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Title Page

BRAIN UPTAKE KINETICS OF NICOTINE AND COTININE AFTER CHRONIC NICOTINE EXPOSURE

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Running Title Page

Running Title: Nicotine and Cotinine Brain Uptake

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Non-standard Abbreviations:
BBB: Blood-brain barrier
CNS: Central nervous system
nAChr: Nicotinic acetylcholine receptor
PA: Cerebrovascular permeability product

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Abstract

Introduction: Blood-brain barrier (BBB) nicotine transfer has been well documented in view of the fact that this alkaloid is a cerebral blood flow marker. However, limited data are available that describe BBB penetration of the major tobacco alkaloids after chronic nicotine exposure. This question needs to be addressed given long-term nicotine exposure alters both BBB function and morphology. In contrast to nicotine, it has been reported that cotinine (the major nicotine metabolite) does not penetrate the BBB, yet cotinine brain distribution has been well documented after nicotine exposure. Surprisingly therefore, the literature indirectly suggests that CNS cotinine distribution occurs secondarily to nicotine *brain* metabolism. The aims of the current report are to define BBB transfer of nicotine and cotinine in naive and nicotine exposed animals. **Methods:** Using an *in situ* brain perfusion model we assessed the BBB uptake of [^{3}H]nicotine and [^{3}H]cotinine in naive animals and in animals exposed chronically to *S*-(-)nicotine (4.5 mg/kg/d) through osmotic minipump infusion. **Results:** Our data demonstrate that: 1) [^{3}H]nicotine BBB uptake is not altered in the *in situ* perfusion model after chronic nicotine exposure, 2) [^{3}H]cotinine penetrates the BBB and 3) similar to [^{3}H]nicotine, [^{3}H]cotinine BBB transfer is not altered by chronic nicotine exposure. To our knowledge this is the first report detailing the uptake of nicotine and cotinine after chronic nicotine exposure and quantifying the rate of BBB penetration by cotinine.

Introduction

The blood to brain transfer kinetics of nicotine and its primary metabolite cotinine is a determinant factor for central nervous system (CNS) concentrations. For CNS access, nicotine and/or cotinine must traverse the specialized neurovascular endothelium, which theoretically can limit plasma to brain distribution. Kinetic transfer at the blood-brain barrier (BBB) is limited by endothelium that is connected through tight junctions (*zonulae occludens,* ZO), the absence of paracellular openings, a lack of pinocytocis activity, enzymatic restrictions, and significant protein-mediated efflux (Begley and Brightman, 2003).

Once nicotine penetrates the CNS, it acts as an agonist at the α4β2 and α3β2 subtypes of nicotinic acetyl choline receptors (nAChRs) in the ventral tegmental area, an action that evokes dopamine release in the nucleus accumbens (Di Chiara, 2000). While the addictive mechanisms of nicotine are under intensive investigation, the degree to which cotinine modifies nicotine addiction is controversial (Buccafusco and Terry, 2003). In support of cotinine addiction theories, cotinine has been shown to activate the superior cervical ganglion (Schroff et al., 2000), act as an agonist (with weak affinity) at the human α7 nAChR (Briggs and McKenna, 1998) and stimulate dopamine release from rat striatal synaptosomes at doses 30 to 50 times greater than nicotine (Crooks et al., 1997). Yet other data demonstrate that cotinine lacks significant whole brain cholinergic effect (Linville et al., 1993; Radek, 1993).

The rapid blood-brain transfer of nicotine in naive animals has been well documented due to the fact that it is a known cerebral blood flow marker (Ohno et al., 1979; Suzuki et al., 1984; Todd and Weeks, 1996; Tomiyama et al., 1999). However,

limited data are present in the literature regarding nicotine BBB penetration after chronic nicotine exposure. Such studies are of significant importance given long-term nicotine exposure changes both BBB function and morphology. Specifically, nicotine has been shown to increase BBB endothelium microvilli formation (Booyse et al., 1981), decrease *in vitro* ZO-1 expression (Abbruscato et al., 2002), and diminish the levels and/or function of nAChRs located at the BBB (Abbruscato et al., 2002), downregulate Na,K,2Cl-cotransporters (Abbruscato et al., 2004), and downregulate α2 Na,K-ATPase (Wang et al., 1994).

In contrast to that of nicotine, the rate of uptake for cotinine across the BBB is poorly defined. Literature reports on the ability of cotinine to penetrate the BBB to any significant degree are conflicting (Halldin et al., 1992; Riah et al., 1998). Cotinine has been detected in brain after nicotine exposure (Crooks et al., 1997; Riah et al., 1998) but indirect data suggest that this CNS presence may be the result of central nicotine metabolism by CYP2B1, a monooxygenase enzyme which has been detected in rat brain and shown to be induced by chronic nicotine (Jacob et al., 1997; Miksys et al., 2000). The current report documents the blood-brain transfer of cotinine using the *in situ* brain perfusion model and compares the kinetics with that of nicotine. Further, the degree and rate of cotinine (as well as nicotine) brain uptake was evaluated in naive and chronic nicotine-exposed animals using the same model.

