

ACTIVE TRANSPORT OF HIGH AFFINITY CHOLINE AND NICOTINE ANALOGS INTO  
THE CENTRAL NERVOUS SYSTEM BY THE BLOOD-BRAIN BARRIER CHOLINE  
TRANSPORTER

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Abbreviations:

BBB, Blood-brain barrier; CNS, Central nervous system; *N*-methylpyridinium iodide (NMPI); *N*-*n*-octylpyridinium iodide (NOPI); *N*-methylnicotinium iodide (NMNI); *N*-*n*-butylnicotinium iodide (NBNI); *N*-*n*-octylnicotinium iodide (NONI) and *N*-*n*-decylnicotinium iodide (NDNI)

## ABSTRACT

Cigarette smoking is strongly implicated in the development of cardiovascular disorders. Recently identified nicotinium analogs may have therapeutic benefit as a smoking cessation therapy, but may have restricted entry into the CNS by the blood-brain barrier (BBB) due to their physicochemical properties. Using the *in situ* perfusion technique lobeline, choline and nicotinium analogs were evaluated for binding to the BBB choline transporter. Calculated apparent  $K_i$  values (in  $\mu\text{M}$ ) for the choline transporter were: *N*-*n*-octyl choline, 1.7; *N*-*n*-hexyl choline, 2.2; *N*-*n*-decylnicotinium iodide, 27; *N*-*n*-octylpyridinium iodide, 31.9; *N*-*n*-octylnicotinium iodide (NONI), 49; lobeline, 393; and *N*-methylnicotinium iodide,  $\geq 1000$ . However, nicotine and *N*-methylpyridinium iodide do not apparently interact with the BBB choline transporter. Given NONI's apparent  $K_i$  value determined in this study and its ability to inhibit nicotine-evoked dopamine release from superfused rat brain slices, potential brain entry of NONI via the BBB choline transporter was evaluated. [ $^3\text{H}$ ]-NONI exhibited a BBB transfer coefficient value of  $\sim 1.6 \times 10^{-3} \text{ ml/s/g}$  and a  $K_m$  of  $\sim 250 \mu\text{M}$ . Unlabeled choline addition to the perfusion fluid reduced [ $^3\text{H}$ ]-NONI brain uptake. We hypothesize the *N*-*n*-octyl group on the pyridinium nitrogen of NONI facilitates brain entry via the BBB choline transporter. Thus, NONI may have utility as a smoking cessation agent, given its ability to inhibit nAChRs mediating nicotine-evoked dopamine release centrally, and to be distributed to brain via the BBB choline transporter.

Tobacco smoking is strongly associated with the development of cardiovascular disease, and is the number one preventable cause of death in the USA. Specifically, smoking has been associated with both hemorrhagic and non-hemorrhagic stroke (Gill et al., 1989). Smoking cessation therapies include abstinence ('cold turkey'), nicotine replacement, lobeline, clonidine and certain antidepressant drugs. Yet in spite of the availability of several treatments for smoking cessation, failure rates remain as high as 70%, even with the most effective protocols (Haustein, 2000). Recently developed quaternary ammonium analogs of nicotine may hypothetically have therapeutic benefit as smoking cessation agents, as suggested by their ability to act as subtype-selective nicotinic receptor antagonists and to inhibit nicotine-evoked dopamine release from superfused brain slices (Crooks et al., 1995; Dwoskin et al., 2000; Wilkins et al., 2002).

Blood-brain barrier (BBB) permeability of synthetic quaternary ammonium nicotine analogs may be limited, due to their physicochemical properties, since these molecules contain a positively charged *N*-n-alkylpyridinium moiety. The BBB is comprised of brain capillary endothelial cells connected by tight junctions circumferentially surrounding the cell margin (Butt et al., 1990), which prevents such charged molecules from entering the brain by passive permeation. The BBB presents drug permeation restrictions similar to a continuous cell membrane, allowing lipid-soluble molecules transport across the membrane; whereas molecules that are hydrophilic, charged, protein bound, or of large molecular weight, have restricted permeation (Smith, 1990). Attempts have been made to increase brain drug delivery across the BBB either by increasing a drug's lipid solubility or by causing a temporary "opening" of the BBB (Greig, 1989) using osmotic methods or specific solutes including RMP-7. Additional methods employed to augment brain drug delivery include direct brain or cerebrospinal fluid injection, intracarotid infusion to maximize brain arterial concentrations, inhibition of active

removal from brain, or blocking drug metabolism (Smith, 1993). Consequently, assessment of drug delivery to the brain and the development of novel strategies to deliver such therapeutic agents to the CNS, is of paramount importance in the design and discovery of drugs as CNS therapeutic agents.

The BBB choline transporter may be utilized to overcome restricted permeation of positively-charged quaternary ammonium compounds across the BBB (Metting et al., 1998). Choline enters the CNS via a native nutrient BBB transport system (Diamond, 1971; Cornford et al., 1978; Metting et al., 1998; Allen and Smith, 2001). This transport system may be an effective strategy as a CNS delivery vector for drugs which have positively charged quaternary ammonium grouping within their structure, such as the quaternized ellipticines that are cytotoxic against isolated human brain tumor cells (Vistica *et al.*, 1994). It has already been demonstrated that lymphoblast choline transporters are effective in delivering nitrogen mustard alkylating agents intracellularly (Goldenberg and Begleiter, 1979). These latter studies suggest that choline transporters can be used opportunistically to deliver drugs across cell membranes. As such, the BBB choline transporter may offer a similar opportunity for the transport of charged molecules, and may have wide application for the delivery of therapeutics to treat CNS disorders.

