Nicotinic Receptor Modulation of Dopamine Transporter Function

in Rat Striatum and Medial Prefrontal Cortex

LISA S. MIDDLETON[1], WAYNE A. CASS[2] and LINDA P. DWOSKIN[1]

[1]College of Pharmacy and [2]Department of Anatomy and Neurobiology, College of Medicine

University of Kentucky

Lexington, KY 40536-0082

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Corresponding Author: Linda P. Dwoskin

College of Pharmacy

University of Kentucky

Rose Street

Lexington, KY 40536

Phone: 859-257-4743

Fax: 859-257-7564

E-mail: ldwoskin@uky.edu

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ABSTRACT

Nicotine activates nicotinic receptors (nAChRs) on dopamine (DA) terminals to evoke DA release, which subsequently is taken back up into the terminal via the DA transporter (DAT). nAChRs may modulate DAT function thereby contributing to the regulation of synaptic DA concentrations. The present study determined the dose-response for nicotine (0.1 – 0.8 mg/kg; s.c.) to modulate DA clearance in striatum and medial prefrontal cortex (mPFC) using in vivo voltammetry in urethane anesthetized rats, and determined if this effect was mediated by nAChRs. Exogenous DA (200 µM) was pressure ejected at 5 min intervals until reproducible baseline signals were obtained. Subsequently, nicotine or saline was administered, and DA pressure ejection continued at 5 min intervals for 60 min. In both striatum and mPFC, signal amplitude decreased by ~20% across the 60-min session in saline-injected rats. A monophasic dose-response curve was found in striatum, with a maximal 50% decrease in signal amplitude after 0.8 mg/kg. In contrast, a U-shaped dose response curve was found in mPFC, with a maximal 50% in signal amplitude after 0.4 mg/kg. Onset of nicotine response occurred at 10-15 min after injection in both brain regions, however, the time to maximal response was 45 and 30 min in striatum and mPFC, respectively. Mecamylamine (1.5 mg/kg) completely inhibited the nicotine (0.8 and 0.4 mg/kg)-induced decrease in signal amplitude in striatum and mPFC, respectively, indicating mediation by nAChRs. Thus, nicotine enhances DA clearance in striatum and mPFC in a mecamylamine-sensitive manner, indicating that nAChRs modulate DAT function in these brain regions.

Previous research on the neurobiology of reward and drug addiction has focused on the mesocorticolimbic and nigrostriatal dopamine (DA) systems, emphasizing the role of the nucleus accumbens, medial prefrontal cortex (mPFC) and striatum. The accumbens shell, which is innervated by dopaminergic projections from the ventral tegmental area, and its associated neurocircuitry are believed to encode primary appetitive stimuli associated with unconditioned drug reward (Wise and Bozarth, 1987; Robbins and Everitt, 1996; Bardo, 1998; Koob, 1999; DiChiara, 2000; Kelley and Berridge, 2002). The mPFC, which includes the anterior cingulate cortex and is innervated by dopaminergic projections from the ventral tegmental area, is believed to encode secondary conditioned stimuli associated with environmental cues paired with drug, leading to reward expectancy, which is recognized as important to the process of addiction and relapse to drug use (Berridge and Robinson, 1998; Shima and Tanji, 1998; Kelley, 1999; DiChiara, 2000; Kelley and Berridge, 2002; Peoples, 2002). Integration of the motivational information from the mPFC occurs at least in part in striatal neurons, which are innervated by dopaminergic projections from the nigra, leading to the initiation and execution of movement in reward expectancy and detection of reward (Martin-Soelch et al., 2001).

Tobacco dependence is the most common drug of abuse and the leading preventable cause of death in the USA (Surgeon General's Report, 1988; Jaffe, 1990). Nicotine, the most abundant alkaloid in tobacco, has intrinsic rewarding properties, which are believed to be responsible for tobacco dependence (Koob,1992; Corrigall et al., 1992; Stolerman and Jarvis, 1995; Garrett et al., 2003). The mechanisms underlying the reinforcing properties of nicotine are not well understood, although the mesocorticolimbic and nigrostriatal DA systems are believed to be involved. For example, c-fos activation of the anterior cingulate cortex, nucleus accumbens and striatum has been demonstrated in nicotine self-administering rats (Pagliusi et al., 1996; Merlo-Pich et al., 1997), and cortical c-fos has been shown to be activated following exposure to environmental cues associated with repeated nicotine administration (Schroeder et al., 2001).   The latter results from animal studies are consistent with findings showing that in tobacco smokers, nicotine dose-dependently increased neuronal activity in the cingulate cortex, frontal lobe and nucleus accumbens, as determined using functional MRI (Stein et al., 1998).

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Extracellular DA concentrations are increased following nicotine stimulation of nicotinic acetylcholine receptors (nAChRs) in terminal regions of the mesocorticolimbic and nigrostriatal systems. mRNA for nine subunits (α2–α7 and β2–β4) of nAChRs have been identified in substantia nigra and ventral tegmental dopaminergic neurons (Klink et al., 2001, Zoli et al., 2002; Azam et al., 2002; Wooltorton et al., 2003), indicating that potentially a large number of heteromeric nAChR subtypes of pentameric structure may be expressed by these neurons. Specific subunit combinations of nAChRs mediating nicotine-evoked DA release (Imperato et al., 1986; Vezina et al., 1992; Nisell et al., 1996, 1997; Teng et al., 1997; Zhou et al., 2001) have not been elucidated conclusively.

Extracellular DA concentrations are the net result of exocytotic DA release from presynaptic terminals and reuptake of DA into presynaptic terminals (clearance) via the DA transporter (DAT). DA is also removed from the extracellular space by metabolism and diffusion, the latter factor playing a larger role in brain regions with sparse dopaminergic innervation and lower DAT density, such as the mPFC. DAT is the major presynaptic terminal protein regulating extracellular DA concentrations and is a presynaptic target for psychostimulant drugs of abuse, as well as for several antidepressant agents. Psychostimulants, such as amphetamine, increase extracellular DA concentrations by reverse transport of DAT (Liang and Rutledge, 1992; Sulzer et al., 1995). Cocaine inhibits DAT function, which results in increased extracellular DA concentrations (Kuhar et al., 1991). The antidepressant and tobacco use cessation agent, bupropion, inhibits DAT function, but also is a nAChR antagonist (Hurt et al., 1997; Slemmer et al., 2000; Miller et al., 2002). Surprisingly, nicotine (0.4 mg/kg) has been reported to increase DA clearance (enhance DAT function) in the nucleus accumbens in anesthetized rats (Hart and Ksir, 1996). The latter results also contrast findings in vitro in which nicotine and another nicotinic agonist, 1,1-dimethyl-4-phenyl-piperazinium, have been reported to decrease [^{3}H]DA uptake into chopped striatum and PC12 cells, respectively (Izenwasser et al., 1991; Huang et al., 1999). However, these in vitro findings have not been replicated (Kramer et al., 1989; Carr et al., 1989; Rowell and Hill 1993; Zhu et al., 2003).

The observation that nicotine enhances DAT function in nucleus accumbens in vivo (Hart and Ksir, 1996) is not expected considering the electrogenic nature of the transporter.

Translocation of DA by DAT across the membrane is coupled to the cotransport of two Na^+ ions and one Cl^- ion down their electrochemical gradients, generating a small inward current (Kanner and Schuldiner, 1987; Rudnick and Clark, 1993). Moreover, the DA neuron membrane potential influences DAT function, such that hyperpolarization increases the velocity of DA transport by DAT and depolarization decreases DA transport velocity (Sonders et al., 1997). As such, nicotine-induced stimulation of nAChRs, which results in depolarization of the plasma membrane (Calabresi et al., 1989), would be expected to decrease DAT function and decrease DA clearance, thereby increasing extracellular DA concentrations.

With respect to nAChR modulation of DAT function, nicotine has also been reported to enhance amphetamine-evoked [³H]DA release from mPFC slices incubated in assay buffer in the absence of calcium (Drew et al., 2000). These assay conditions precluded nicotine-evoked exocytotic DA release, suggesting the involvement of DAT in the nicotine-induced augmentation of the response to amphetamine. Surprisingly, nicotine did not enhance amphetamine-evoked [³H]DA release in striatum or nucleus accumbens (Drew et al., 2000), indicating that this effect was specific to mPFC. The observed nicotine enhancement of the effect of amphetamine in mPFC was inhibited by nAChR antagonists, mecamylamine and dihydro-β-erythroidine, but not α-bungarotoxin, indicating that specific nAChR subtypes are involved. The latter findings suggest that nAChRs may modulate DAT function under physiological conditions, i.e., in the presence of extracellular calcium, at least in mPFC.