Methods:

The brain uptake of [^{3}H]nicotine and [^{3}H]cotinine was assessed using the *in situ* rat brain perfusion technique with modifications described (Smith, 2003). In this study,

perfusions of 15-60 s were used to determine tracer brain uptake rates in naive rats and those exposed to nicotine for 28 d. Integrity of the blood-brain barrier was verified in all experiments using [^{14}C]sucrose. All studies were approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Radiochemicals

High specific activity [^{3}H]nicotine (10 Ci/mmol, >98% purity) was obtained from Tocris Cookson Inc. (Ellisville, MO.); [^{3}H]cotinine (56 Ci/mmol) from American Radiolabeled Chemicals, Inc. (St Louis, MO.), [^{14}C]sucrose (4.75 mCi/mmol) and [^{3}H]diazepam (86 Ci/mmol) from Dupont-New England Nuclear (Boston, MA.). [^{3}H]Nicotine, [^{3}H]cotinine and [^{3}H]diazepam were dried prior to being dissolved in perfusion buffer to remove volatile tritium contaminants including [^{3}H]$H_2$0.

Nicotine administration by osmotic minipump

Chronic nicotine administration was comparable to previous methodology that provided nicotine and cotinine blood levels similar to those found in heavy smokers (nicotine ~ 80-100 ng/mL; cotinine > 250 ng/mL) (Hawkins et al., 2004; Lockman et al., 2005). Briefly, osmotic minipumps (Alzet 2ML4, 28 d capacity: 2000 µL) were loaded with *S*-(-) nicotine (42 mg; sterile; free base/dissolved in sterile saline) and released over 28 d at a rate of 2.5 µL/hr. Prior to implanatation, minipumps were primed in sterile saline at 37 ^{o}C for 24 h according to the manufacturer's specifications. Pumps were then removed and immediately implanted interscapularly (Castagnoli et al., 2002) into male Fischer-344 rats (220-330 g; Charles River Laboratories, Kingston, N.Y., U.S.A.). The

surgical procedure consisted of anesthesia (sodium pentobarbital; 50 mg/kg) and rectal temperature monitoring and maintainance of core body temperature at 37 °C by a heating pad feedback device (YSI Indicating Controller, Yellow Springs, Ohio). A small thoracolumbar subcutaneous incision (~4 cm) was made and a pocket projecting rostrally, large enough to accommodate the minipump, was opened using a blunt hemostat. Manual insertion of the minipump was completed under sterile conditions and the wound closed with staples. Rats were monitored during recovery after which they were allowed *ad libitum* access to food and water. Nicotine levels (~72 ng/mL) were verified by HPLC in a subset of animals on day 28. Cotinine levels (469 $\pm$ 27 ng/mL) were also verified by enzyme immunoassay (Cozart Bioscience Ltd, Oxfordshire, UK) prior to *in situ* perfusion procedures to ensure adequate nicotine exposure.

Perfusion procedure

After 28 d of nicotine exposure, animals were anesthetized with sodium pentobarbital (50 mg/kg; intraperitoneal). A PE-60 catheter filled with heparinized saline (100 units/mL) was placed into the left common carotid artery after ligation of the left external carotid, occipital, and common carotid arteries (common carotid artery ligation was accomplished caudally to the catheter implantation site). The pterygopalatine artery was left open. Rat body temperature was monitored and maintained at 37 °C by a heating pad and feedback device (YSI Indicating Controller, Yellow Springs, Ohio). The buffered physiologic perfusion fluid used was titrated to pH of 7.4 (osmolarity ~290 mOsm; verified) and contained (in mM): NaCl 128, $NaPO_3$ 2.4, $NaHCO_3$ 29.0, KCl 4.2, CaCl 1.5, MgCl 0.9, and D-glucose 9 with 0.33 μCi/ml [^{14}C]sucrose, and either 0.5 μCi/ml

[^{3}H]nicotine, or 1.0 μCi/mL [^{3}H]cotinine. Immediately prior to perfusion, the fluid was filtered and warmed to 37 °C, and gassed with 95% air and 5% CO_2

The perfusion fluid was infused into the left carotid artery via infusion pump for 15-60 s at 10 mL/m (Harvard Apparatus, South Natick, MA.). This level of flow maintained carotid artery pressure at ~120 mm Hg. Rats were decapitated, the brain rapidly removed from the skull, and the perfused hemisphere dissected on ice after removal of the arachnoid membrane and meningeal vessels. Brain regions and perfusion fluid samples were digested overnight at 50 °C in 1 mL of 1 M piperidine. Dual labeled scintillation counting of brain and perfusate samples was then accomplished with correction for quench, background and efficiency (Beckman Coulter, LS6500 Multipurpose scintillation counter, Fullerton, CA).