In the present report, the ability of synthetic choline and nicotine analogs to inhibit BBB choline transport was evaluated, providing initial information on the ability of this transporter to deliver potential drug candidates, such as *N*-n-octylnicotinium iodide (NONI), to brain, as well as providing information about the choline transporter pharmacophore. The results suggest that charged, polar compounds can be designed that have high affinity for the BBB choline transporter, and that can be delivered via this transporter to the CNS.

## MATERIALS AND METHODS

### Subjects

Male Fischer-344 rats (220-330 g) were obtained from Charles River Laboratories (Kingston, N.Y., U.S.A.) and were used for all experiments described herein. All studies were approved by the Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### In Situ Rat Brain Perfusion Technique

The *in situ* rat brain perfusion technique was utilized to evaluate BBB transporter binding and uptake of nicotine and choline analogs into brain. Analog access to brain via the BBB choline transporter was evaluated indirectly by assessment of analog-induced inhibition of [<sup>3</sup>H]-choline uptake into brain. Promising candidates were evaluated directly by determining the CNS distribution parameters of the [<sup>3</sup>H]-labeled analog (i.e., [<sup>3</sup>H]-NONI), and by determining the ability of natural substrates for the choline transporter to inhibit uptake of the [<sup>3</sup>H]-labeled analog.

[<sup>3</sup>H]-NONI and [<sup>3</sup>H]-choline uptake into brain were determined using a modification of the *in situ* rat brain perfusion technique (Takasato et al., 1984; Smith 1996; Allen et al., 1997). Perfusions were conducted by carotid artery infusion of buffered physiological saline to substitute for cerebrovascular flow to the ipsilateral cerebral hemisphere for 0-60 s. Briefly, the method consists of Male Fischer-344 rats (220-330 g; Charles River Laboratories, Kingston, N.Y., U.S.A.) being anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal). After anesthesia was induced the perfusion cannula (PE-60 tubing) was implanted into the left common carotid artery, just proximal to the bifurcation with the external and internal carotid arteries. The left external carotid artery was ligated to reduce flow to noncerebral tissues, and the left pterygopalatine artery remained open according to the modified technique. Immediately prior

to perfusion, the heart was stopped by cutting the cardiac muscle to eliminate flow from the systemic circulation (Smith, 1996). Subsequently, fluid was infused into the common carotid artery cannula at a constant rate of 10 ml/min (perfusion pressure, 75-100 mm Hg) using a Harvard Model 944 dual channel infusion pump (Harvard Apparatus, South Natick, MA). As fluid infused into the left carotid artery, the left cerebral hemisphere received the majority of the fluid flow. To quantify cerebral perfusion flow (F) [ $^3\text{H}$ ]-diazepam (0.15  $\mu\text{Ci/ml}$ ) uptake for 15 s was determined in a discrete experimental group (Takasato et al., 1984). As such, data are presented for left brain regions.

The perfusion fluid consisted of  $\text{HCO}_3^-$  buffered physiological saline, containing 128 mM NaCl, 24 mM  $\text{NaHCO}_3$ , 4.2 mM KCl, 2.4 mM  $\text{NaH}_2\text{PO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 0.9 mM  $\text{MgSO}_4$  and 9 mM glucose (pH  $\sim 7.35$ ;  $[\text{Na}] = 154.4$  mM). All solutions were filtered, oxygenated, warmed to 38° C and adjusted to pH 7.35 before perfusion. The Harvard pump allowed input from two syringes that were connected via a four-way valve allowing conduct of a brief vascular washout (Allen and Smith, 2001). To determine initial brain uptake baseline parameters, perfusion fluid containing [ $^3\text{H}$ ]-choline (1.0  $\mu\text{Ci/ml}$ ), [ $^3\text{H}$ ]-NONI (1.0  $\mu\text{Ci/ml}$ ) or [ $^{14}\text{C}$ ]-sucrose (0.3  $\mu\text{Ci/ml}$ , as a vascular marker) was infused into the cerebral circulation for 5-60 s.

Inhibition of [ $^3\text{H}$ ]-choline brain uptake was determined by including unlabeled choline or nicotine analog in the perfusion fluid. Structures of the compounds evaluated are illustrated in Figure 1. To determine  $K_i$ , compounds were evaluated at initial concentrations of 250  $\mu\text{M}$ , unless specifically stated otherwise, as described previously (Smith et. al, 1987) and detailed below. Compounds which were examined included: lobeline, *N*-n-hexyl choline, *N*-n-octyl choline, nicotine, *N*-methylpyridinium iodide (NMPI), *N*-n-octylpyridinium iodide (NOPI), *N*-methylnicotinium iodide (NMNI), *N*-n-butylnicotinium iodide (NBNI), *N*-n-octylnicotinium iodide (NONI) and *N*-n-decylnicotinium iodide (NDNI). In the experiments evaluating inhibition of [ $^3\text{H}$ ]-choline brain uptake, the perfusion was changed subsequently to tracer-free perfusion fluid for 15 s to wash out [ $^3\text{H}$ ]-choline, which had not been taken up into brain (Allen and Smith, 2001). Inhibition of [ $^3\text{H}$ ]-NONI uptake into brain was determined by addition of unlabeled

substrates to the perfusion fluid in separate experiments (choline 500  $\mu$ M and 5 mM; NONI 250  $\mu$ M).

At the end of each experiment, rats were decapitated and the brain was removed from the skull. Brain was dissected to obtain cortical tissue samples from the left cerebral hemisphere (25-90 mg) (Takasato et al., 1984). Perfusate samples (50  $\mu$ l, in duplicate) were collected for determination of perfusate tracer concentrations. All samples were placed in glass vials, weighed and then digested overnight at 50 °C in 1 ml piperidine (1.0 M). Subsequently, 10 ml scintillation fluid (Scintisafe, Fisher Scientific, Pittsburgh, PA) was added to each vial. The amount of tracer was determined by dual-label liquid scintillation counting (LS 6500; Beckman Coulter, Fullerton, CA) with appropriate quench and efficiency corrections (efficiency for each label with dual label counting was:  $^3\text{H} \geq 60\%$ ,  $^{14}\text{C} \geq 95\%$ ).