The goal of the present study was to characterize the dose-response relationships for nicotine enhancement of DAT function in striatum and mPFC. Furthermore, nAChR mediation was determined by assessing mecamylamine inhibition of the nicotine effect on DAT function in both striatum and mPFC. Under physiological conditions, clearance of exogenously applied DA was assessed with msec resolution using in vivo voltammetry, a technique which has been previously shown to reliably evaluate DAT function (Cass et al., 1992).

METHODS

Materials.   S(-)-Nicotine ditartrate (nicotine), 3-hydroxytyramine hydrochloride (dopamine, DA), mecamylamine HCl (mecamylamine) and sodium phosphate dibasic were purchased from Sigma/RBI (Natick, MA). Sodium phosphate monobasic, sodium chloride, ascorbic acid and urethane were purchased from Fisher Scientific (Pittsburgh, PA). Nafion perfluorinated ion-exchange resin was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dental wax was purchased from Patterson Dental Supply, Inc. (Louisville, KY). Dental acrylic was purchased from CMA Microdialysis (Acton, MA). Epoxylite and PX grade Graphpoxy were purchased from Epoxylite Corp. (Irvine, CA) and Dylon Industries, Inc. (Cleveland, OH), respectively. Carbon fibers of 30 μm diameter were purchased from Textron, Inc. (Lowell, MA), and 28 gauge lacquer-coated copper wire was purchased from Radio Shack (Lexington, KY).

Animals. Male Sprague Dawley rats (200 – 250 g) were obtained from Harlan Laboratories (Indianapolis, IN) and were housed two per cage with free access to food and water in the Division of Lab Animal Resources at the College of Pharmacy, University of Kentucky. Experimental protocols involving the animals were in strict concordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

***In vivo* Electrochemical Measurements**. Rats were anesthetized with urethane (1.25 - 1.5 g/kg, i.p.) and placed into a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). Body temperature was maintained at 37 °C with a heating pad coupled to a rectal thermometer (Harvard Apparatus, Holliston, MA). The scalp was reflected, and a section of the skull and dura overlying the frontal cortex were removed. At a location remote from the recording site, a small hole was drilled in the skull above the posterior cortex for placement of two Ag/AgCl reference electrodes, which were cemented into place with dental acrylic.

Electrochemical electrodes were prepared and electrochemical experiments conducted using previously described methods (Cass et al., 1992; Gratton et al., 1989). Electrochemical recording electrodes contained a single carbon fiber (33 μm diameter) in a pulled glass capillary

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(4 mm outer diameter, 0.80 mm wall diameter) sealed with Epoxylite. Graphite epoxy resin and a 28 gauge lacquer-coated copper wire were inserted into the glass capillary to establish electrical contact with the carbon fiber. The exposed carbon fiber extended 50-150 μm beyond the tip of the glass capillary. Electrodes for some experiments were purchased from Quanteon (Lexington, KY). To enhance selectivity for DA, the carbon fiber electrode was coated with Nafion polymer (5% solution, 6 - 8 coats) and cured by heating at 250 °C for 5 min. Electrodes were calibrated *in vitro* to determine the sensitivity and selectivity for DA. Calibration curves were generated using a range of DA concentrations (1.0 – 10 μM, at 22 °C in 0.1 M PBS solution, pH 4.0). Electrodes showed good sensitivity to DA and were relatively insensitive to ascorbic acid, such that the mean selectivity ratio of DA/ascorbic acid was 594 ± 57 to 1 ($n = 108$). Subsequently, each carbon fiber electrode was attached to a single barrel micropipette (tip diameter of 10-15 μm) with dental wax. The tips of the electrode and micropipette were positioned 250 - 300 μm apart. Micropipettes were prepared from monofilament glass (1.0 mm O.D., 0.58 mm I.D.) using a vertical pipette puller (Model 720, David Kopf Instruments, Tujunga, CA). Single barreled micropipettes were filled with DA (200 μM, in 154 mM NaCl and 100 μM ascorbic acid, pH 7.4) immediately prior to conducting the experiment.

The electrode/micropipette assembly was lowered into the dorsal striatum (1.5 mm anterior to bregma, 2.3 mm lateral from midline, and 4.0 - 5.5 mm below the surface of the cortex) or mPFC (cingulate cortex; 2.9 mm anterior to bregma, 1.0 mm lateral from midline, and 2.5 – 5.0 mm below the surface of the cortex), according to the rat brain atlas of Paxinos and Watson (1986). Exogenous DA was pressure ejected (30 - 50 psi, 0.05 ms – 2.5 s) at 5-min intervals using a Picosprizter II (General Valve Corporation, Fairfield, NJ), until reproducible baseline signals (variation in signal amplitude $< \pm 10\%$) were obtained. Using a stereomicroscope fitted with a reticule in one eyepiece, ejection volume (250 nl/mm) was monitored by measurement of the fluid displaced from the micropipette.

In the first series of experiments, dose-response curves for nicotine modulation of DAT function in striatum and mPFC were generated, with nicotine dose and brain region as between-groups factors. Rats were randomly assigned to 5 treatment groups to assess the effect of nicotine (0.1 - 0.8 mg/kg, s.c.) or vehicle (saline) on DAT function in striatum. Rats were

assigned to 6 treatment groups to assess the effect of nicotine (0.1 - 0.8 mg/kg, s.c.) or vehicle (saline) on DAT function in mPFC. In an attempt to generate the full dose-response relationship in mPFC, the effect of an additional nicotine dose (0.3 mg/kg) was determined based on the dose-response curve obtained.

For each experiment, nicotine was administered once reproducible baseline signals were obtained. Pressure ejection of DA continued at 5-min intervals for 60 min following nicotine or vehicle injection. In the majority of mPFC experiments, rats were pretreated with desipramine (25 mg/kg, s.c.) to assure that DAT function was measured and that the norepinephrine transporter (NET) did not play a prominent role. Our previous in vivo voltammetry studies have shown that the mPFC is heterogeneous with respect to transporter regulation of extracellular DA concentrations (Cass and Gerhardt, 1995). Results from the latter study reveal that at deep recording depths (2.5 – 5.0 mm below the cortical surface), DAT primarily clears DA from the extracellular compartment. At more superficial recording depths (0.5 - 2.25 mm the cortical surface), NET plays a greater role. As such, local application of desipramine into mPFC at superficial depths resulted in inhibition of DA clearance. At deep recording depths, desipramine had no effect on DA clearance. In contrast, local application of GBR 12909, a selective DAT inhibitor, produced the reverse effect, i.e., inhibited DA clearance at deep recording depths and had no effect at superficial depths in mPFC. In the current study, recording was at the lower depths of mPFC (2.5 – 5.0 mm below the cortical surface), in an attempt to ensure that the effect of nicotine on DAT function was evaluated. Comparisons of results in the absence and presence of desipramine revealed no significant differences in the effect of nicotine on DA clearance.

To determine if nAChRs mediated the response to nicotine on DA clearance in striatum and mPFC, the effect of mecamylamine, a noncompetitive nAChR antagonist (Dwoskin and Crooks, 2001), was determined. Rats were randomly assigned to 8 treatment groups (4 treatment groups/brain region). Each group was injected s.c. with either mecamylamine or saline, followed 40 min later by a second injection s.c. of nicotine or saline; such that herein, treatment groups are designated as first/second injection (i.e., mecamylamine/saline, mecamylamine/nicotine, saline/nicotine and saline/saline groups). Once reproducible baseline signals were obtained, mecamylamine (1.5 mg/kg) or vehicle (saline) was injected. The dose of mecamylamine was

chosen primarily based on previous behavioral studies from our laboratory and others in which a similar dose of mecamylamine (1.2 mg/kg) inhibited both the acute effects of nicotine on locomotor activity and the sensitization induced by repeated administration of nicotine (Miller et al., 2001). A lower dose of mecamylamine (1.0 mg/kg) was previously shown to inhibit nicotine-induced locomotor stimulation (Clarke and Kumar, 1983). The dose of mecamylamine chosen was also within the range of that inhibiting nicotine self-administration in rats (Rauhut et al., 2002; Rezvani et al., 2002). Mecamylamine (1.0 mg/kg) also was shown to inhibit the effect of nicotine to enhance DA clearance in nucleus accumbens using in vivo voltammetry (Hart and Ksir, 1996). Based on results of the current nicotine dose-response curves in the first series of experiments, nicotine doses (0.8 and 0.4 mg/kg) that produced a maximal effect in striatum and mPFC, respectively, were chosen. DA was pressure ejected every 5 min during the 40 min period following the first s.c. injection and for 60 min following the second s.c. injection. For all experiments, drug dose represents the salt weight, and the injection volume was 1 ml/kg body weight.

