate striatal DA release (Champtiaux et al., 2002; Marks et al., 2014). As noted in the *Introduction*, preclinical studies indicate that METH causes aberrant DA sequestration and release, leading to oxidative stress that, in turn, contributes to the persistent dopaminergic deficits caused by the stimulant (Cubells et al., 1994; for review, see Fleckenstein et al., 2007 for review). Thus, the effects of nicotine and METH on these subtypes were investigated. Of note, preclinical associations between chronic nicotine exposure, the expression of these subtypes, and dopaminergic protection have been reported (Khawaja et al., 2007; Huang et al., 2009). Furthermore, chronic nicotine exposure increases $\alpha 4\beta 2$ nAChR density in human smokers (Benwell et al., 1988).

Results revealed that long-term nicotine exposure increased striatal $\alpha 4\beta 2$ nAChR density in both saline- and METH-treated rats. These data are consistent with reports that chronic nicotine administration upregulates $\alpha 4\beta 2$ nAChR binding in several brain regions (Marks et al., 1992; McCallum et al., 2006; Perez et al., 2008), with the upregulation accompanied by increased function (for review, see Buisson and Bertrand, 2002). Furthermore, and consistent with previous reports (Lai et al., 2005; Khawaja et al., 2007), our study revealed that chronic nicotine administration reduces striatal $\alpha 6\beta 2$ nAChR density. This alteration in the balance between $\alpha 4\beta 2$ and $\alpha 6\beta 2$ receptor subtypes is consistent with the suggestion that nicotine upregulates $\alpha 4\beta 2$ nAChRs by increasing assembly of $\beta 2$ with $\alpha 4$ subunits and consequently reducing assembly of $\beta 2$ with $\alpha 6$ subunits (Kuryatov et al., 2005; Sallette et al., 2005; Colombo et al., 2013).

It is interesting to speculate that an upregulation of $\alpha 4\beta 2$ nAChR expression/signaling afforded by nicotine at the time of METH treatment may have contributed to neuroprotection. Importantly, METH causes acetylcholine release and thus indirectly activates nAChRs (Tsai and Chen, 1994; Taguchi et al., 1998; Dobbs and Mark, 2008). $\alpha 4\beta 2$ nAChRs are found on dopaminergic terminals and increase tonic DA release when they are activated (Meyer et al., 2008). Because it is widely hypothesized that METH causes long-term dopaminergic deficits through accumulation of cytoplasmic DA that readily oxidizes and forms reactive species, $\alpha 4\beta 2$ nAChR activation could protect against METH-induced dopaminergic deficits through increased release of tonic DA (or basal firing) during the high-dose METH treatment. Noteworthy, however, are findings that $\alpha 4\beta 2$ nAChR activation has antioxidant effects (Linert et al., 1999), and nicotine administration to rats suppresses the formation of dihydrobenzoacetic acid (Obata et al., 2002), an index of hydroxyl radical formation that is increased after high-dose METH treatment (Fleckenstein et al., 1997).

An increase in $\alpha 4\beta 2$ nAChR expression/signaling at the time of METH treatment likely occurred at the expense of $\alpha 6\beta 2$ signaling. Thus, neurons expressing a greater $\alpha 6\beta 2/\alpha 4\beta 2$ ratio would be predictably more vulnerable to METH-induced deficits. Consistent with this postulation are findings that METH caused long-term deficits in $\alpha 6\beta 2$ expression, perhaps indicating a loss of dopaminergic neurons that preferentially expressed this subtype at the time of METH exposure.

Of interest are findings that nicotine-induced changes in nAChRs differ between adolescent and adult rats. Particularly, upregulation of the $\alpha 4\beta 2$ subtypes and downregulation of the $\alpha 6\beta 2$ subtypes of nAChRs are more robust in adolescent rats compared with adult rats (Doura et al., 2008). Assuming that these alterations in nAChRs contribute to protection, then the protection observed in our studies would be affected by age and could explain the shorter nicotine exposure necessary for neuroprotection to occur in adolescent versus adult rats.

In conclusion, these data indicate that nicotine protects against METH-induced striatal dopaminergic deficits, and that both age of onset and duration of nicotine exposure affect this protection. These data extend past studies indicating a role for $\alpha 7$ nAChRs in contributing to the neurotoxic effects of METH (Northrop et al., 2011), by implicating $\alpha 4\beta 2$ nAChRs as contributing to this phenomenon. The lack of $\alpha 6\beta 2$ nAChRs, owing to a shift in balance with $\alpha 4\beta 2$ nAChRs, may also affect this phenomenon. Future studies investigating correlations between the timing of, and paradigms displaying or lacking, shifts in the balance of these receptor subtypes will be important for investigating their roles in affording protection. Additional studies involving the impact of nicotine after treatment (as well as selective $\alpha 4\beta 2$ and $\alpha 6\beta 2$ agonists and antagonists) will also be of importance, because these could suggest treatment strategies for METH-induced toxicities as well as degenerative disorders such as PD.

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Authorship Contributions

Participated in research design: Vieira-Brock, Hanson, Fleckenstein.
Conducted experiments: Vieira-Brock, McFadden, Nielsen, Ellis, Walters, Stout.
Performed data analysis: Vieira-Brock, McFadden, Ellis, Walters, Stout, Wilkins, Fleckenstein.
Wrote or contributed to the writing of the manuscript: Vieira-Brock, McFadden, McIntosh, Wilkins, Hanson, Fleckenstein

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Address correspondence to: Dr. Annette E. Fleckenstein, School of Dentistry, University of Utah, 26 South 2000 East, Suite 5900, Salt Lake City, UT 84112. E-mail: fleckenstein@hsc.utah.edu