### Chemicals

[ $^3\text{H}$ -Methyl]choline chloride (~80 Ci/mmol), [ $^{14}\text{C}$ ]-sucrose (4.75 mCi/mmol), and [ $^3\text{H}$ ]-diazepam (79.5 Ci/mmol) were obtained from Dupont-New England Nuclear (Boston, MA, U.S.A.). [ $^3\text{H}$ ]-NONI (10 mCi/mmol) was obtained from Sibtech (Newington, CT; random tritium labeling with >90% of the tritium label incorporated in the *N*-n-octyl chain). All radiolabeled compounds were dried completely before use to eliminate possible contaminants, including [ $^3\text{H}$ ]- $\text{H}_2\text{O}$ . Unlabeled choline chloride, lobeline hydrochloride, S(-)-nicotine and components of the physiologic buffer (NaCl,  $\text{NaHCO}_3$ , KCl,  $\text{NaH}_2\text{PO}_4$ ,  $\text{CaCl}_2$ ,  $\text{MgSO}_4$  and d-glucose) were obtained from Sigma (St. Louis, MO, U.S.A.). Unlabeled NONI, NMNI, NBNI, NDNI, NMPI and NOPI were prepared as previously described (Crooks et al., 1995; Rui et al., 2002).

### Calculations

Concentrations of tracer in brain and perfusion fluid were expressed as dpm/g brain or dpm/ml perfusion fluid, respectively. Blood-brain barrier transport was determined using the initial uptake method, as previously described (Takasato et al. 1984; Smith, 1996, 1989). In



preliminary experiments, linear and unidirectional [<sup>3</sup>H]-NONI uptake into brain was determined by perfusion with [<sup>3</sup>H]-NONI (13.8 μM) for a 5-60 s period. Unidirectional uptake transfer constants ( $K_{in}$ ) were calculated from the following relationship to the linear portion of the uptake curve:

$$Q^*/C^* = K_{in}T + V_o \quad (1)$$

where  $Q^*$  is the quantity of [<sup>3</sup>H]-tracer in brain (dpm/g) at the end of perfusion,  $C^*$  is the perfusion fluid concentration of [<sup>3</sup>H]-NONI (dpm/ml),  $T$  is the perfusion time (s) and  $V_o$  is the extrapolated intercept ( $T = 0$  s; "vascular volume" in ml/g). After determination of a perfusion time that allowed adequate amount of tracer to pass into brain and yet remained in the linear uptake zone,  $K_{in}$  was determined in single time-point experiments as  $K_{in} = [Q^* - V_o C^*] / C^* T$  (Takasato et al., 1984). When [<sup>3</sup>H]-choline uptake was evaluated, vascular tracer was removed in most experiments by a brief intravascular wash (15 s) with tracer-free perfusion fluid.  $K_{in}$  values were converted to apparent cerebrovascular permeability-surface area products (PA) using the Crone-Renkin equation (Smith, 1989):

$$PA = -F \ln (1 - K_{in}/F). \quad (2)$$

where  $F$  is cerebral perfusion fluid flow. In all instances, PA differed by < 2% from  $K_{in}$ , because  $F$  exceeded  $K_{in}$  by > 40 fold.

An apparent inhibition constant ( $K_i$ ) for choline and nicotine analogs was determined assuming competitive kinetics (Smith et al., 1987) from the equation:

$$[(PA_o - K_D)/(PA_i - K_D)] = 1 + C_i/K_i \quad (3)$$

where  $PA_o$  is the [<sup>3</sup>H]-choline PA in the absence of competitor,  $PA_i$  is the [<sup>3</sup>H]-choline PA in the presence of inhibitor, and  $C_i$  is the perfusate concentration of inhibitor. Apparent  $K_i$  is defined as the inhibitor concentration that reduces saturable brain [<sup>3</sup>H]-choline influx by 50% at tracer choline concentration ( $C_{pf} \ll K_m$ ) and in the absence of other competing compounds. Previously, we demonstrated competition at the BBB choline transporter with a 0.25 – 12.5 μM concentration range of hemicholinium-3, the defining substrate for choline transport systems

(Allen et. al., 1996a). Evaluation of  $K_i$  was completed after a one-way ANOVA determined significant differences between [ $^3\text{H}$ ]-choline PA (ml/s/g) in the presence of the nicotinium analogs (data not shown). Compounds that demonstrated a significant reduction in choline PA (ml/s/g) were assumed to have a competitive interaction at the BBB choline transporter, and thus a  $K_i$  was calculated as described previously. For compounds that had a non-significant reduction in choline PA (ml/s/g), a power analysis was performed to determine if the comparison resulted in a P value less than alpha, and thus, physiologic  $K_i$  significance could be achieved if more data points were collected. Power analysis limits for significance were set at: 1) choline PA reduction of 0.23 ml/s/g (corresponding to a  $K_i$  value of 1 mM; i.e. maximum level set for assumed physiologic relevance), and 2) a percent power of 70%. The level of assumed physiologic relevance was selected, since the main goal of the present work was to identify high-affinity ligands, which may be delivered to brain via this transport system. Higher concentrations are considered less relevant for such brain-directed delivery because of affinity and capacity of the BBB transport system (Smith, 1993).