Kinetic Analysis

Concentrations of tracer in brain and perfusion fluid are expressed as dpm/g brain or dpm/mL perfusion fluid, respectively. A brain/perfusate distribution volume was ascertained as described (Smith, 2003) from the following relationship:

$$\text{Brain distribution volume} = Q^*/C^* \qquad \text{Equation (1)}$$

where Q* is the quantity of tracer in brain (dpm/g) at the end of perfusion, and C* is the perfusion fluid concentration of tracer (dpm/mL).

Unidirectional uptake transfer constants, or K_{in}, were then calculated from brain distribution volume versus time, using linear regression analysis (Smith, 2003), by Equation 2:

$$Q^*/C^* = K_{in}T + V_o \qquad \text{Equation (2)}$$

where Q* is the quantity of tracer in brain (dpm/g) at the end of perfusion, C* is the perfusion fluid concentration of tracer (dpm/mL), T is perfusion time (s), and V_O is the intercept of [^{14}C]sucrose (T = 0 s; "vascular volume" in mL/g). Tracer ([^{3}H]nicotine and [^{3}H]diazepam) trapped in the vascular space was accounted for by subtracting the vascular volume (concurrently measured with [^{14}C]sucrose). Similarly, cerebral perfusion flow rate (F) was determined by the uptake of [^{3}H]diazepam using the in situ-perfusion technique in both nicotine exposed and naive animals. Data were in agreement with previously published values (Lockman et al., 2003).

K_{in} values for [^{3}H]nicotine and [^{3}H]cotinine were converted to apparent cerebrovascular permeability-surface area products (PA) using the Crone-Renkin equation (Smith, 2003):

$$PA = -F \ln (1-K_{in}/F) \qquad \text{Equation (3)}$$

Statistical Analysis

Data presented are from total left hemispheric brain, unless otherwise specified. Brain PA and K_{in} were evaluated by Student's t-test, and regional data were evaluated by one-way ANOVA analysis followed by Bonferroni's multiple comparison test. Regional differences in Kin or PA were evaluated individually by a students t-test (two-tailed). Differences were considered statistically significant at $p<0.05$. Errors are reported as standard error of mean (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA).

Results

Brain uptake rates of [^{3}H]nicotine and [^{3}H]cotinine were evaluated in both naive rats and rats exposed to nicotine (28 d at 4.5 mg/kg/d; plasma levels ~ 72 ng/mL) and cotinine (the presence of cotinine was from endogenously metabolized nicotine; 469 $\pm$ 27 ng/mL).

Brain uptake of [^{3}H]nicotine was evaluated with short perfusions of 15 s (**Figs. 1A and 2**) to prevent significant brain to blood efflux (i.e., unilateral uptake) and CNS metabolism of nicotine to cotinine. No significant difference in total brain [^{3}H]nicotine uptake were noted between naive (3.11 $\pm$ 0.42 x 10^{-2} mL/s/g) and nicotine exposed (3.31 $\pm$ 1.1 x 10^{-2} mL/s/g) rats (**Fig. 1A**). To assess if chronic nicotine exposure altered flow in our model, we evaluated brain uptake of [^{3}H]diazepam (Lockman et al., 2003). As seen in **Fig. 1B,** no apparent flow alterations were noted between control (5.11 $\pm$ 0.98 x 10^{-2} mL/s/g), and nicotine exposure (4.61 $\pm$ 0.81 x 10^{-2} mL/s/g). The increased cerebral perfusion flow rate with [^{3}H]diazepam compared to [^{3}H]nicotine was found to be consistent with previously published perfusion flow data (Lockman et al., 2003; Smith, 2003).

Regional brain analyses confirmed the lack of significant differences in [^{3}H]nicotine uptake into brain between naive and nicotine exposed animals (**Fig. 2**). [^{3}H]Nicotine uptake in this study showed a pattern of uptake influenced by cerebral perfusion flow commonly found in the *in situ* perfused brain, where flow rates are found to be highest in the cortical regions (4.02 $\pm$ 0.59 x 10^{-2} mL/s/g), and lower in the cerebellum (1.39 $\pm$ 0.40 x 10^{-2} mL/s/g) and pons medulla (0.94 $\pm$ 0.21 x 10^{-2} mL/s/g) (Smith, 2003).

