SMOKING AND BRAIN
DOPAMINERGIC NEUROCHEMISTRY

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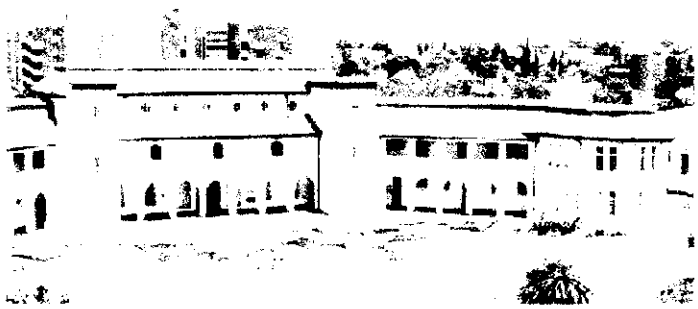

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Dedicated to

God

and

my husband, parents and sister

PREFACE

First and foremost, this work was accomplished through the mercy and love of my Lord, the Great Creator. I am blessed to have completed this work for His glory. I stand in awe of His greatness.

My intend was to give a brief overview of available literature, to the extent that the reader will comprehend the rational behind each aim, which we set out to accomplish (see chapter 2, section 2.5). Therefore to inspire the reader, you will find referrals to more comprehensive articles on that particular subject in the literature review chapter. An index and list of abbreviations are provided for each chapter (chapter 2 – 6), as well as for the complete thesis. The references are listed at the end of each chapter, in order to provide convenient cross-referencing.

This thesis was accomplished with the motivation, guidance and assistance of the following people, which I would like to thank.

- The four most important people in my life who supported me unconditionally. You are truly wonderful blessings in my life.

  I would like to extend my deepest and sincerest gratitude to my **husband, Dr. James McAfee**, for inspiring me every minute of every day, not only within the realms of this thesis, but above and beyond. I thank you for your unwavering belief in me, your ongoing patience, support and encouragement. You have helped maintain my sanity not to mention my sustenance throughout the whole process. Without you, this thesis would not have reached its end so gracefully. You are a "ongelooflike, verskriklike" amazing person and I am so proud to have you as my husband. I love you dearly and thank God everyday for you, my 'perfect' blessing, which was given to me by virtue of His mercy and greatness! I look forward to many more years of friendship and marriage.

  To my **parents, Mr and Mrs Kobus and Gisela Robbertse**. You inspired me from the day I was born to be exactly who I am today, and for that I am eternally grateful to you. Thank you for your endurance and perseverance throughout my life, in teaching me the values of life. I stand in great appreciation of every sacrifice you had to make, to get me where I am today. You are the best people God picked for me as parents. Thank you for your encouragement in faith during this journey of my thesis.

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"It is the heart which perceives God, and not the reason."

Blaise Pascal
(1623-1662) French Scientist

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COMPLETE LIST OF ABBREVIATIONS

[^{3}H]DA	[^{3}H]Dopamine
AChRs	cholinergic receptors
ATR/FT-IR	Attenuated total reflectance Fourier transform infrared spectroscopy
BBB	blood-brain barrier
BH_4	6R-L-erythro-tetrahydrobiopterin
C	concentration of tracer in perfusion fluid (dpm/ml)
CDC	Centers for Disease Control and Prevention
CNS	central nervous system
COMT	catechol-O-methyl transferase
CSE	cigarette smoke extract
CYP	cytochrome P450
DA	dopamine
DAT	dopamine transporter
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxy-phenyl-acetic acid
ECL	enhanced chemiluminescence
F	cerebral perfusion flow rate
GABA	gamma-aminobutyric acid
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
K_{in}	unidirectional uptake transfer constants

LC	locus coeruleus
L-DOPA	3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAc	nucleus accumbens
nAChRs	nicotinic cholinergic receptors
N-CSE	nicotine containing cigarette smoke extract (Marlboro®)
NF-CSE	nicotine-free cigarette smoke extract (Quest®)
NKA	Na^+–K^+-ATPase
NO	nitric oxide
NOS	nitric oxide synthase
PA	cerebrovascular permeability-surface area products
PC12	pheochromocytoma
PD	Parkinson's disease
PG	propylene glycol
PVDF	polyvinylidene difluoride
Q	quantity of tracer in brain (dpm/g) at the end of perfusion
r^2	correlation coefficient
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SN	substantia nigra
SNc	substantia nigra pars compacta
SOD	superoxide dismutase
T	perfusion time (s)
TH	tyrosine hydroxylase