### Statistics

Data presented are generated from the frontal cerebral cortex with comparable data seen in parietal and occipital cortical regions, as well as the hippocampal, striatal, thalamic, cerebellum and midbrain regions (data not shown). Inhibition of brain [ $^3\text{H}$ ]-choline uptake by choline analogs and [ $^3\text{H}$ ]-NONI brain uptake values were expressed as means  $\pm$  SEM for  $n = 3-5$  independent determinations for each compound evaluated. Data were analyzed by ANOVA with Bonferroni correction for multiple comparisons (Instat, GraphPad Software, San Diego, CA). Differences between the means were considered significant at  $p < 0.05$ . Power analyses were calculated using GraphPad StatMate version 1.01i, GraphPad Software, San Diego CA).

## RESULTS

### Inhibition of [<sup>3</sup>H]-Choline Brain Uptake

Binding of choline and nicotine analogs to the BBB choline transporter was determined by evaluation of their ability to inhibit [<sup>3</sup>H]-choline uptake into brain. These studies were conducted to provide insight into the potential ability of these compounds not only to bind to the transporter, but to determine their potential ability to be transported across the BBB from the vasculature compartment into brain. Baseline [<sup>3</sup>H]-choline PA values ( $1.26 \pm 0.04 \times 10^{-3}$  ml/s/g) obtained in the present experiments were similar to previously published values (Allen and Smith, 2001; Lockman et al, 2001). Given that nicotine and lobeline are currently utilized as smoking cessation therapies, both were assessed for their ability to inhibit choline uptake into brain at a single concentration of 250  $\mu$ M. While nicotine did not significantly reduce choline PA ( $PA_i \sim 1.10 \pm 0.06 \times 10^{-3}$  ml/s/g), lobeline did inhibit brain [<sup>3</sup>H]-choline uptake with an apparent  $K_i$  value of  $393 \pm 51$   $\mu$ M, which is within an order of magnitude of the  $K_m$  for choline (Table 1). It is important to note from previous studies using the capillary depletion method evaluating brain [<sup>3</sup>H]-choline uptake that no significant endothelial cell association of choline occurred (Allen and Smith, 2001). Further, washout studies using the capillary depletion method indicated that no differences in endothelial cell sequestration or binding occurred. Taken together, this uptake represents true [<sup>3</sup>H]-choline brain penetration.

In the current study, we assessed the ability of *N*-n-hexyl and *N*-n-octyl choline to inhibit BBB [<sup>3</sup>H]-choline transport. At a concentration of 10  $\mu$ M, *N*-n-hexyl and *N*-n-octyl choline afforded apparent  $K_i$  values of  $2.2 \pm 0.1$   $\mu$ M and  $1.7 \pm 0.3$   $\mu$ M, respectively. These studies demonstrate that increasing the length of one of the *N*-methyl groups of the choline molecule significantly increases (~25 fold) the ability of choline analogs to bind the BBB choline

transporter, as indicated by inhibition of [<sup>3</sup>H]-choline brain uptake. As such, it appears that a lipophilic binding pocket proximal to the choline-binding site is present which can significantly enhance binding to the transporter.

The ability of *N*-n-alkylpyridinium compounds (i.e. NMPI and NOPI) to inhibit [<sup>3</sup>H]-choline uptake into brain was also investigated. NMPI inhibited brain [<sup>3</sup>H]-choline uptake with an apparent  $K_i$  value of  $3990 \pm 474 \mu\text{M}$ , whereas NOPI was a much more potent inhibitor with an apparent  $K_i$  value of  $32 \pm 22 \mu\text{M}$  (Table 1).

A series of *N*-n-alkylnicotinium analogs have been previously demonstrated to exert effects in dopaminergic systems (Crooks et al., 1995; Wilkins et al., 2002). The *N*-n-alkyl chain moiety in these compounds was varied from C<sub>1</sub> to C<sub>8</sub>. The C<sub>8</sub> compound, NONI, inhibited [<sup>3</sup>H]-choline uptake with an apparent  $K_i$  value of  $49 \pm 24 \mu\text{M}$ , whereas the C<sub>1</sub> compound, NMNI, showed significantly lower affinity for the transporter with an estimated apparent  $K_i$  value of  $\geq 1000 \mu\text{M}$ . The C<sub>10</sub> compound, NDNI, inhibited [<sup>3</sup>H]-choline uptake into brain with an apparent  $K_i$  value of  $27 \pm 2 \mu\text{M}$ , similar to that of NONI and choline. The C<sub>4</sub> compound, NBNI, was much less potent in blocking uptake, with an estimated apparent  $K_i$  value of  $777 \pm 588 \mu\text{M}$ . These results suggest that increasing the length of the *N*-n-alkyl chain in these nicotine analogs may facilitate binding, and thus, potentially their brain uptake by the BBB choline transporter.

### **[<sup>3</sup>H]-NONI Brain Uptake**

Given the initial  $K_i$  values for NONI, experiments were performed to verify that this compound gains access to brain via the BBB choline carrier. The brain distribution parameters of [<sup>3</sup>H]-NONI were evaluated using the rat brain perfusion method. Uptake of [<sup>3</sup>H]-NONI (1  $\mu\text{Ci/ml}$ ) into brain was evaluated from 0-60 s in the absence of unlabeled NONI. Brain/perfusion fluid ratios (i.e., volume of distribution or 'space') were plotted as a function of time and are

illustrated in Figure 2. The transfer coefficient value ( $K_{in}$ ) for [ $^3\text{H}$ ]-NONI uptake was determined to be  $1.59 \pm 0.14 \times 10^{-3}$  ml/s/g, calculated according to the slope of the compound accumulating in brain versus time (Smith, 1989). An uptake time of 45 s was chosen as within the linear portion of the brain uptake curve to evaluate [ $^3\text{H}$ ]-NONI brain uptake in the presence of unlabeled NONI. Unlabeled NONI (250  $\mu\text{M}$ ) in the perfusion fluid resulted in 46% inhibition of [ $^3\text{H}$ ]-NONI brain uptake. The inhibition suggests saturable kinetic parameters associated with transporter-mediated BBB transport is present for NONI (Figure 3).