Considering [^{3}H]cotinine brain uptake is approximately 10 fold less than that of [^{3}H]nicotine, evaluation of [^{3}H]cotinine uptake required the plotting of distribution volume per time, subtraction of vascular volume, and linear regression analysis to accurately estimate uptake rate (K_{in}). As shown in **Fig. 3A and 3B**, the total brain uptake of [^{3}H]cotinine in animals subjected to chronic nicotine exposure (K_{in}: 2.07 $\pm$ 0.25 x 10^{-3} mL/s/g; PA: 2.11 $\pm$ 0.25 x 10^{-3} mL/s/g) was found not to be significantly altered from control in whole brain (K_{in}; 2.03 $\pm$ 0.17 x 10^{-3} mL/g; PA: 2.06 $\pm$ 0.17 x 10^{-3} mL/s/g) or regionally (**Fig. 4**). In contrast to [^{3}H]nicotine regional data where flow differences may result in changes of uptake between cortical and subcortical regions, [^{3}H]cotinine is permeability limited and regional alterations of [^{3}H]cotinine brain uptake are minimized (cortex K_{in}: 2.13 $\pm$ 0.26 x 10^{-3} mL/s/g and PA: 2.17 $\pm$ 0.27 x 10^{-3} mL/s/g; pons medulla K_{in}: 1.60 $\pm$ 0.19 x 10^{-3} mL/s/g and PA 1.62 $\pm$ 0.19 x 10^{-3} mL/s/g).

Vascular volume measurements after chronic nicotine exposure (using the impermeant marker [^{14}C]sucrose) were also assessed concurrently in all experiments to determine BBB integrity (i.e., increased vascular volumes would indicate BBB disruption). With regard to nicotine and cotinine exposure, total brain vascular volume did not significantly vary between control (1.33 $\pm$ 0.34 x 10^{-2} mL/g) and nicotine exposed groups (1.31 $\pm$ 0.30 x 10^{-2} mL/g) (**Fig. 3A and B**)**.** Also, no regional alterations in vascular volume were noted (**Fig. 5**).

Discussion

Data presented in the current study demonstrate: 1) [^{3}H]nicotine blood-brain transfer is not altered after chronic nicotine exposure as measured in the *in situ* perfusion

model, 2) [^{3}H]cotinine penetrates the BBB, and 3) similar to the data found for [^{3}H]nicotine, [^{3}H]cotinine BBB transfer is not altered by chronic nicotine exposure (**Fig. 6**). To our knowledge this is the first report detailing brain uptake of nicotine and cotinine following chronic *in vivo* nicotine exposure and also a rate determination study of cotinine BBB penetration.

Chronic tobacco exposure decreases cerebral blood flow (Rogers et al., 1983) and accordingly diminishes BBB transfer of compounds that are flow dependent (i.e., extraction approxmately ≥ 80%). Therefore, to determine if nicotine has a similar effect we evaluated the uptake of [^{3}H]diazepam in both naive and nicotine exposed animals. It appears from our data cerebral perfusion flow values are not significantly altered in the presence of chronic nicotine exposure. However, our data do not preclude the influence of effects on cerebral blood flow due to *in situ* model limitations, including secondary loss of cerebral blood flow (such as the absence of auto-regulatory factors in a pump driven organ perfusion flow system).

Earlier work by the group of Ghosheh on the brain distribution of nicotine found increased levels (i.e., distribution) of nicotine in brain following chronic nicotine exposure. The authors suggested several rationales for the increased nicotine brain distribution including alterations in BBB transfer rates, increased nAChr binding, and sequesteration of nicotine in glial cytosol through its protonation (pKa = 9.13, glial cytosol pH ~ 6.5 - 7.0) (Ghosheh et al., 2001). Our data explored the first rationale proposed by Ghosheh's group. In our experiments, the BBB transfer of [^{3}H]nicotine was found to be ~70 – 80% of flow ([^{3}H]diazepam uptake) and consequently may be influenced by flow alterations. Therefore, if chronic nicotine exposure resulted in BBB

alterations that may influence blood-brain transfer of [^{3}H]nicotine such changes should be apparent using the *in situ* model. Our findings suggest there is no alteration of [^{3}H]nicotine brain uptake after chronic nicotine exposure either in whole brain or in any measured brain region. We believe the increased CNS distribution of nicotine seen in previous studies may be from the result of either increased neuronal binding or glial sequestration.