High-speed chronoamperometric electrochemical measurements were made continuously (5 times/s, 5 Hz and averaged to 1 Hz), using an electrochemical recording system (IVEC 10; Medical Systems Corporation, Greenvale, NY). The oxidation potential was a square wave of +0.55 volts applied for 100 ms (versus the Ag/AgCl reference electrode), and the resting potential was 0.0 volts for 100 ms. Oxidation currents were digitally integrated during the last 80 ms of each 100-ms pulse.

Data and Statistical Analysis. Data are represented as mean values $\pm$ S.E.M., and n represents the number of animals in a treatment group. Three parameters were determined from the DA oxidation currents; maximal signal amplitude, which is defined by the maximal change in extracellular DA concentration; T_{80}, the time for the signal to decay by 80% minus the rise time of the signal; and DA clearance rate, the slope of the initial pseudolinear portion of the decaying signal (between the T_{20} and T_{60} time points; Fig. 1), integrating signal amplitude and time course of decay.

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For first series of experiments generating nicotine dose-response curves in striatum and mPFC, time courses for the nicotine dose effect were analyzed by two-way mixed-factor ANOVA, with dose as a between-groups factor and time as within-subjects factor. A separate ANOVA was performed to analyze the time course for each of the three parameters assessed, maximal signal amplitude, T_{80} and clearance rate for each brain region. To determine the dose-related effect of nicotine, one-way ANOVAs were conducted on the signal amplitude data from striatum at each 5-min time point during the 30-60 min period following nicotine injection. One-way ANOVAs were also conducted on the dose response for signal amplitude and clearance rate data from mPFC at each time point during the 30-60 min period following nicotine injection. The time period chosen for the latter analyses was based on previous results in rat nucleus accumbens following a single dose (0.4 mg/kg, s.c.) of nicotine (Hart and Ksir, 1996). Dunnett's post hoc analysis was performed to determine differences from control. Additionally, specific within-subject contrasts determined the time point at which nicotine significantly decreased signal amplitude compared to baseline (prior to drug injection). Furthermore, for both striatum and mPFC, signal amplitude data were summed across the 60-min recording period to assess nicotine dose-response relationships, and these data were analyzed by one-way ANOVA and Tukey test to determine the nicotine dose that significantly decreased signal amplitude.

Analysis of mecamylamine-induced inhibition of the effect of nicotine on each of the three parameters (maximal signal amplitude, T_{80} and clearance rate) for each brain region was accomplished using separate three-way mixed-factor ANOVAs, with mecamylamine and nicotine as between-group factors and time as a within-subjects factor. Significant interactions were found for the data expressed as signal amplitude and clearance rate, such that one-way ANOVAs were utilized to assess the effect of nicotine and mecamylamine at individual time points. Additionally, specific within-subjects contrasts were performed for the group administered saline followed by nicotine to determine the time point at which nicotine significantly decreased signal amplitude compared to baseline.

ANOVA, specific contrasts and post hoc analyses were performed using SPSS (standard version 11.0, Chicago, IL). $p < 0.05$ was considered significant. Based on the apriori hypothesis being tested, one-tailed tests were considered statistically significant; however, two-tailed tests

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were reported herein unless otherwise indicated. Nonlinear curve fitting of the nicotine dose-

response data was performed by Prism through a nonweighted iterative process (Prism v3.0,

GraphPad software, Inc., San Diego, CA).

RESULTS

Effect of Nicotine on Exogenous DA Clearance in Striatum. The effect of systemic administration of nicotine (0.1 – 0.8 mg/kg) on exogenous DA clearance in the medial dorsal striatum of urethane-anesthetized rats was determined. Pressure ejection of 200 μM DA every 5 min resulted in stable baseline signals, with a maximal signal amplitude of 4.21 μM (± 0.22 μM; mean ± S.E.M.; range, 1.51 – 9.90 μM), T_{80} value of 32.6 s (± 3.1 s) and clearance rate of 0.307 μM/s (± 0.056 μM/s; n = 32 independent experiments). Maximal signal amplitude during measurement of basal DA clearance varied with the placement of the electrode/micropipette assembly in the striatum. Fig. 1 illustrates the reproducible pattern of a representative series of baseline signals obtained prior to systemic injection of nicotine (0.8 mg/kg) or saline (Fig. 1, top panel). Maximal signal amplitude under basal conditions was 3.64 μM, which was near the median response and within one SD of the mean response of the group. Once baseline signals had stabilized, rats were injected with either nicotine or saline (control), and pressure ejection of DA continued every 5 min for 60 min. The effect of nicotine on maximal signal amplitude, T_{80} and clearance rate was determined at 5 min intervals and compared to that after saline injection. An overlay of representative signals, obtained in striatum at the 45-min time point after injection when the maximal effect of nicotine (0.8 mg/kg) was observed, reveals a large decrease (45%) in maximal signal amplitude (Fig. 1, bottom panel). Rise time, duration and decay of the DA signal were not different between nicotine- and saline-treated rats.

Analysis of the data from the control group (saline injected) reveals that DA signal amplitude gradually decreased by ~15% of baseline during the 55-min period after saline injection; however, the decrease (~23%) in signal amplitude was significant only at the 60-min time point (Fig. 2, top panel). T_{80} did not change across the time course of the experiment in the saline-injected group (Fig. 2, middle panel). Similar to signal amplitude, DA clearance rate also gradually decreased by ~15% of baseline across the time course of the session (Fig. 2, bottom panel). The latter panel illustrates that at the 60-min time point, DA clearance rate decreased by 40%; however, there were no significant main effects or interactions, such that posthoc analyses were not performed. Thus, repeated DA application at 5-min intervals resulted in a small increase in DAT function across the 60-min session after saline injection.

The dose response for nicotine-induced modulation of DAT function in striatum was determined and the results are shown in Fig. 2. The parameters of DAT function (maximal signal amplitude, T_{80} and clearance rate) were analyzed using separate two-way ANOVAs. With respect to signal amplitude, a significant interaction of dose and time was found ($F_{52,351} = 1.58$, $p < 0.05$). To further analyze the interaction, separate one-way ANOVAs of signal amplitude were conducted on the data from each 5-min time point during the 30-60 min after injection (Fig. 2, top panel). Dose-related decreases in signal amplitude were observed at various time points. Significant main effects of nicotine dose were found at 40-45 min ($p < 0.05$), and at the 35 min and 50-55 min time points ($p < 0.05$, one-tailed) after injection. Dunnett's post hoc analysis revealed that DA signal amplitude was decreased 35-55 min in the 0.8 mg/kg group compared to the control group. Signal amplitude decreased by a maximum of 48% of control at 45 min after the 0.8 mg/kg dose of nicotine (between-groups comparison); however, when signal amplitude at this time point was compared to the baseline response (within-subject comparison), a 60% decrease in signal amplitude was found. To determine the onset of the effect of nicotine, specific within-subjects contrasts were performed comparing DA signal amplitude at each time point following nicotine injection to baseline signal amplitude. A significant decrease in signal amplitude occurred 15 min following nicotine injection (0.8 mg/kg, $p < 0.01$). In contrast to the effect of nicotine on signal amplitude, no significant main effects or interactions were found for T_{80} or clearance rate, when the data were analyzed by two-way ANOVA (Fig 2, middle and bottom panels, respectively). With respect to clearance rate, the highest dose (0.8 mg/kg) of nicotine tended to increase clearance rate, but did not reach significance. Thus, compared to T_{80} and clearance rate, DA signal amplitude is the more sensitive parameter for detection of the effect of nicotine on DAT function.

To further evaluate the nicotine dose-response relationship, DA signal amplitude data for each dose were cumulated across the 60-min sampling period to generate a dose-response curve (Fig. 3). The nicotine dose-response curve was monophasic, and nonlinear regression revealed a significant fit to the data ($r^2 = 0.99$). Thus, as the nicotine dose increased, DA signal amplitude decreased, indicating that in a dose-related fashion nicotine enhances DAT function in striatum.