The ability of choline to inhibit [ $^3\text{H}$ ]-NONI uptake into brain and [ $^3\text{H}$ ]-NONI distribution parameters were determined. Figure 4 illustrates the [ $^3\text{H}$ ]-NONI brain uptake profile in the presence of choline. The PA (ml/s/g) for [ $^3\text{H}$ ]-NONI with no inhibitors present was  $1.64 \pm 0.37 \times 10^{-3}$  ml/s/g, determined as a single time point PA value, as described in the methods. If NONI is transported in total or in part by the BBB choline transporter, then addition of choline to the perfusion fluid should reduce brain uptake. When 500  $\mu\text{M}$  choline was added to the perfusion fluid, the PA tended to decrease ( $\sim 25\%$  to  $1.24 \pm 0.5 \times 10^{-3}$  ml/s/g), but did not reach significance. A higher concentration (5 mM) of choline further reduced the uptake of [ $^3\text{H}$ ]-NONI to  $7.55 \pm 3.30 \times 10^{-4}$  ml/s/g ( $p < 0.05$ ), which was  $< 50\%$  of the uptake in the absence of choline. These results suggest that a significant component of NONI uptake occurs via the BBB choline transporter.

## DISCUSSION

Pharmacological intervention in neurological diseases is often limited by poor access of therapeutic agents into the CNS. Approaches to successful therapy in CNS diseases must consider the BBB as a potential impediment. Numerous methods have been used with limited success to deliver polar drugs to the CNS (Pardridge, 1998; 1997). Use of antibodies, drug lipidization, prodrug development have been used with some success. Use of small molecules that directly target transport proteins to overcome BBB restrictions eliminates the need for the drug to be biotransformed in brain and linking to antibodies. Lipidization can result in an undesirable pharmacokinetic profile (Grieg, 1989). We have proposed to use BBB transport proteins such as the choline transporter as brain drug delivery vectors for polar drugs (Metting et al., 1998); this approach has been successful with regard to the amino acid transporter and the transport of L-DOPA and gabapentin into brain (Smith, 1993). In this respect, the present study focused on the BBB choline transporter for drug delivery, as this transporter may offer the advantage of delivering cholinergic and nicotine-like drugs to brain (Metting et al., 1998). Of significance in this report is that the BBB choline transporter efficiently transports choline, a small charged molecule with minimal passive permeation, to brain. Evaluation of this transporter system may afford a possible brain entry portal for similarly charged therapeutic molecules. To date, this system has not been established as a vector for such CNS delivery.

To utilize a BBB transporter protein as a CNS drug delivery vector, multiple factors must be considered. These factors include: 1) kinetic availability to transport physiologic molecules, 2) structural binding requirements of the transporter, 3) therapeutic compound manipulation so that the compound binds, but also remains active *in vivo* and 4) actual transport of the molecule (not just binding to the transporter) into brain. This discussion will address each issue with

regard to the novel nicotine analogs studied herein, and will demonstrate delivery of NONI to brain via the BBB choline transporter.

In vivo choline transport at the BBB has been demonstrated to be carrier-mediated and saturable (Cornford et al., 1978; Allen and Smith, 1999; Allen and Smith, 2001). Endogenous choline plasma levels are less than the calculated  $K_m$  for the transporter, and thus, with respect to availability of the transporter, it is not saturated under physiological conditions. These characteristics coupled with the high transport capacity of the choline transporter and the adequate blood-to-brain transfer rate suggests this carrier has the necessary characteristics to deliver therapeutic molecules to brain (Smith, 1993).

Structural binding requirements of the choline transporter have been extensively characterized in multiple tissues using binding inhibition studies (for a review, see Lockman and Allen, 2002). Previous studies reveal that the principal structural moiety in the choline molecule required for recognition by the transporter binding site is the quaternary ammonium group, which is believed to interact with a corresponding anionic group on the transporter binding site. Simon et al. (1975) demonstrated this relationship, and observed that simple quaternary ammonium cations such as tetraethylammonium and tetramethylammonium were inhibitors of choline uptake in striatal synaptosomes. Furthermore, when longer alkyl moieties were substituted for the *N*-methyl and *N*-ethyl groups, increased binding affinity to the choline transporter was observed (Dowdall et al., 1976). The increased affinity of these modified compounds was hypothesized to result from a hydrophobic interaction with the lipid membrane (Lerner, 1989). The present results with the *N*-*n*-alkylnicotinium and *N*-*n*-alkylpyridinium analogs are consistent with these reports (Table 1), and demonstrate that: 1) *N*-*n*-alkylazaaromatic compounds containing long chain *n*-alkyl groups, and which are charged at

physiologic pH, bind significantly to the choline transporter, 2) the hydroxyl group of the choline molecule is not an absolute requirement for binding to the choline transporter, and 3) when the 3-hydroxy group in the choline molecule is removed, replacement of one of the *N*-methyl groups with a longer *N*-*n*-alkyl group results in compounds with good affinity for the choline transporter, and appears to completely overcome the hydroxy group requirement that has been previously observed (Allen et al., 1996b). As indicated in Table 1, the transporter affinity of these compounds are close to that of choline itself suggesting comparable binding. As these compounds are required in much lower concentrations in brain to exert effects, the potential to deliver these agents to brain in sufficient quantities to be therapeutic seems likely. Taken together, these findings suggest the *N*-*n*-alkyl chains may be interacting with a proximal lipophilic domain and facilitating binding and/or transport.