As a major metabolic pathway in the periphery nicotine primarily (~80%) undergoes liver aldehyde oxidation via CYP2A6 to form cotinine in humans (Messina et al., 1997; Yamanaka et al., 2004) and in rats (homologue CYP 2B1) (Hammond et al., 1991; Nakayama et al., 1993). There is controversy as to whether cotinine penetrates the BBB from plasma (Halldin et al., 1992; Riah et al., 1998) or whether the detection of cotinine in brain is solely the result of central nicotine metabolism. Recently, nicotine has been shown to be metabolized in rat brain (Jacob et al., 1997) via CYP2B1 (human homologue CYP 2B6) and this process is upregulated after chronic nicotine exposure (Miksys et al., 2000) (though the amount of nicotine metabolism in brain has not been elucidated). Therefore, in order to determine if brain cotinine concentrations are also altered by BBB transfer in naive and nicotine exposed animals, we evaluated the brain uptake of [^{3}H]cotinine.

Contrary to previous reports our data demonstrate there is significant BBB transfer of cotinine that is generally homogeneous among brain regions. Comparison of the PA and logP for [^{3}H]cotinine suggests [^{3}H]cotinine crosses the BBB by passive diffusion (Ghosheh et al., 2001; Smith, 2003). This rate of [^{3}H]cotinine BBB penetration

is significant considering it is comparable to other neuro-active molecules including theophylline, adenosine and choline (Smith, 2003).

Our evaluation of unidirectional influx of cotinine and nicotine into brain further suggests that the nicotine metabolite penetrates the BBB significantly. The average cotinine plasma levels measured in heavy smokers were found to be fairly stable and range from 250-350 ng/mL (Benowitz et al., 1983; Paoletti et al., 1996). Calculation of influx (influx = C_{pl} x PA) reveals that cotinine enters brain at a rate of 0.5-0.7 ng/s/g or ~43-61 μg/g/d. On the other hand, nicotine plasma levels vary significantly between 10-50 ng/ml in smokers (Russell and Feyerabend, 1978; Benowitz et al., 1982), following increment peaks of 5-30 ng/ml per cigarette ($t_{½}$ ~ 2 h) (Isaac and Rand, 1972; Armitage et al., 1975). Therefore, assuming that the highest average nicotine plasma level is 40 – 50 ng/mL over a 24 h period, the BBB influx of nicotine would approximate 1.32 - 1.65 ng/s/g or 114 – 143 μg/g/d. Comparison of influx data measured for the two compounds suggests that cotinine enters brain at amounts approximately 40% than that of nicotine regardless of prior nicotine exposure. While our studies did not include measurement of pharmacological activity, the data suggest that cotinine may penetrate the BBB to a degree that would allow central action.

Data presented in the current study also demonstrate that vascular volumes are not altered after chronic nicotine exposure. These data are consistent with our recently published work (Lockman et al., 2005) and other reports demonstrating that chronic pharmacological relevant nicotine exposure does not alter functional BBB integrity of epithelium (Minty et al., 1984) or endothelium *in vivo* (Booyse et al., 1981; Allen et al.,

1988; Myers et al., 1988). Taken together, these data suggest that chronic nicotine exposed animals retain an operative BBB.

In summary, our data demonstrate that both cotinine and nicotine significantly penetrate the BBB, and that transfer rates are not affected by chronic heavy nicotine exposure. This current report will be expanded further by compartmental (both central and peripheral) pharmacokinetic modeling for both nicotine and cotinine. Care must be taken to elucidate the distribution sites in such modeling studies (e.g., nicotine/cotinine accumulation in plasma, endothelium, glia, neurons and extracellular fluid as measured in the central compartment). Such future work may significantly increase our understanding of the distribution of free drug in brain and may help elucidate the complex action of nicotine and its major metabolite in a tobacco consuming human population.

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Legends for Figures

Figure 1

Cerebral perfusion flow (brain uptake of [^{3}H]diazepam) (A) and the PA for [^{3}H]nicotine (B) in naive and nicotine-exposed rats (28 d x 4.5 mg/kg/d). No significant differences are noted in either group. Data suggest chronic nicotine exposure does not result in increased cerebral perfusion flow or movement of [^{3}H]nicotine across the BBB. All data represent mean $\pm$ SEM for total brain; n = 6 for both points.

Figure 2

Regional brain uptake of [^{3}H]nicotine in naive rats and in nicotine-exposed rats (28 d x 4.5 mg/kg/d). No significant changes were noted throughout all brain regions with the exception of the caudate putamen region (* = $p<0.05$). These data support those shown in Fig. 1 in that chronic nicotine exposure does not appear to increase [^{3}H]nicotine uptake across the BBB. All data represent mean $\pm$ SEM; n = 6 for all points.