TMN	2,3,6-trimethyl-1,4-naphthoquinone
TTBS	Tween Tris buffer saline
Vo	"vascular volume" in ml/g at T = 0 s
VTA	ventral tegmental area
ZO-1	zonula occludens-1

ABSTRACT

Tobacco use is not only a major health concern worldwide but also a grotesque economic burden on the smoker as well as the health care system. The most well-known and most researched constituent of tobacco products is nicotine. There are a variety of products on the market that ensure nicotine intake, including cigarettes, cigars, pipe tobacco and smokeless tobacco.

Once absorbed by the body, nicotine undergoes phase I metabolism by cytochrome P450 (CYP) 2A6 (humans) or CYP2B1 (rat) to cotinine, the major metabolite. Since nicotine is a blood flow marker, its transport across the blood-brain barrier (BBB) has been well documented. However, data on the BBB penetration of nicotine and cotinine in animals subject to chronic nicotine exposure are limited. This gap in literature was identified and subsequently the focus of our first objective. Our data indicate that neither nicotine or cotinine uptake by the BBB is altered after chronic nicotine exposure in rat.

Nicotine exerts its effect by binding to nicotinic cholinergic receptors (nAChRs) on dopaminergic neurons in the striatum and the ventral tegmental area (VTA). The addictive property of nicotine is attributed to its effects on the mesocorticolimbic system, which serves a fundamental role in the acquisition of behaviors. Smoking not only plays a role in addiction but also in Parkinson's disease (PD), where epidemiological studies have shown that smokers have a lower incidence of PD as opposed to non-smokers. Dopamine (DA) is one of the major neurotransmitters that plays a critical role in addiction and PD. Centrally, the biosynthesis of DA occurs intraneuronally through the rate-limiting enzyme, tyrosine hydroxylase (TH). DA undergoes metabolism by monoamine oxidase (MAO) intraneuronally. DA, that is not metabolized by MAO, is subsequently transported into the storage vesicles. After stimulation of nAChRs, DA is released into the synaptic cleft after membrane depolarization. Released DA stimulates post-synaptic dopaminergic receptors, is metabolized by catecholamine-O-methyl-transferase or transporter back into the pre-synaptic neuron by DA transporter (DAT).

Little is known about the effects of whole cigarette smoke on the dopaminergic system. Therefore, our second objective of this study was to determine the effect of whole cigarette smoke extract (nicotine-containing and nicotine-free smoke extract), nicotine and cotinine on TH and DAT expression in undifferentiated pheochromocytoma cells. Our third objective was closely developed from our second. After investigating the effect *in vitro*, we determined the effect *in vivo* in rats after 28 day exposure of whole cigarette smoke extract (nicotine-containing and nicotine-free smoke

extract), nicotine and cotinine on TH and DAT regulation. Both the *in vitro* and *in vivo* TH as well as the *in vivo* DAT regulation data implicated nicotine to be responsible for TH and DAT upregulation.

It is known that nicotine releases DA from rat striatal synaptosomes.[1] We therefore aimed to determine whether a component of tobacco leaf extracts which is a MAO-A and MAO-B inhibitor, 2,3,6-trimethyl-1,4-naphthoquinone (TMN) release DA from rat striatal synaptosomes. We found that TMN releases DA from synaptosomes, to a greater extent when compared to nicotine.

Our data conclude that cotinine does cross the BBB and that both nicotine and cotinine transport do not vary after chronic nicotine exposure. We also found that nicotine, as the major constituent of tobacco smoke, is responsible for increased DA synthesis and DA transport back into the presynaptic neuron. TMN, is not only a MAO-A and MAO-B inhibitor but experiments from our laboratory indicate that in striatal synaptosomes, TMN releases DA to a greater extent than nicotine.

References

1. Sakurai, Y., Takano, Y., Kohjimoto, Y. & others. 1982. Enhancement of [3H]dopamine release and its [3H]metabolites in rat striatum by nicotinic drugs. *Brain Res*, 242: 99 - 106.

UITTREKSEL

Sigaretrook is nie net 'n gesondheidsrisiko nie, maar ook 'n finansiële las op beide die roker en die mediese skemas. Nikotien is die bekendste en mees nagevorste verbinding wat in rook voorkom. Daar is verskeie produkte op die mark wat nikotien inname verseker, insluitend sigarette, sigare, pyp tabak en rookvrye tabak.