Effect of Nicotine on Exogenous DA Clearance in mPFC. To assess the effect of nicotine on DAT function in the mesocorticolimbic system, a range of nicotine doses (0.1 – 0.8 mg/kg) was administered s.c. to separate groups of rats, and exogenous DA clearance in mPFC was determined. Pressure ejection of DA (200 μM) resulted in stable baseline signals with a maximal signal amplitude of 4.54 μM ($\pm$ 0.18 μM; mean $\pm$ S.E.M), T_{80} value of 76.8 s ($\pm$ 4.62 s), and clearance rate of 0.085 μM/s ($\pm$ 0.011 μM/s; n = 33 independent experiments). Once baseline signals had stabilized, groups of rats were injected with either a dose of nicotine (0.1 – 0.8 mg/kg, s.c.) or saline and DA pressure ejection continued at 5-min intervals for 60 min. The effect of nicotine on maximal signal amplitude, T_{80} and clearance rate was determined at 5-min intervals and compared to the saline-injected control group (Fig. 4).

Analysis of the data from the saline-injected control group revealed that DA signal amplitude gradually decreased by ~13% during the first 55 min of the session (Fig. 4, top panel). At 60 min after the injection of saline, signal amplitude was significantly decreased by 15% ($p <$ 0.001). T80 was not different from baseline across the duration of the session (Fig. 4, middle panel). DA clearance rate tended to decrease (~15% of baseline) across the session time course, but did not reach significance (Fig. 4, bottom panel). Thus, under control conditions, repeated DA application at 5-min intervals resulted in a small increase in DAT function across the 60-min session, similar to that observed in striatum.

When the effect of nicotine on both signal amplitude and clearance rate were analyzed by two-way ANOVA, significant interactions of dose and time were found ($F_{65,351} = 1.95$, $p < 0.001$ and $F_{65,351} = 1.74$, $p < 0.01$, respectively). No significant main effects or interactions were observed for T_{80}. Signal amplitude data were analyzed further by one-way ANOVAs at individual 5-min time points, revealing dose-related decreases in signal amplitude at 15-30 min and 50-60 min ($p < 0.05$; at 35 min, $p < 0.05$, one-tailed, Fig. 4, top panel). Dunnett's post hoc analysis revealed that compared to the control group, DA signal amplitude was decreased in the 0.4 mg/kg group at 15-30 min ($p < 0.05$), and at the 35 min and 50-55 min time points ($p < 0.05$, one-tailed). Compared with the control group, a maximal decrease of 46% in signal amplitude was observed 30 min after injection of nicotine (0.4 mg/kg); and when the data at this time point were compared to the within-subject baseline, a 53% decrease in signal amplitude was observed.

To assess the onset of nicotine effect, specific within-subjects contrasts were performed comparing signal amplitude at each time point after injection to that at baseline. A significant ($p < 0.001$) decrease in signal amplitude was observed 10 min following nicotine injection.

With respect to clearance rate, one-way ANOVAs were conducted to assess the nicotine dose-response at each 5-min time point after injection (Fig. 4, bottom panel). Dose-related decreases in clearance rate were observed at 15-25 min and 60 min ($p < 0.05$), and at the 30 min and 50-55 min time points ($p < 0.05$, one-tailed) post injection. Dunnett's post hoc analysis revealed that DA clearance rate was decreased in the 0.4 mg/kg group compared to the control group at 15-25 min ($p < 0.05$) and at 30 min ($p < 0.05$, one-tailed) after injection. Compared to the control, clearance rate after 0.4 mg/kg decreased by a maximum of 33% at the 45-min time point; whereas a maximum decrease of 44% was observed when compared to the within-subject baseline response. The onset of the nicotine effect on clearance rate occurred at 10 min following nicotine injection (0.4 mg/kg, $p < 0.01$).

DA signal amplitude was also expressed as a cumulative change across the 60-min sampling period to evaluate the nicotine dose-response relationship (Fig. 5). Surprisingly, a U-shaped dose-response relationship was apparent. Only the 0.4 mg/kg dose of nicotine decreased DA signal amplitude in mPFC. Similarly, a U-shaped dose-response relationship was observed for clearance rate (data not shown). Thus, nicotine also modulates DAT function in mPFC; however, the dose-response pattern was different from that observed in striatum, and the time of onset and of maximal response occurred more rapidly after systemic nicotine injection in mPFC than in striatum.

Effect of Mecamylamine on Nicotine-induced Modulation of DAT Function in Striatum. To ascertain if the effect of nicotine on DAT function is mediated by nAChRs, the ability of mecamylamine to inhibit the nicotine-induced increase in DA signal amplitude was determined. Pressure ejection of DA (200 μM) resulted in a maximal signal amplitude of 6.75 μM ($\pm$ 0.43 μM; mean $\pm$ S.E.M.), T_{80} value of 23.8 s ($\pm$ 4.60 s), and clearance rate of 0.902 μM/s ($\pm$ 0.119 μM/s; n = 26 independent experiments). Once baseline signals stabilized, groups of rats were injected with mecamylamine (1.5 mg/kg, s.c.) or saline, and 40 min later with nicotine (0.8

mg/kg, s.c.) or saline. The dose of nicotine was chosen based on the above results from the dose-response analysis. DA pressure ejection continued every 5 min after mecamylamine or saline and every 5 min for 60 min after nicotine or saline. Data for the three parameters of DA clearance are illustrated in Fig. 6.

Since signal amplitude was the only parameter which detected the dose relationship for nicotine to enhance DAT function in striatum, a three-way ANOVA on signal amplitude data was performed to determine if mecamylamine inhibited the effect of nicotine. A significant three-way interaction of mecamylamine x nicotine x time was found ($F_{13,286} = 1.82$, $p < 0.05$). As previously observed, DA signal amplitude tended to gradually decrease across the 60-min session when compared to baseline in the Saline-Saline control group; however, only at the 60-min time point did the decrease (25%) in signal amplitude reach significance. In the Saline-Nicotine group, the onset of the effect of nicotine to decrease signal amplitude (30%) occurred at 25 min after nicotine injection (within-subject comparisons, $p < 0.01$). At the 55-min time point, nicotine maximally decreased signal amplitude by 56% compared to baseline. At the latter time point, comparison of the Saline-Nicotine and Saline-Saline groups revealed that nicotine decreased signal amplitude by a maximum of 36%. With respect to the Mecamylamine-Saline group, no within-subject differences were observed across the time course of the session compared to baseline, indicating that mecamylamine alone had no effect on DAT function. To determine if mecamylamine inhibited the effect of nicotine, one-way ANOVAs compared the treatment groups at each time point beginning at the onset of nicotine's effect (i.e., 25-60 min). At the 40, 45, 55 and 60 min time points, signal amplitude in the Mecamylamine-Nicotine group was significantly greater than that in the Saline-Nicotine group ($p < 0.05$, one-tailed) and not different from that in the Saline-Saline group, indicating that mecamylamine completely inhibited the effect of nicotine on DAT function in striatum.

Effect of Mecamylamine on Nicotine-induced Modulation of DAT Function in mPFC. To ascertain if nAChRs also mediate nicotine-induced modulation of DAT function in mPFC, the ability of mecamylamine to inhibit the nicotine-induced decrease in signal amplitude in urethane-anesthetized rats was determined. Stable baseline signals, obtained in response to pressure ejection of 200 μM DA, exhibited a maximal signal amplitude of 4.14 μM (± 0.27 μM;

mean ± S.E.M.), T_{80} value of 80.5 s (± 6.02 s), and clearance rate of 0.066 µM/s (± 0.008 µM/s; n = 24 independent experiments). Once baseline signals stabilized, experiments were performed as described above, except that electrochemical measurements were made in mPFC (Fig. 7). A three-way ANOVA was performed to determine if mecamylamine inhibited the nicotine-induced decrease in DA signal amplitude in mPFC. A significant interaction of mecamylamine x nicotine x time ($F_{13,260}$ = 2.08; $p < 0.02$) was found. DA signal amplitude decreased to a maximum of 20% compared to baseline at 60 min following the second saline injection in the Saline-Saline group. Within-subjects comparison in the Saline-Nicotine group revealed that the onset of the nicotine effect occurred 15 min after nicotine injection, at which time a significant ($p < 0.001$) decrease in signal amplitude was observed compared to baseline response. At the 35 min time point, nicotine produced a maximal decrease in signal amplitude (45%) compared to baseline; between-groups comparison of the response in the Saline-Nicotine and Saline-Saline groups revealed a maximum decrease of 25% at this time point. Within-subjects comparisons in the Mecamylamine-Saline group revealed no differences across the session compared to baseline, indicating that mecamylamine alone had no effect on DAT function in mPFC. One-way ANOVA followed by Dunnett's test compared data from the Mecamylamine-Nicotine and Saline-Nicotine groups at each time point from 15-60 min, when nicotine decreased signal amplitude compared to baseline. Signal amplitude was greater in the Mecamylamine-Nicotine group than that in the Saline-Nicotine group at 20-30, 40 and 50 min ($p < 0.05$) and 35, 45 and 60 min ($p < 0.05$, one-tailed) of the session. Importantly, the response in the Mecamylamine-Nicotine group was not different from that in the Saline-Saline group at these time points, indicating that mecamylamine completely inhibited the effect of nicotine on DAT function in mPFC. Thus, these results suggest that nicotine modulates DAT function *via* a nAChR-mediated mechanism in mPFC.