Figure 3

Time course of [^{3}H]cotinine brain uptake and cerebrovascular volume in naive rats (**A**) and in nicotine-exposed rats (**B**) (28 d x 4.5 mg/kg/d). Calculation of [^{3}H]cotinine K_{in} is based upon linear regression of brain distribution volume per time. No significant differences are noted between groups. Data suggest chronic nicotine exposure does not result in increased movement of [^{3}H]cotinine across the BBB. All data represent mean $\pm$ SEM for total brain; n = 3-5 for all points.

Figure 4

Regional brain uptake of [^{3}H]cotinine in naive rats and in nicotine-exposed rats (28 d x 4.5 mg/kg/d). Similar to whole brain studies, no significant changes were noted with the exception of the hippocampus region (* = $p<0.05$).. All data represent mean $\pm$ SEM; n = 3-5 for all points.

Figure 5

Regional vascular volume (measured by [^{14}C]sucrose) in naive rats and in nicotine-exposed rats (28 d x 4.5 mg/kg/d). [^{14}C]sucrose does not penetrate the BBB in the time frames evaluated and therefore accurately measures BBB integrity. No significant changes were noted between naive rats and in nicotine-exposed groups. All data represent mean $\pm$ SEM; n = 3-5 for all points. Max Vv on the Y-axis indicates the typical maximum vascular volume measurement seen using in situ perfusions.

Figure 6:

Title: Blood to brain penetration of nicotine and cotinine

A summary of the blood to brain transfer of nicotine and its major metabolite cotinine. Data are from previous literature and this current study. A * indicates results of this study in naive rats and ^{O} for nicotine exposed rats.