Nikotien ondergaan fase I metabolisme deur sitochroom P450 (CYP) 2A6 (mens) of CYP2B1 (rot) om kotinien, nikotien se hoofmetaboliet, te vorm nadat nikotien geabsorbeer is deur die liggaam. Omrede nikotien 'n bloedvloeimerker is, is die transport van nikotien oor die bloedbreinskans (BBS) goed gedefinieer in die literatuur. Daarenteen is inligting beperk oor die transport van nikotien en kotinien oor die BBS by diere wat blootgestel is aan chroniese nikotien. Die leemte in die literatuur is geïdentifiseer en was gevolglik ons eerste doel van die studie. Ons data toon dat nie kotinien of nikotien se transport oor die BBS geaffekteer word deur chroniese nikotien blootstelling nie.

Nikotien oefen sy effekte uit deur binding met nikotieniese cholinergiese reseptore (nAChRs) op dopaminergiese neurone in die striatum en ventrale tegmentale area (VTA). Die verslawende eienskap van nikotien is as gevolg van nikotien se effekte op die mesokortikolimbiese sisteem, wat 'n fundamentele rol speel in die uitdrukking van gedrag. Sigaretrook speel nie net 'n rol by verslawing nie, maar ook by Parkinson se siekte (PD) waar epidemiologiese studies getoon het dat rokers 'n laer insidensie van PD het vergelyke met nie-rokers. Dopamien (DA), 'n belangrike neurotransmitter, speel 'n kritiese rol by verslawing en PD. Sentraal vind DA biosintese interneuronaal plaas deur die snelheidsbepalende ensiem, tirosien hidroksilase (TH). DA ondergaan interneuronale metabolisme deur die inwerking van monoamien oksidase (MAO). Onafgebreekte DA word getransporteer na stoorvesikels. DA word vrygestel in die sinapspleet na stimulasie van nAChRs en membraan depolarisasie. Vrygestelde DA stimuleer postsinaptiese dopaminergiese reseptore, word gemetaboliseer deur catechol-O-metieltransferase, of word terug getransporteer in die presinaptiese neuron in deur DA transporter (DAT).

Min is bekend oor die effekte van heel sigaretrook op die dopaminergiese sisteem. Dus, ons tweede doel van die studie was om die invloed van sigaretrook (nikotienbevattende en nikotienvrye sigaretrook ekstrak), nikotien en kotinien op TH regulasie in ongedefinieerde feochromositoom selle te bepaal. Ons derde doel het uit die tweede doel voortgevloei. Na die ondersoek van die effek *in vitro*, het ons die effek *in vivo* in rotte bepaal wat vir 28 dae blootgestel is aan sigaretrook (nikotienbevattende en nikotienvrye sigaretrook ekstrak), nikotien en kotinien op TH en DAT

regulasie. Beide die *in vitro* en *in vivo* TH asook die *in vivo* DAT regulasie data dui daarop dat nikotien verantwoordelik is vir die opregulering van TH en DAT.

Dit is bekend dat nikotien DA vrystel uit rot striatale sinaptosome.[1] Ons het gevolglik bepaal of 'n komponent van tabakblaarekstrak wat 'n MAO-A en MAO-B inhibeerder is, 2,3,6-trimetiel-1,4-naftakinoon (TMN), DA vrystel uit rot striatale sinaptosome. Ons het bevind dat TMN DA in 'n groter mate as nikotien uit sinaptosome vrystel.

Ons data toon dat kotinien wel die BBB kruis en dat nòg nikotien, nòg kotinien transport oor die BBB verander na chroniese nikotien blootstelling. Addisioneel het ons gevind dat nikotien, as die hoofverbinding in sigaretrook, verantwoordelik is vir die toename in DA sintese en DA transport terug in die presinaptiese neuron in. Voorts, TMN is nie alleen 'n MAO-A en MAO-B inhibeerder nie, maar eksperimente van ons laboratorium toon aan dat TMN ook DA vrystel vanuit rot striatale sinaptosome.

Bibliografie

1. Sakurai, Y., Takano, Y., Kohjimoto, Y. & andere. 1982. Enhancement of [3H]dopamine release and its [3H]metabolites in rat striatum by nicotinic drugs. Brain Res, 242: 99 - 106.