The *N*-*n*-alkylnicotinium compounds evaluated in this study were previously examined for their ability to inhibit nicotine-evoked dopamine release from superfused rat striatal slices (Crooks et al., 1995; Dwoskin and Crooks, 2001; Wilkins et al., 2002). Importantly, these nicotine analogs were observed to selectively inhibit the  $\alpha 3\beta 2^*$  neuronal nicotinic acetylcholine receptor, which has been suggested to mediate nicotine-evoked dopamine release from its presynaptic terminals in striatum. Specifically, given the ability of NONI to inhibit the effect of nicotine on dopaminergic systems in vitro ( $IC_{50} \sim 1.1 \mu M$ ; Crooks et al., 1995; Wilkins et al., 2002) and the calculated apparent  $K_i$  at the BBB choline transporter (Table 1), the ability of NONI to access the brain via active transport through the BBB choline carrier was further explored.

The results of the present study show that NONI, the *N*-*n*-octyl derivative of nicotine, is transported into brain via the BBB choline transporter. This is a significant finding, since from



structural and physicochemical considerations, one would predict that the ability of NONI to penetrate the BBB and enter the CNS would be poor, due to the charged, polar nature of the compound. [<sup>3</sup>H]-NONI brain/perfusion fluid ratios were plotted versus time (Figure 2), and the calculated transfer coefficient value based upon slope values (Smith, 1989) was determined to be  $1.59 \times 10^{-3}$  ml/s/g. These data suggest that, despite NONI being a charged quaternary ammonium compound, significant brain penetration occurs that is greater than what would be expected from passive permeation alone, indicating an active transport process.

To further confirm that [<sup>3</sup>H]-NONI penetration into brain is an active process, unlabeled NONI (250  $\mu$ M) was added to the perfusion fluid. If [<sup>3</sup>H]-NONI were to enter the brain via active transport, unlabeled NONI would theoretically compete with the transport of the radiolabeled component, and thus reduce [<sup>3</sup>H]-NONI brain uptake. Indeed, Figure 3 illustrates that [<sup>3</sup>H]-NONI brain uptake was reduced by ~46% in the presence of 250  $\mu$ M unlabeled NONI. These data provide further evidence that [<sup>3</sup>H]-NONI is not entering brain by passive permeation alone. To determine the role of the BBB choline transporter in [<sup>3</sup>H]-NONI brain uptake, unlabeled choline was added to the perfusion buffer. As with the previous experiment, if [<sup>3</sup>H]-NONI enters the brain via the BBB choline transporter, the presence of unlabeled choline should diminish [<sup>3</sup>H]-NONI brain entry. Given that the  $K_m$  of choline for the transporter is ~ 45  $\mu$ M (Allen and Smith, 2001), the unlabeled choline concentrations would be sufficient to saturate the transporter and effectively block the transport of [<sup>3</sup>H]-NONI via this carrier. In fact, the presence of 500  $\mu$ M choline tended to decrease uptake by ~ 25%, whereas 5 mM significantly reduced uptake to ~50% of control (Figure 4). Taken together, the present results indicate that the BBB choline transporter has a substantive role to play in the active transport of [<sup>3</sup>H]-NONI into brain. It is important to note that, although the  $K_i$  for NONI is ~49 $\mu$ M, the amount of choline required

for inhibition is higher. However, the concentrations of NONI required to inhibit the effect of nicotine on dopaminergic systems is significantly lower in the range of 1.1  $\mu\text{M}$  as noted above. As such, delivery of sufficient NONI to brain to cause effect can occur with these physiologic conditions. Further, considering brain distribution, and the previous demonstration that NONI inhibits nicotine-evoked dopamine release by selectively interacting with the nicotinic receptor subtype mediating this effect, it is likely that NONI may have significant potential as a CNS therapeutic agent, and if it can be shown that NONI has a good brain distribution profile, it may have significant utility in smoking cessation therapy and stroke prevention. The current observations that the charged, polar *N*-n-alkylnicotinium analogs studied in this report not only inhibited the effect of nicotine on dopamine systems in the CNS, but also gained access to the brain from the periphery, in spite of potential physicochemical limitations, is a key finding in the development of NONI as a potential smoking cessation agent.

Previously, we have identified derivatives of lobeline and isoarecolone that bind to the BBB basic amine transporter (Metting et al., 1998). In light of the findings that NONI is transported in to brain via the BBB choline transporter, further studies are warranted on lobeline and isoarecolone derivatives to determine if the BBB choline transporter can act as vector-mediated uptake system to also deliver these drugs into the brain.

This report provides for the first time, evidence that the BBB choline transporter can be used as a brain drug delivery vector for nicotine and choline analogs. Furthermore, the results suggest that other structurally related charged quaternary ammonium compounds containing lengthy *N*-n-alkyl groups may also exhibit increased brain distribution via active choline transport.



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### Footnotes

1. These studies were supported by NIH Grants NS41933, DA10934, DA00399, the Burroughs Wellcome Fund, the American Foundation for Pharmaceutical Education, and Texas Tech University Health Sciences Center School of Pharmacy.

2. The University of Kentucky owns the patent on NONI, and a royalty stream may become available to LPD and PAC through University arrangements.



## Figure Legends

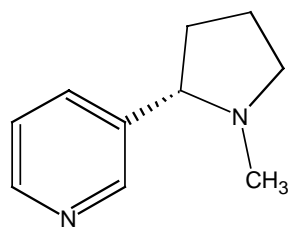
1. Structures of nicotine, lobeline, choline and the analogs of choline and nicotine.
2. Linear time course of [<sup>3</sup>H]-NONI (1 μCi/ml) uptake into rat brain. Calculated transfer coefficient is related to slope values (Smith 1996);  $K_{in} \sim 1.59 \times 10^{-2}$  ( $r^2 = 0.985$ ). Data are frontal cortex brain perfusion concentration ratios (i.e., brain distribution spaces) versus time. Each point is mean  $\pm$  SEM; n = 3.
3. Inhibition of brain [<sup>3</sup>H]-NONI (1 μCi/ml) permeability surface-area (PA) coefficient (control) by the addition of 250 μM NONI to the perfusion fluid on frontal cortex. Data are mean  $\pm$  SEM; n = 6-8. “\*” indicates significant difference ( $p < 0.05$ ) from control.
4. Inhibition of brain cortex [<sup>3</sup>H]-NONI permeability surface-area (PA) coefficient (control) by the addition of unlabeled choline (500 μM and 5 mM). Data are mean  $\pm$  SEM; n = 4-8. “\*” indicates significant difference ( $p < 0.05$ ) from control.