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Figure 1

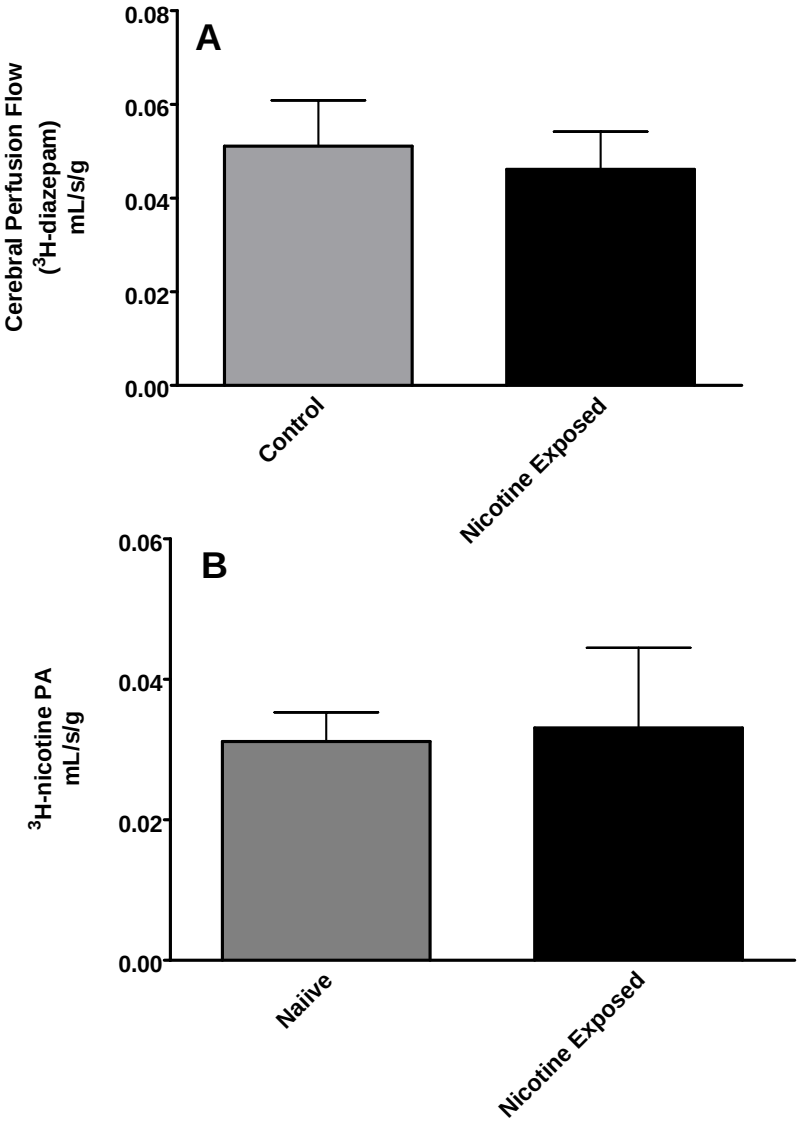

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Figure 2

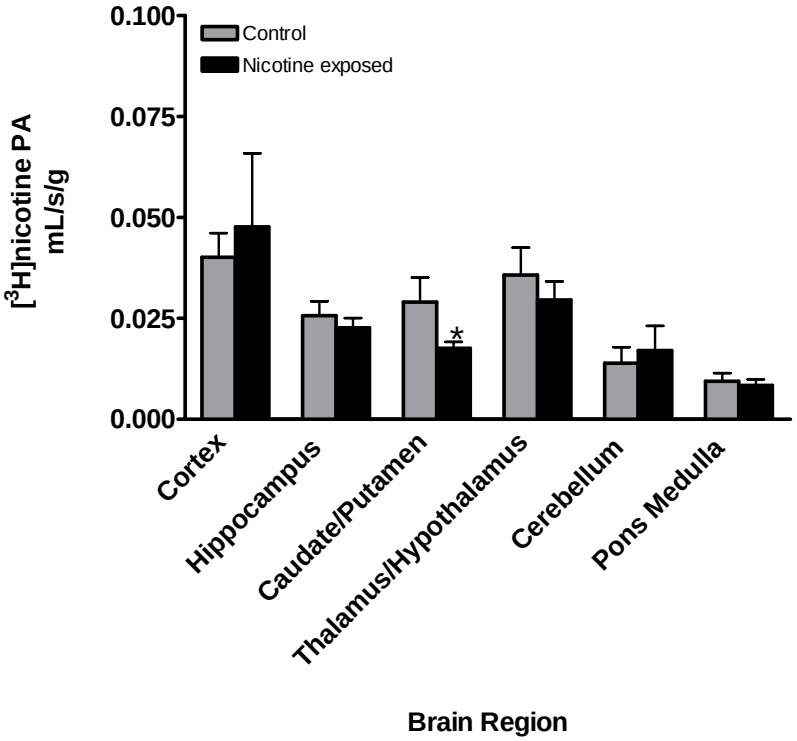

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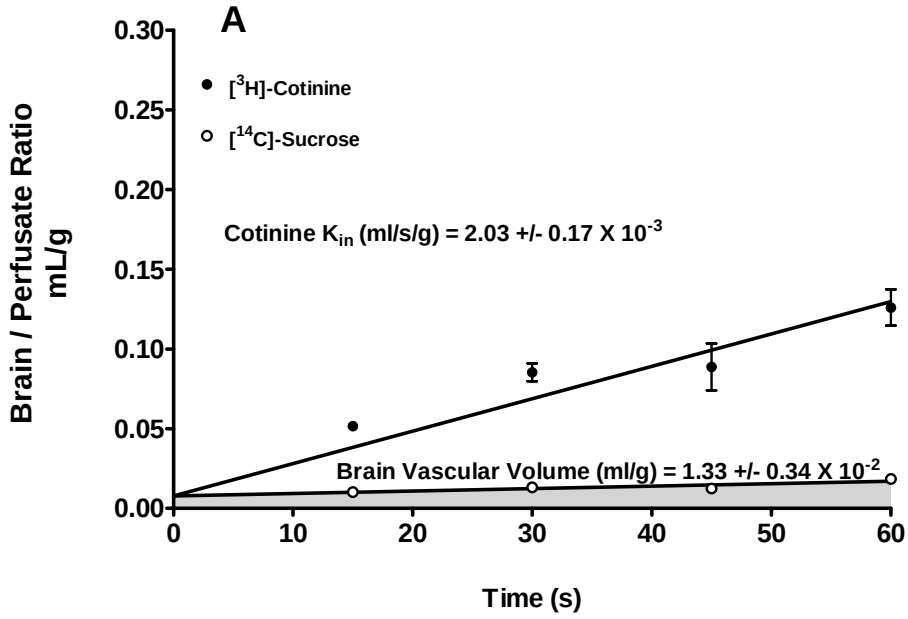
Figure 3