CHAPTER 1:

INTRODUCTION

Cigarette smoking is of great importance from a societal and medico-ethical perspective, since it has been described as the leading preventable cause of morbidity and mortality in developed countries.[1] Approximately a quarter of all adult Americans smoke cigarettes.[2] Cigarette smoking is the major cause of a variety of cancers as well as cardiac, vascular and pulmonary diseases. Regardless of these health risks, there is little doubt that a majority of people, who smoke cigarettes, do so in order to experience the psychopharmacological properties of this habit.

The first epidemiological study, published by Nefzger *et al.* in 1968, stated that smokers are less likely to develop Parkinson's disease (PD) as oppose to non-smokers.[3] Since 1968, four other studies have documented a lower incidence of PD among smokers where the latest article was published in 1982.[4-7] Characterized by selective dopaminergic neuronal loss in the substantia nigra, PD is a debilitating disorder where patients experience symptoms that include muscular rigidity, tremor and bradykinesia.

The discovery of the inverse correlation between cigarette smoking and PD, created an explosion of research in this field in order to elucidate the cause of such a significant finding. Certain aspects important to neurodegeneration came to the foreground, where significant progress has been made during the past 5 years.[8]

PD and cigarette smoke addiction both involve dopaminergic pathways. In PD the nigrostriatal pathway plays an important role in the control of motor activity as opposed to addiction, where the mesocorticolimbic system is associated with reward and pleasure centers.

Our contribution to this field of research focuses on the effect of cigarette smoke and cigarette smoke components, on biomarkers of the dopaminergic pathway. This research will help to elucidate the complex relationship between cigarette smoke, addiction and PD.

1.1. References

1. Peto, R., Lopez, A.D., Boreham, J. & others. 1992. Mortality from tobacco in developed countries: indirect estimation from national vital statistics. *Lancet*, 339: 1268 - 1278.

2. Cent.Dis.Control Prev. 1999. Cigarette smoking among adults. *Morbid Mortal Wkly*, 48: 993 - 996.

3. Nefzger, M.D., Quadfasel, F.A. & Karl, V.C. 1968. A retrospective study of smoking in Parkinson's disease. *Am J Epidemiol*, 88: 149 - 158.

4. Baumann, R.J., Jameson, H.D., McKean, H.E. & others. 1980. Cigarette smoking and Parkinson disease: 1. Comparison of cases with matched neighbors. *Neurology*, 30: 839 - 843.

5. Godwin-Austen, R.B., Lee, P.N., Marmot, M.G. & others. 1982. Smoking and Parkinson's disease. *J Neurol Neurosurg Psychiatry*, 45: 577 - 581.

6. Kessler, I.I. 1972. Epidemiologic studies of Parkinson's disease. 3. A community-based survey. *Am J Epidemiol*, 96: 242 - 254.

7. Kessler, I.I. & Diamond, E.L. 1971. Epidemiologic studies of Parkinson's disease. I. Smoking and Parkinson's disease: a survey and explanatory hypothesis. *Am J Epidemiol*, 94: 16 - 25.

8. Quik, M. 2004. Smoking, nicotine and Parkinson's disease. *Trends Neurosci*, 27: 561 - 568.

CHAPTER 2:

LITERATURE REVIEW

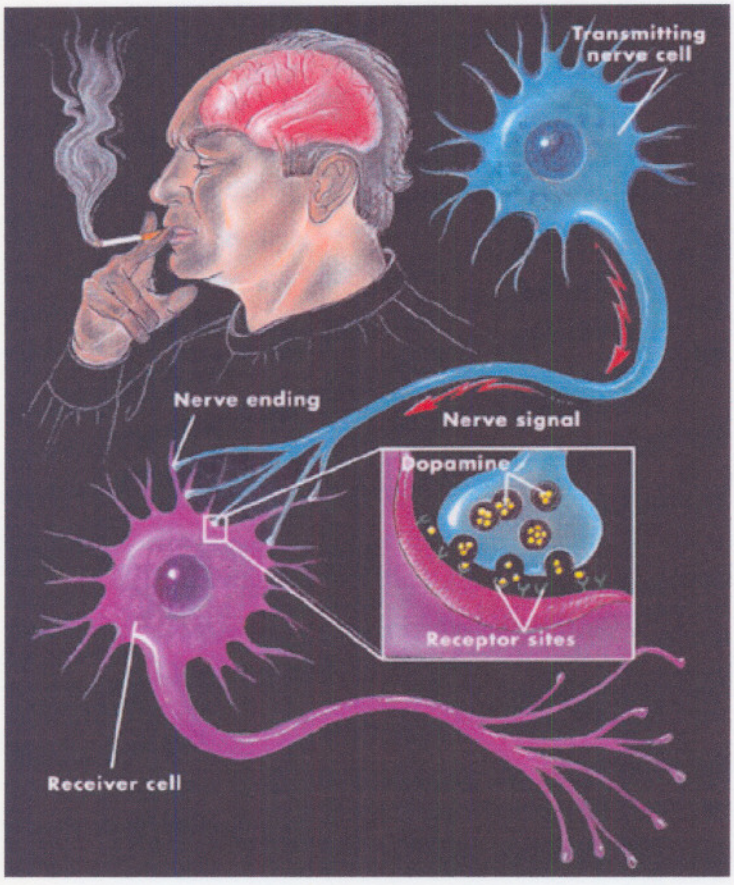

Illustration by Kibiuk, 1998.[1]

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LIST OF ABBREVIATIONS

AChRs	cholinergic receptors
BBB	blood-brain barrier
BH_4	6R-L-erythro-tetrahydrobiopterin
CDC	Centers for Disease Control and Prevention
COMT	catechol-O-methyl transferase
CYP	cytochrome P450
DA	dopamine
DAT	dopamine transporter
DOPAC	3,4-dihydroxy-phenyl-acetic acid
GABA	gamma-aminobutyric acid
GSH	glutathione
GSSG	glutathione disulfide

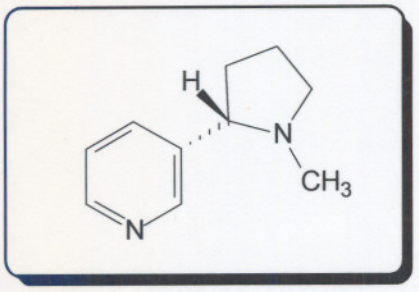
H_2O_2	hydrogen peroxide
LC	locus coeruleus
L-DOPA	3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAc	nucleus accumbens
nAChRs	nicotinic cholinergic receptors
NKA	Na^+–K^+-ATPase
NO	nitric oxide
NOS	nitric oxide synthase
PD	Parkinson's disease
SN	substantia nigra
SNc	substantia nigra pars compacta
SOD	superoxide dismutase
TH	tyrosine hydroxylase
TMN	2,3,6-trimethyl-1,4-naphthoquinone
VTA	ventral tegmental area
ZO-1	zonula occludens-1

According to the Centers for Disease Control and Prevention (CDC), tobacco use remains the leading preventable cause of death in the United States of America, causing almost 450,000 deaths each year and more than $75 billion in direct medical costs.[2] However, world-wide, almost half of the male population (47%) continue to smoke.[3] Every day, about 4,000 teenagers under the age of 18 try their first cigarette. This might start out as an innocent venture for acceptance among peers, but 80% of these teenagers will subsequently become addicted to cigarettes and continue to smoke into adulthood.[2]

There are more than 4,000 chemicals in tobacco products such as cigarettes, cigars and pipe tobacco, of which nicotine is the best known and the most researched.[4] Nicotine (figure 2.1) is recognized as one of the most frequently used addictive drugs.[5] A report released by United

States Surgeon General C. Everett Koop on May 16, 1988 stated that the addictive properties of nicotine are similar to those of cocaine and heroin.[6]

A



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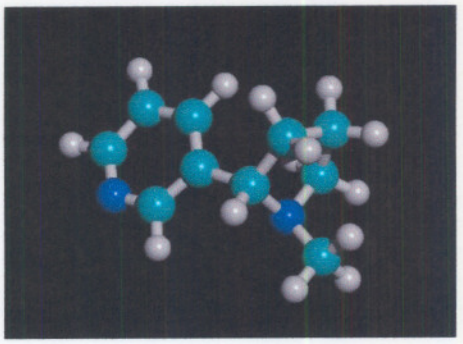

Figure 2.1: **A.** Molecular structure of (*S*)-nicotine.