DISCUSSION

The results from the current in vivo voltammetry study demonstrate that nicotine in a dose-related manner decreases DA signal amplitude in both striatum and mPFC, indicating that nicotine enhances DA clearance in both brain regions. However, across the same nicotine dose range, differential patterns in the nicotine dose-response curve were observed in striatum and mPFC. That is, a monophasic dose-response curve was observed in striatum, whereas a U-shaped curve was found in mPFC, both curves having a maximal 50% effect. Maximal effect occurred at a lower dose in mPFC than in striatum (0.4 and 0.8 mg/kg, respectively). In both brain regions, the onset of a significant effect on DA clearance occurred 10-15 min after nicotine injection; however, DA clearance tended to increase by 5 min after nicotine injection. Additionally, the time to maximal response was more rapid in mPFC compared to striatum (30 and 45 min, respectively). The time course of the response to nicotine in both brain regions is in good agreement with pharmacokinetic data showing a maximal nicotine concentration in rat brain at 5 min after peripheral nicotine injection and a brain $t_{1/2}$ of 52 min (Ghosheh et al., 2001). Nevertheless, the pattern of the nicotine dose-response curve in the present study was different between mPFC and striatum, with mPFC showing greater sensitivity to nicotine.

The current results support and extend previous findings, showing that systemically administered nicotine increases DA clearance in several dopaminergic terminal regions, including mPFC and striatum (current study), and nucleus accumbens (Hart and Ksir, 1996). The latter results are surprising since stimulation of nAChRs results in depolarization of the plasma membrane (Calabresi et al., 1989) and since depolarization of the membrane generally decreases DA transport velocity (Sonders et al., 1997). However, this was not the case with nicotine. The current results demonstrate a dose-related nicotine-induced enhancement of DAT function in striatum and mPFC, and extend the findings of Hart and Ksir (1996) in nucleus accumbens.

The present study also demonstrates that the nicotine-induced enhancement of DA clearance in both mPFC and striatum was inhibited by pretreatment of the rats with mecamylamine, a nonselective nAChR antagonist (Varanda et al., 1985). These results suggest that nicotine stimulates nAChRs to increases DAT function in these brain regions. In the current

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study, mecamylamine had no effect on its own, but inhibited the effect of nicotine on DA clearance in both striatum and mPFC. The current results are in agreement with the work of Hart and Ksir (1996), who also reported no effect of mecamylamine alone on DAT function in nucleus accumbens. The observation that mecamylamine had no effect alone, suggests that nAChRs, which modulate DAT function in these brain regions, are not tonically activated.

The involvement of distinct nAChR subtypes in striatum and mPFC provides a likely explanation for the differential nicotine dose-response pattern observed in the current study. Recent studies have demonstrated the presence of multiple mRNAs for nAChR subunits ($\alpha2$-$\alpha7$ and $\beta2$-$\beta4$) and their respective proteins in DA cell bodies in both substantia nigra and ventral tegmental area; however, differences in the relative abundance of these subunits in substantia nigra and ventral tegmental area have also been reported (Klink et al., 2001; Azam et al., 2002; Zoli et al., 2002; Wooltorton et al., 2003). The nAChRs expressed in the nigrostriatal and mesocorticolimbic DA systems depend on the specific combinations of subunits forming functional nAChRs. Pairwise expression of nAChR subunits in *Xenopus* oocytes initially revealed characteristic pharmacological profiles, i.e., relative sensitivity and responsiveness to a range of nicotinic ligands (Luetje and Patrick, 1991).   Inclusion of a third type of subunit (e.g., $\alpha5$ with $\alpha3$ and $\beta2$ subunits) in similar expression systems further altered the physiological response, calcium permeability and desensitization characteristics of the expressed nAChR subtypes (Gerzanich et al., 1998). Characterization of the physiological response of individual neurons in midbrain slices revealed four different patterns of nAChR-mediated currents, revealing the complexity of native nAChRs which purportedly contained as many as four different subunits (Klink et al, 2001).

Although the specific subunit composition of nAChR modulating DAT function in either striatum or mPFC is not known, the nAChR subtype modulating DAT function in mPFC may be more sensitive to nicotine than the specific nAChR subtype modulating DAT function in striatum, since the nicotine dose producing an enhancement of DA clearance is lower in mPFC than in striatum. The current results show that in both striatum and mPFC, 0.1 and 0.3 mg/kg produce no effect on DAT function; however, 0.4 mg/kg enhances DAT function in mPFC, whereas 0.8 mg/kg enhances DAT function in striatum. Thus, the descending portion of the

dose-response in mPFC resembles the dose-response in striatum, but the curve from mPFC is shifted to the left of that from striatum. Doses of nicotine above 0.8 mg/kg were not examined in the current study, since such doses elicit seizures that may have confounded the results. Thus, the differential pattern of response observed in the present study in mPFC and striatum with respect to nAChR-mediated modulation of DAT function is likely the result of stimulation of distinct nAChR subtypes.

An alternative explanation for the differential nicotine dose-response relationships in striatum and mPFC is the lower dopaminergic terminal density in mPFC compared to striatum, and the decreased number of DAT per terminal in mPFC compared to striatum (Sesack et al., 1998). It may be that a higher dose of nicotine is required to observe the modulation of DAT function in striatum simply due to the greater number of DAT protein in striatum compared to mPFC.

Another potential explanation for the difference in regional dose-response is that the local neuronal circuitry is different between these two brain regions, i.e., different afferents impinge on the dopaminergic terminals in striatum and mPFC potentially providing differential regulation of DAT function. In this regard, the U-shaped function in mPFC may be the result of nicotine-induced stimulation of an additional neurotransmitter system in the mPFC. The result of activation of the additional neurotransmitter may have opposed the nAChR-mediated enhancement of DAT function. For example, high concentrations of nicotine have been shown to activate alpha7 nAChRs, resulting in glutamate release in frontal cortex (Schilstrom et al., 2000; Marchi et al., 2002). For example, stimulation of metabotropic glutamate receptors have been reported to decrease DAT function (Page et al., 2001). Thus, activation of alpha7 receptors indirectly through glutamate neurotransmission could result in inhibition of DAT function, counteracting activation of the high affinity heteromeric nAChRs, which enhance DAT function.

Alternatively, nAChRs may indirectly modulate DAT function through activation of neural circuitry at the cell body level. Local administration of nicotine into the substantia nigra and ventral tegmental area has been shown to evoke DA release in striatum and nucleus accumbens, respectively, via stimulation of nAChRs in the cell body region (Blaha and Winn,

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1993; Sziraki et al., 2002). Additionally, the effect of peripheral administration of nicotine to increase DA release in the nucleus accumbens determined using microdialysis was inhibited by local administration of mecamylamine into the ventral tegmental area (Sziraki et al., 2002). Thus, it seems plausible that in the current study, peripherally administered nicotine may be acting at nAChRs at the level of the cell body to modulate DAT function at the terminal. Furthermore, different nAChR subtypes expressed at the cell body may be responsible for the different dose-response patterns observed in mPFC and striatum with respect to DA clearance.

In both striatum and mPFC, the mechanism by which nicotine modulates DAT function may be via nAChR-induced augmentation of DAT trafficking to the presynaptic terminal membrane consistent with an increase in DA clearance. The relatively rapid nicotine-induced increase in DA clearance suggests that new synthesis of DAT protein is not responsible. Rather, the time course of response is consistent with trafficking of intracellular stores of DAT protein to the terminal membrane. Cocaine, another drug of abuse, has been shown to dynamically regulate DAT function by increasing DAT trafficking to the plasma membrane in cells expressing hDAT (Little et al., 2002). In contrast, amphetamine diminished DAT localization at the plasma membrane in hDAT expressing cells (Saunders et al., 2000) and in rat striatal dopaminergic terminals (Fleckenstein et al., 1997). Investigation of the effect of nicotine on DAT trafficking is warranted based on the enhanced DA clearance using in vivo voltammetry in the current study.