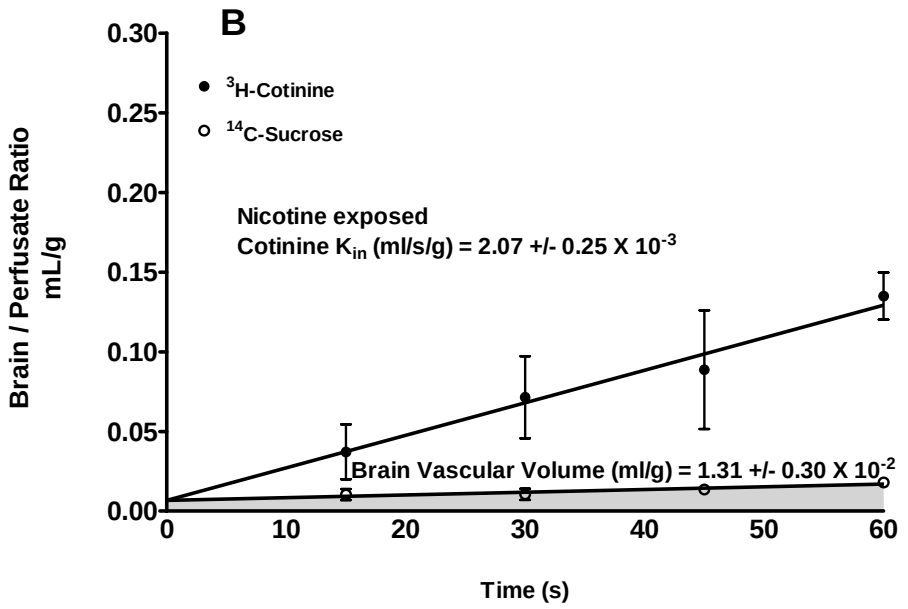


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Figure 4

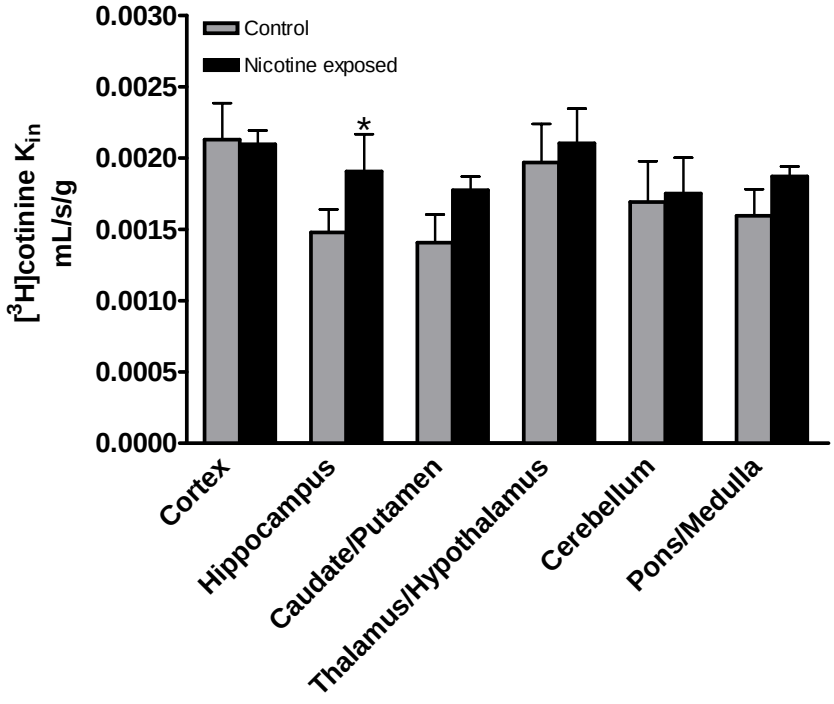

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Figure 5

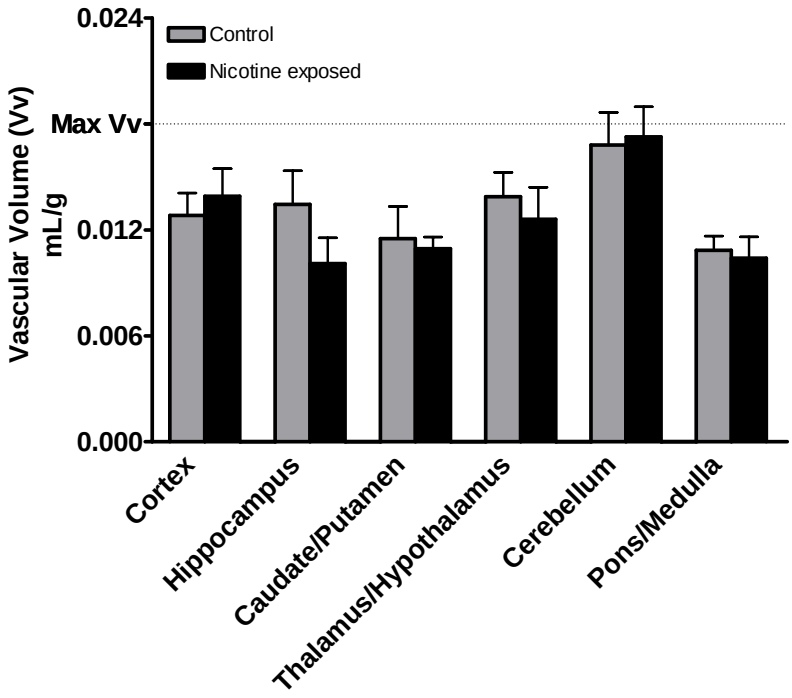

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Figure 6