**Table 1**

Summary of [<sup>3</sup>H]-choline inhibition (K<sub>i</sub>) by choline and nicotine analogs. Table is arranged in order of decreasing affinity for the choline transporter.

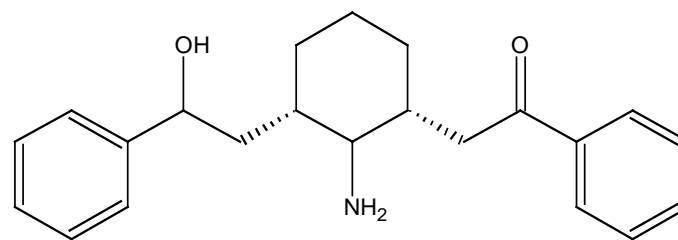
<b>Compound</b>	<b>Concentration (<math>\mu</math>M)</b>	<b>K<sub>i</sub> (<math>\mu</math>M)</b>
<i>N</i> -n-octylcholine	10	1.7 $\pm$ 0.3
<i>N</i> -n-hexylcholine	10	2.2 $\pm$ 0.1
NDNI	250	27.0 $\pm$ 0.1
NOPI	250	31.9 $\pm$ 22
NONI	250	49 $\pm$ 24
Lobeline	250	393 $\pm$ 51
NBNI	250	777 $\pm$ 588
NMNI	250	
Nicotine	250	$\geq$ 1000
	250	No apparent binding

Figure 1



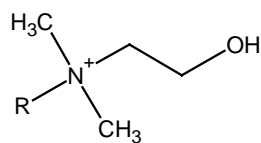
**A**

Nicotine;  $K_i \sim 2040\mu M$



**B**

Lobeline;  $K_i \sim 393\mu M$

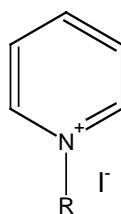


**C**

Choline: R= CH<sub>3</sub>;  $K_m \sim 40mM$

N-Hexyl choline: R= C<sub>6</sub>H<sub>13</sub>;  $K_i \sim 2.2mM$

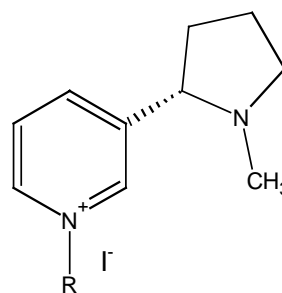
N-Octyl choline: R= C<sub>8</sub>H<sub>17</sub>;  $K_i \sim 1.7mM$



**D**

NMPI: R= CH<sub>3</sub>;  $K_i \sim 4000mM$

NOPI: R= C<sub>8</sub>H<sub>17</sub>;  $K_i \sim 32mM$



**E**

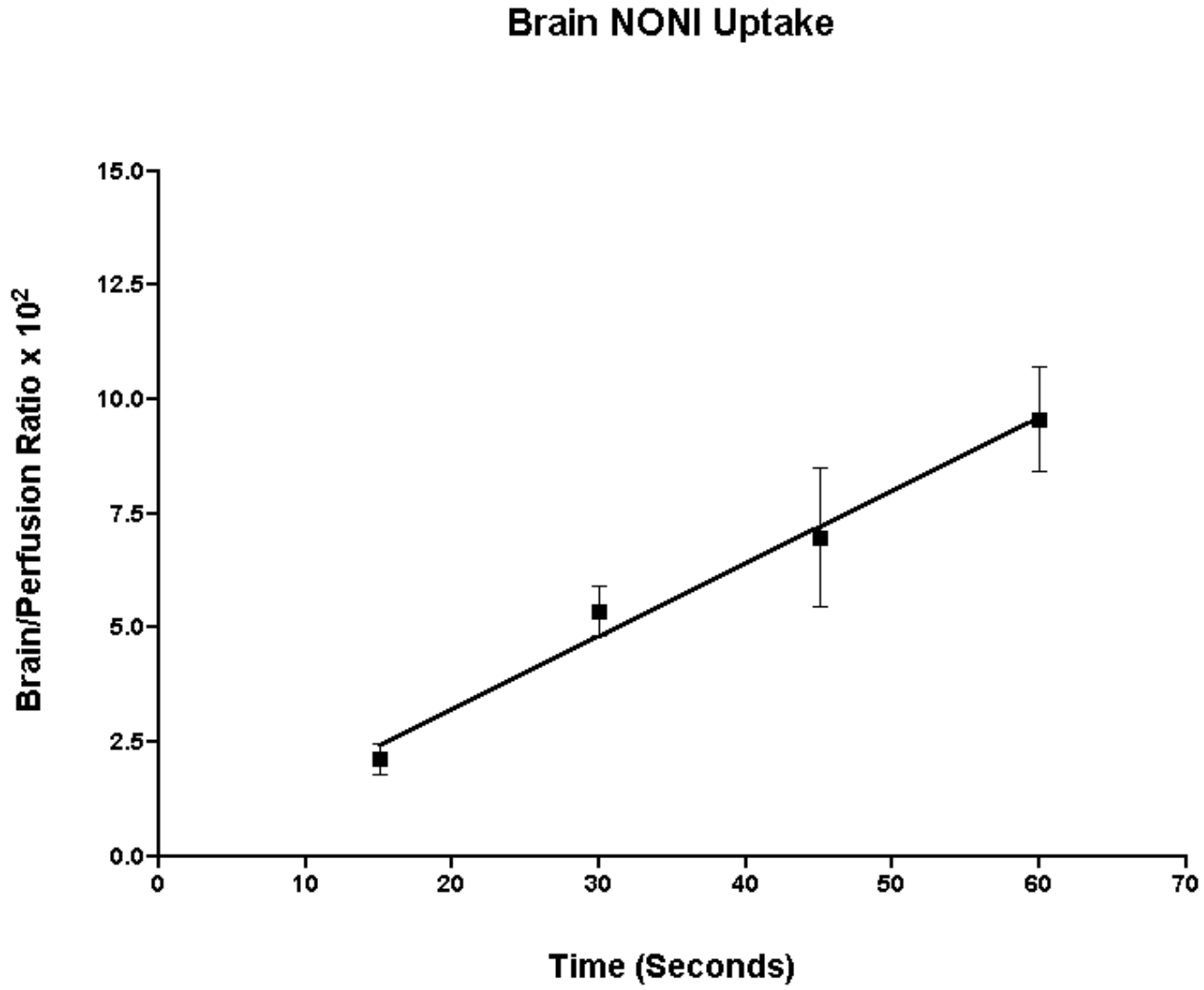
NMNI: R= CH<sub>3</sub>;  $K_i \sim 2000mM$