Due to the lower DAT density and decreased number of DAT per terminal in mPFC compared to striatum, metabolism and diffusion likely play a larger role in clearing DA from the extracellular space in mPFC under physiological conditions. However, following pharmacological treatment with nicotine, enhanced DAT function in mPFC would be predicted to have a large impact on dopaminergic transmission. Nicotine enhancement of DAT function would result in more efficient DA clearance from the extracellular space, and cortical function would be disinhibited. Thus, the ability of nAChRs to modulate DAT function, and thereby extracellular DA concentration, may have physiological importance with respect to nicotine enhancement of cognitive processes such as attention, learning and memory, as well as important clinical relevance with respect to schizophrenia and drug abuse.

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FOOTNOTES

Reprint requests should be directed to Linda P. Dwoskin

        College of Pharmacy

        University of Kentucky

        Rose Street

        Lexington, KY 40536

        Phone: 859-257-4743

        Fax: 859-257-7564

        E-mail: ldwoskin@email.uky.edu

FIGURE LEGENDS

Fig. 1. Representative DA signals in striatum of urethane-anesthetized rats prior to (top panel) and 45 min following nicotine or saline injection (bottom panel).   Reproducible electrochemical signals were obtained following pressure ejection of DA (200 μM) and represent the baseline response (top panel). Representative DA signals 45 min following injection of nicotine (NIC; 0.8 mg/kg, s.c.) or saline are represented by dashed and solid lines, respectively (bottom panel).   Data are expressed as μM DA as a function of time (sec) after DA pressure ejection. DA concentrations were calculated based on calibration curves generated in vitro.

Fig. 2. Dose-response for systemically administered nicotine to alter DA signal amplitude (top panel), T_{80} (middle panel), and clearance rate (bottom panel) in striatum of urethane-anesthetized rats. Nicotine in a dose-related manner decreased signal amplitude, but did not significantly alter T_{80} or clearance rate across 60-min recording sessions. After stable baseline signals were obtained in response to pressure ejection of DA (200 μM) at 5-min intervals, either a dose of nicotine (NIC; 0.1 – 0.8 mg/kg) or saline was injected s.c. (indicated by the arrow), and pressure ejection of DA at 5-min intervals continued for 60 min. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). Mean baseline values for each parameter are provided in the Results section. (n = 5 - 8 rats/group) * difference from saline control at the corresponding time point ($p < 0.05$); [+] first time point at which nicotine decreased signal amplitude compared to the corresponding basal.

Fig. 3. In a dose-related manner, nicotine decreased signal amplitude cumulated across the 60-min sampling period. For each experiment, the change in signal amplitude was cumulated across the 60-min period of electrochemical recording in striatum. Data are expressed as mean $\pm$ S.E.M. as a percent of baseline. (n = 5 – 8 rats/group)

Fig. 4. Dose-response for systemically administered nicotine to alter DA signal amplitude (top panel), T_{80} (middle panel) and clearance rate (bottom panel) in mPFC of urethane-

anesthetized rats. Nicotine in a dose-related manner decreased signal amplitude (top panel) and DA clearance rate (bottom panel), but did not alter the T_{80} (middle panel) across the 60-min recording session. After stable baseline signals were obtained in response to pressure ejection of DA (200 μM) at 5-min intervals, either a dose of nicotine (NIC; 0.1 – 0.8 mg/kg) or saline was injected s.c. (indicated by the arrow), and DA pressure ejection continued at 5-min intervals for 60 min. Data are expressed as mean, S.E.M. as a percentage of within-subject baseline values as a function of time (min). Baseline values for each parameter are provided in the Results section. (n = 5 - 7 rats/group) * difference from saline control at the corresponding time point ($p < 0.05$); # difference from saline control at the corresponding time point ($p < 0.05$; one-tailed); + first time point at which nicotine decreased signal amplitude compared to the corresponding basal.

Fig. 5. In a dose-related manner, nicotine decreased signal amplitude cumulated across the 60-min sampling period. For each experiment, the change in signal amplitude was cumulated across the 60 min period of electrochemical recording in mPFC. Data are expressed as mean $\pm$ S.E.M. as a percent of baseline. (n = 5 – 7 rats/group)

Figure 6. Effect of mecamylamine to inhibit the nicotine-induced decrease in signal amplitude in striatum. Signal amplitude is shown in the top panel; T_{80} and clearance rate are shown in middle and bottom panels, respectively, for comparison. No significant differences in were observed in T_{80} or clearance rate. After stable baseline signals were obtained in response to pressure ejection of DA (200 μM) at 5-min intervals, either mecamylamine (MEC; 1.5 mg/kg) or saline was injected s.c. and 40 min later nicotine (NIC; 0.8 mg/kg) or saline was injected s.c.(second injection indicated by the arrow). DA continued to be pressure ejected at 5-min intervals for 60 min following the second injection. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). Baseline values for each parameter are indicated in the Results section. Legend indicates treatment group designating first and second injections. (n = 6 - 7 rats/group) # difference between Mecamylamine/Nicotine group and Saline/Nicotine group at the corresponding time point ($p < 0.05$; one-tailed); + first time point at which nicotine decreased signal amplitude compared to the corresponding basal.