NBNI: R= C<sub>4</sub>H<sub>9</sub>;  $K_i \sim 777mM$

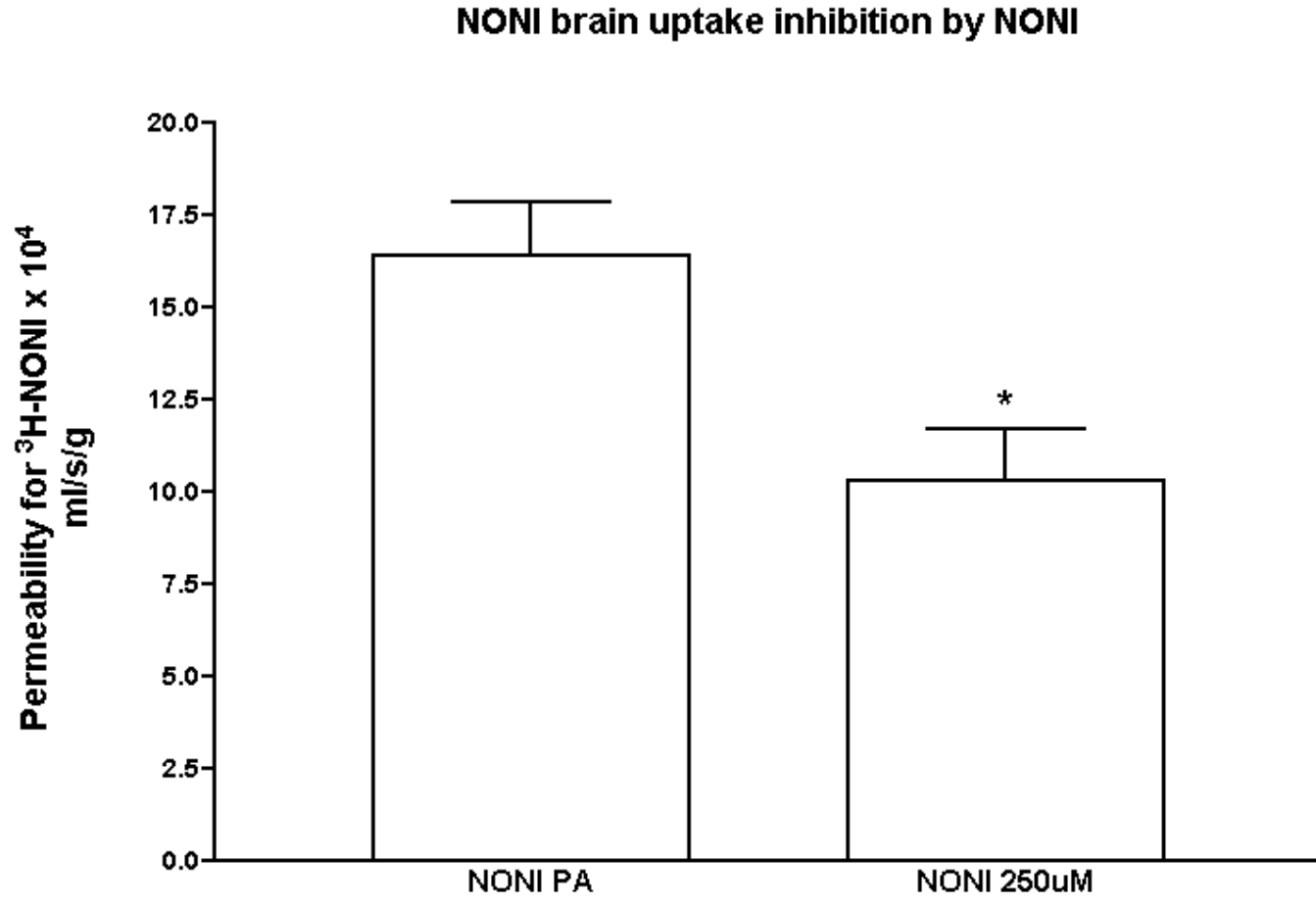
NONI: R= C<sub>8</sub>H<sub>9</sub>;  $K_i \sim 49mM$

NDNI: R= C<sub>10</sub>H<sub>21</sub>;  $K_i \sim 27mM$

**Figure 2**



**Figure 3**



**Figure 4**