Figure 7. Effect of mecamylamine to inhibit the nicotine-induced decrease in signal amplitude in mPFC. Signal amplitude is shown in the top panel; T_{80} and clearance rate are shown in middle and bottom panels, respectively, for comparison. No significant differences in were observed in T_{80} or clearance rate. After stable baseline signals were obtained in response to pressure ejection of DA (200 μM) at 5-min intervals, either mecamylamine (MEC; 1.5 mg/kg) or saline was injected s.c. and 40 min later injected s.c. with nicotine (NIC; 0.4 mg/kg) or saline (second injection indicated by the arrow). DA was pressure ejected at 5-min intervals for 60 min following the second injection. Data are expressed as mean, S.E.M. as a percentage of the respective baseline values as a function of time (min). Baseline values for each parameter are indicated in the Results section. Legend indicates treatment group designating first and second injections. (n = 5 - 7 rats/group) * difference between Mecamylamine/Nicotine group and Saline/Nicotine group at the corresponding time point ($p < 0.05$); [#] difference between Mecamylamine/Nicotine group and Saline/Nicotine group at the corresponding time point ($p < 0.05$; one-tailed); [+] first time point at which nicotine decreased signal amplitude compared to the corresponding basal.
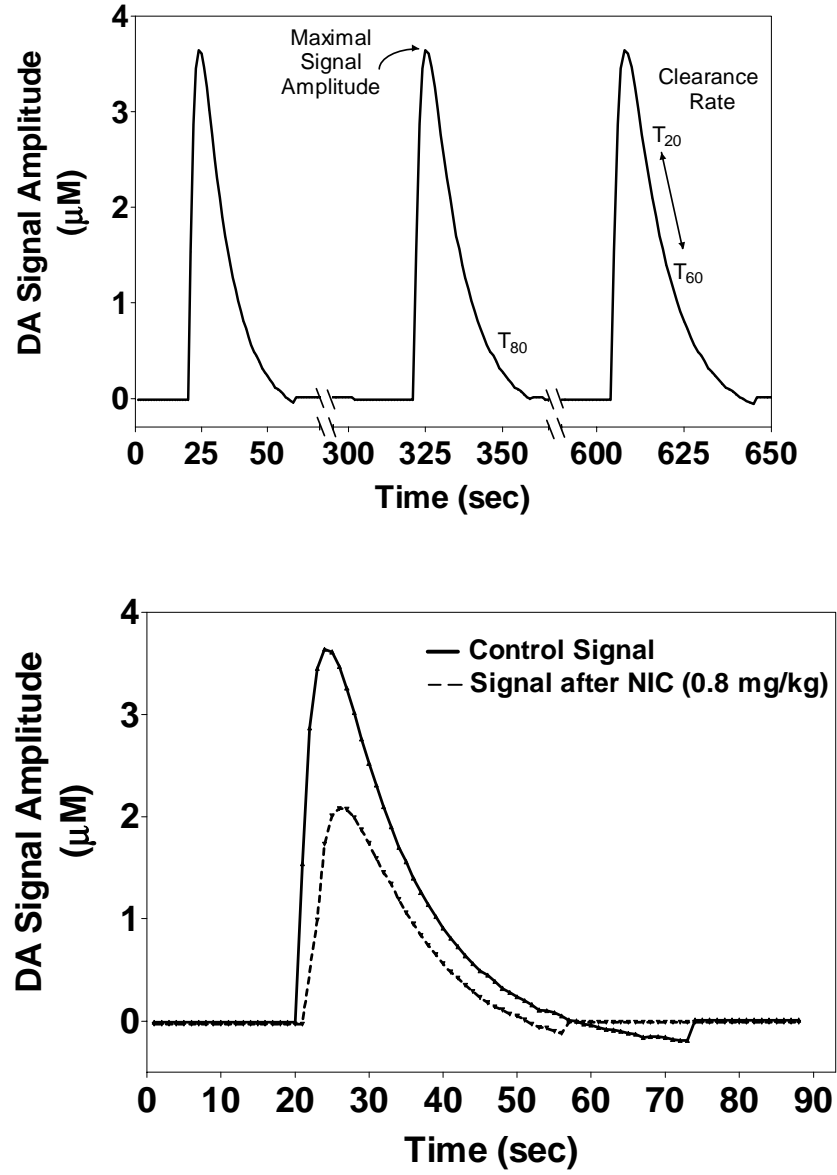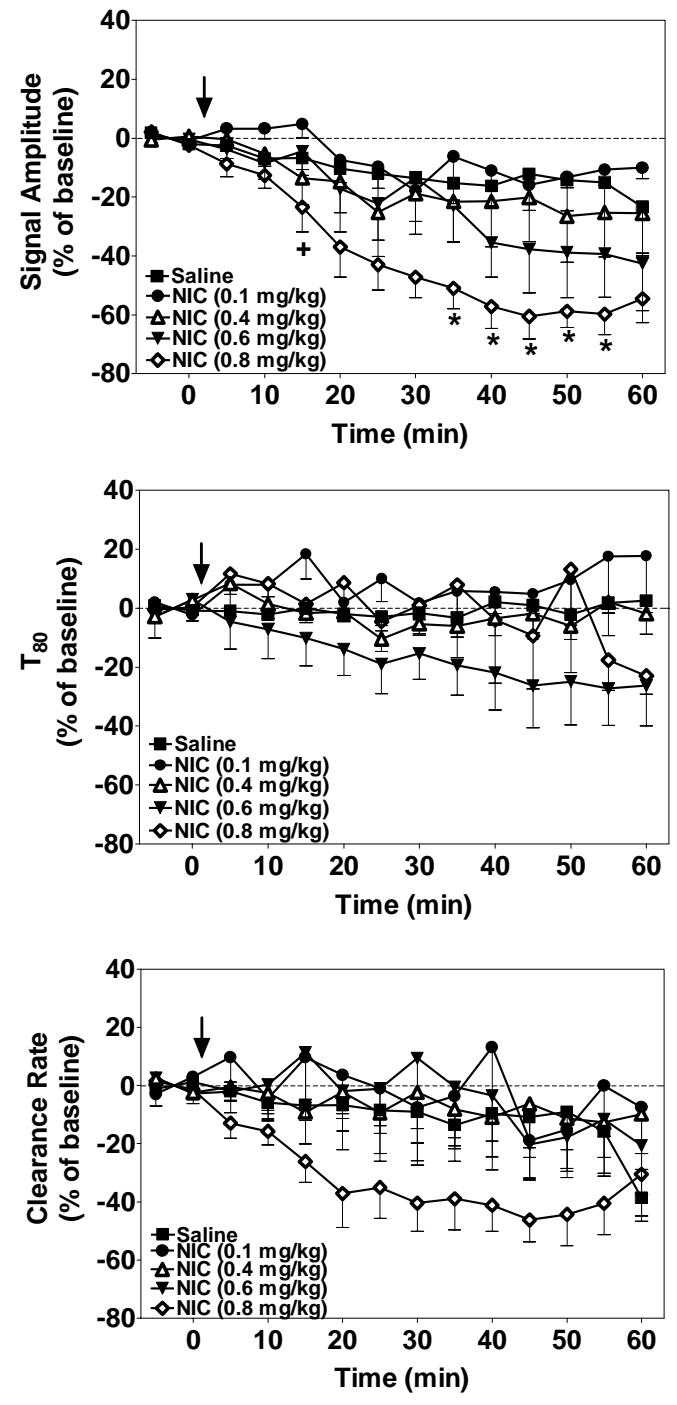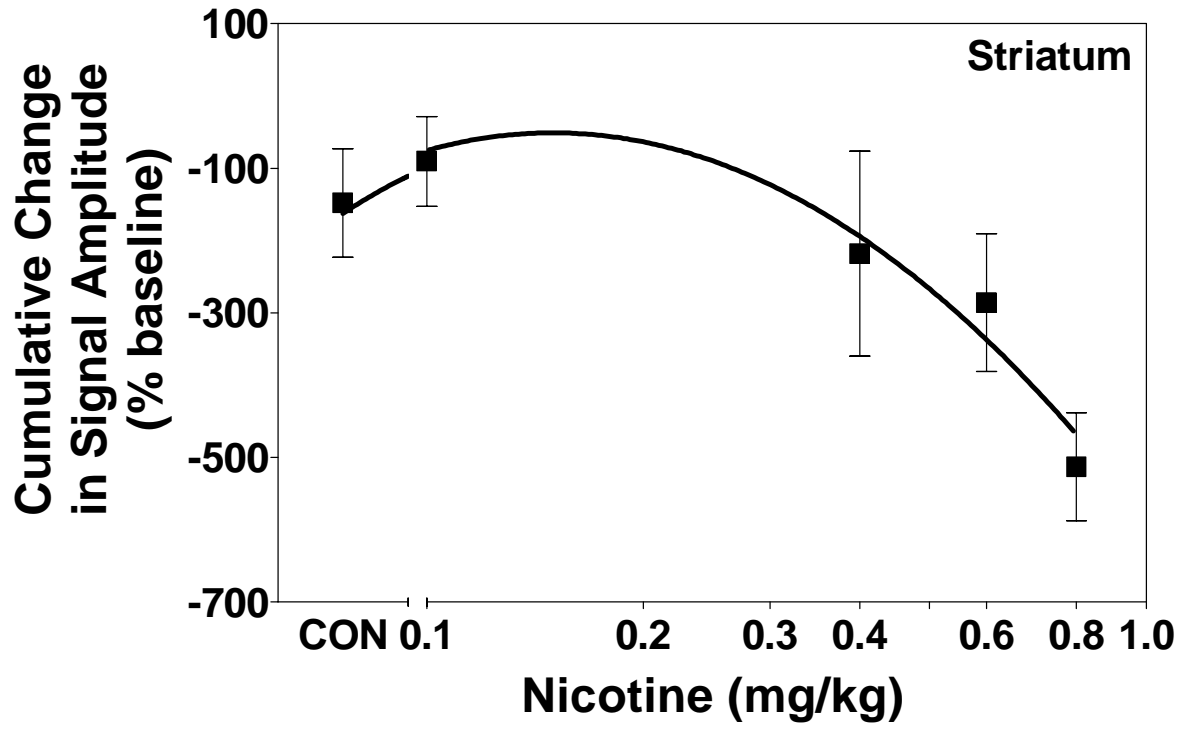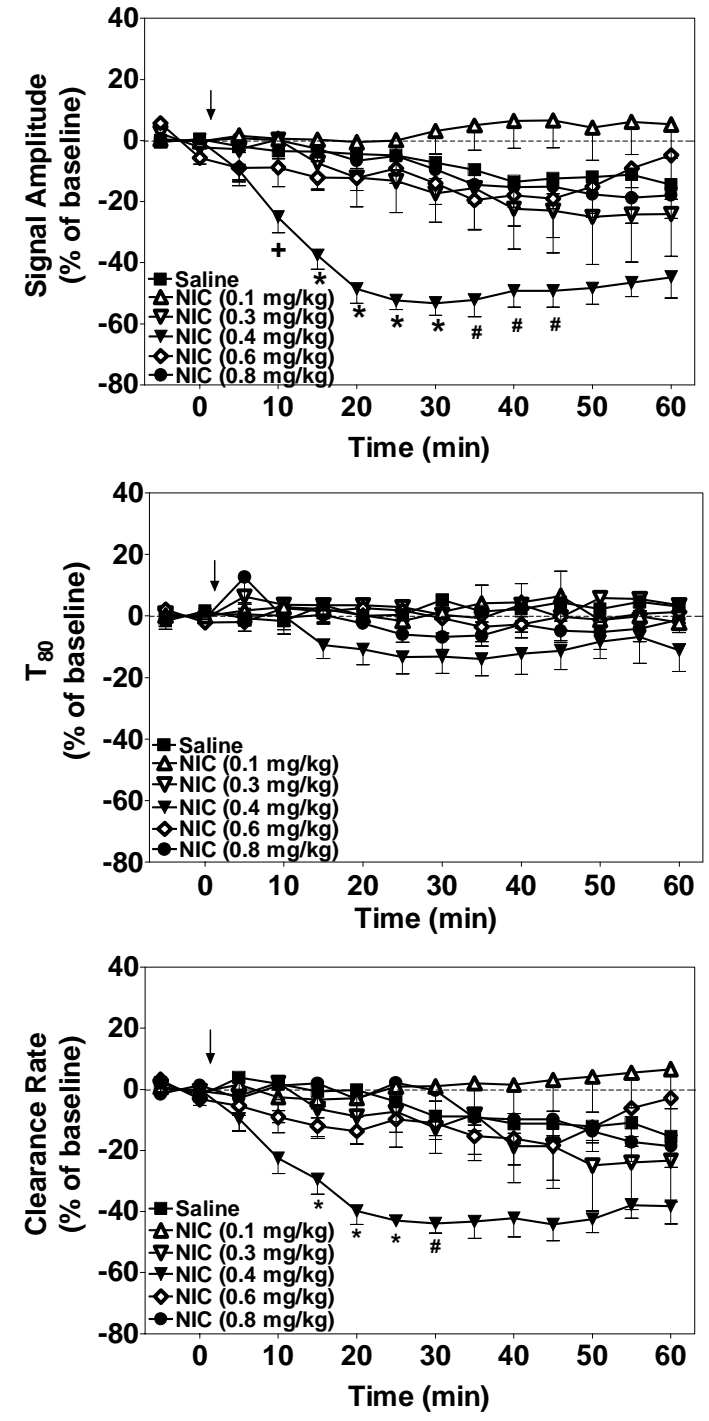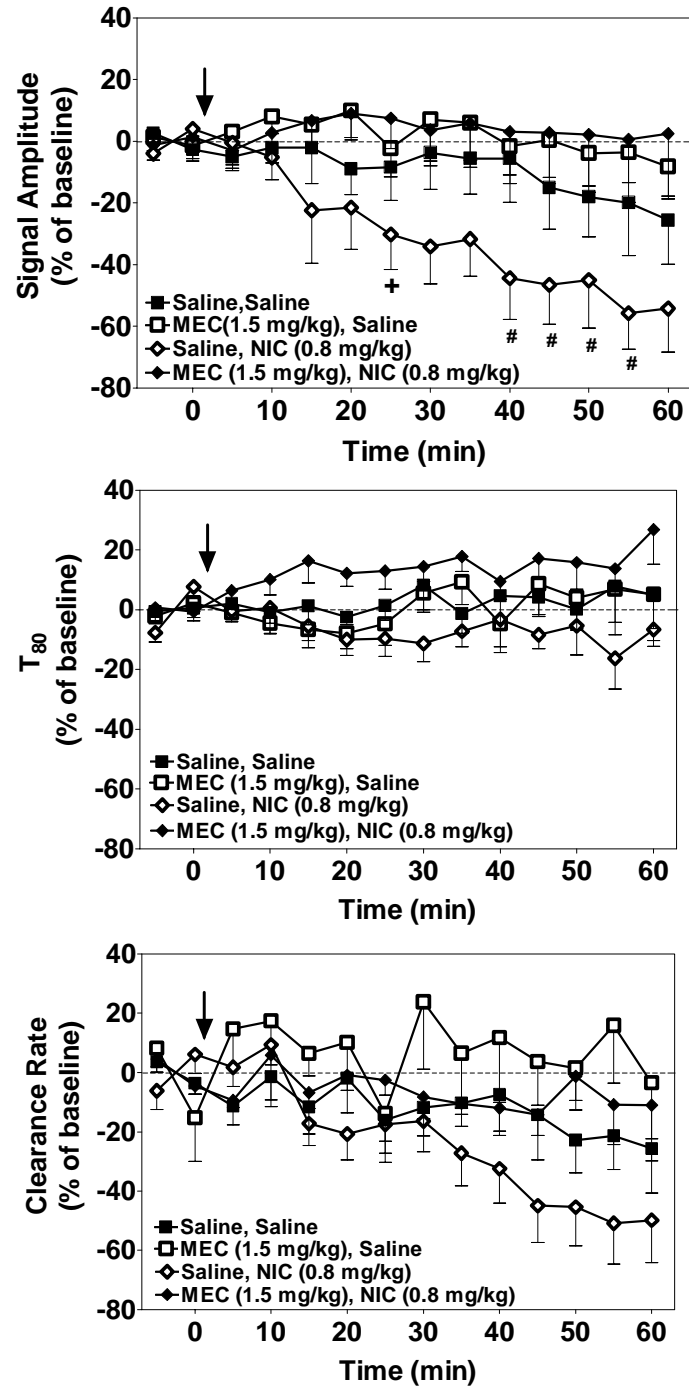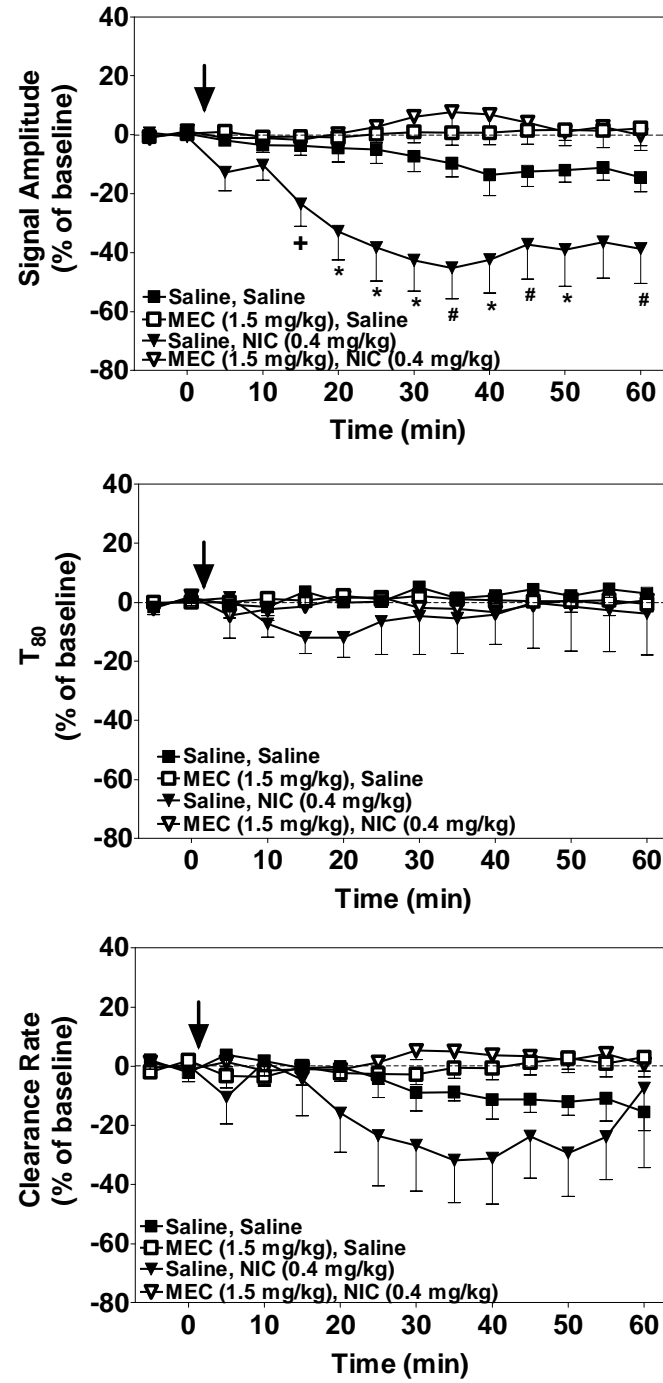
Fig. 1

Fig. 2

Fig. 3

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Fig. 4

Fig. 5

Fig. 6

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Fig. 7