B. Ball and stick representation of (*S*)-nicotine generated by Hyperchem version 7.[7]

2.1. Metabolism of nicotine

Nicotine, a naturally occurring alkaloid, was first isolated and determined to be the major constituent of tobacco in 1828.[8] Nicotine absorption can occur through the oral cavity, skin, lung and gastrointestinal tract.[9] For this reason, a variety of tobacco products, including cigarettes, cigars, pipe tobacco and smokeless tobacco such as snuff and chewing tobacco,[10] exist on the market that are used by tobacco users to provide them with nicotine intake. Tobacco product users resort to nicotine replacement therapies such as nicotine containing gum and transdermal nicotine patches to aid them in the cessation of tobacco use.[11]

Once absorbed, nicotine is extensively metabolized by the liver to a number of major and minor metabolites.[12] Metabolism of nicotine and its metabolites in living organisms involve phase I (microsomal oxidation) and phase II (N-glucuronidation and O-glucuronidation) metabolism.[13] Nicotine is hydroxylated at the 5' position to an unstable intermediate, 5'-hydroxynicotine which exists in equilibrium with the $\Delta^{1'(5')}$iminium ion (figure 2.2).[14] 5'-Hydroxylation of nicotine is catalyzed by cytochrome P450 2A6 (CYP2A6) in humans.[15-17] Nicotine is not only metabolized by CYP2A6 but it has been shown that long-term nicotine administration also down-regulates hepatic CYP2A6, causing inhibition of its own metabolism.[18-20]

CYP2B6 contributes minimally to the metabolism of nicotine since the alkaloid has lower affinity for this enzyme and CYP2B6 has variable expression in human liver.[21] However, it has been shown that polymorphic variation in CYP2B6 affects smoking cessation rates.[22] CYP2B6 has been detected in the human brain,[23] and has been shown to be induced by nicotine.[22] The rat homologue of CYP2B6 is CYP2B1 which has been identified in rat brain and neuronal tissue. In rats, hepatic CYP2A enzymes do not metabolize nicotine as in humans, instead CYP2B1 is the major CYP responsible for the metabolism of nicotine to its major metabolite, cotinine by a similar pathway,[24,25] although to a lesser extent.[26]

CYP2D6 has been shown to be involved in the metabolism of other drugs of abuse, including the formation of morphine from codeine and ethylmorphine, among others.[27-29] However, there remains controversy concerning the importance of CYP2D6 in nicotine metabolism.[15] Unlike CYP2D6 that has been reported to be present in human brain, there is no evidence of the presence of CYP2A6 in human brain.[29,30] Thus far, CYP2A6 has only been documented to exist peripherally in human liver, nasal mucosa and lung.[31-39] Rat CYP2A3, the ortholog of human CYP2A6, is expressed in the olfactory mucosa[34] and lung and to a lesser extent in the breast and esophagus, but not in the liver.[34,40-42] To date, there is no literature on the expression of CYP2A3 in rat brain, as with its ortholog, CYP2A6 in humans.

Controversy exists surrounding which compound undergoes aldehyde oxidation to form cotinine, $\Delta^{1'(5')}$iminium ion or 5'-hydroxynicotine.[13,14,43,44] Nevertheless, in most people nicotine is 70 – 80% metabolized to cotinine through 5'-hydrolyation.[13,17,45,46] Nicotine and cotinine forms quaternary N-glucuronides, whereas trans-3'-hydroxycotinine forms an O-glucuronide (figure 2.2).[43] Figure 2.3 indicates the commonly accepted pattern of nicotine metabolism and urinary recovery based on a study in individuals receiving nicotine at steady state through transdermal nicotine patches.

As an indicator of direct or passive exposure to cigarette smoke, cotinine levels are measured in urine, blood and saliva.[47-53] There are, however, variations in cotinine levels among smokers who smoke the same number of cigarettes.[54] This interindividual variation in the metabolism of xenobiotics, such as nicotine, can be attributed to polymorphism of the genes that encode metabolic enzymes.[55] Among the enzymes that are induced by smoking and involved in cotinine production, CYP1A1, CYP2A6, CYP2D6 and CYP2E1 are known to be genetically polymorphic.[56-60] In addition, life style factors, e.g. alcohol drinking and coffee or tea consumption may induce or inhibit expression of these enzymes and affect nicotine metabolism.[61]

It has been suggested that racial differences may contribute to varying patterns of cigarette smoking.[62-64] Race may therefore be partially accountable for variations in cotinine levels and

elimination. After controlling for the number of cigarettes smoked per day, nicotine content of cigarette and frequency of inhalation patterns, it was found that African-American smokers have higher cotinine levels when compared to Caucasians.[65]

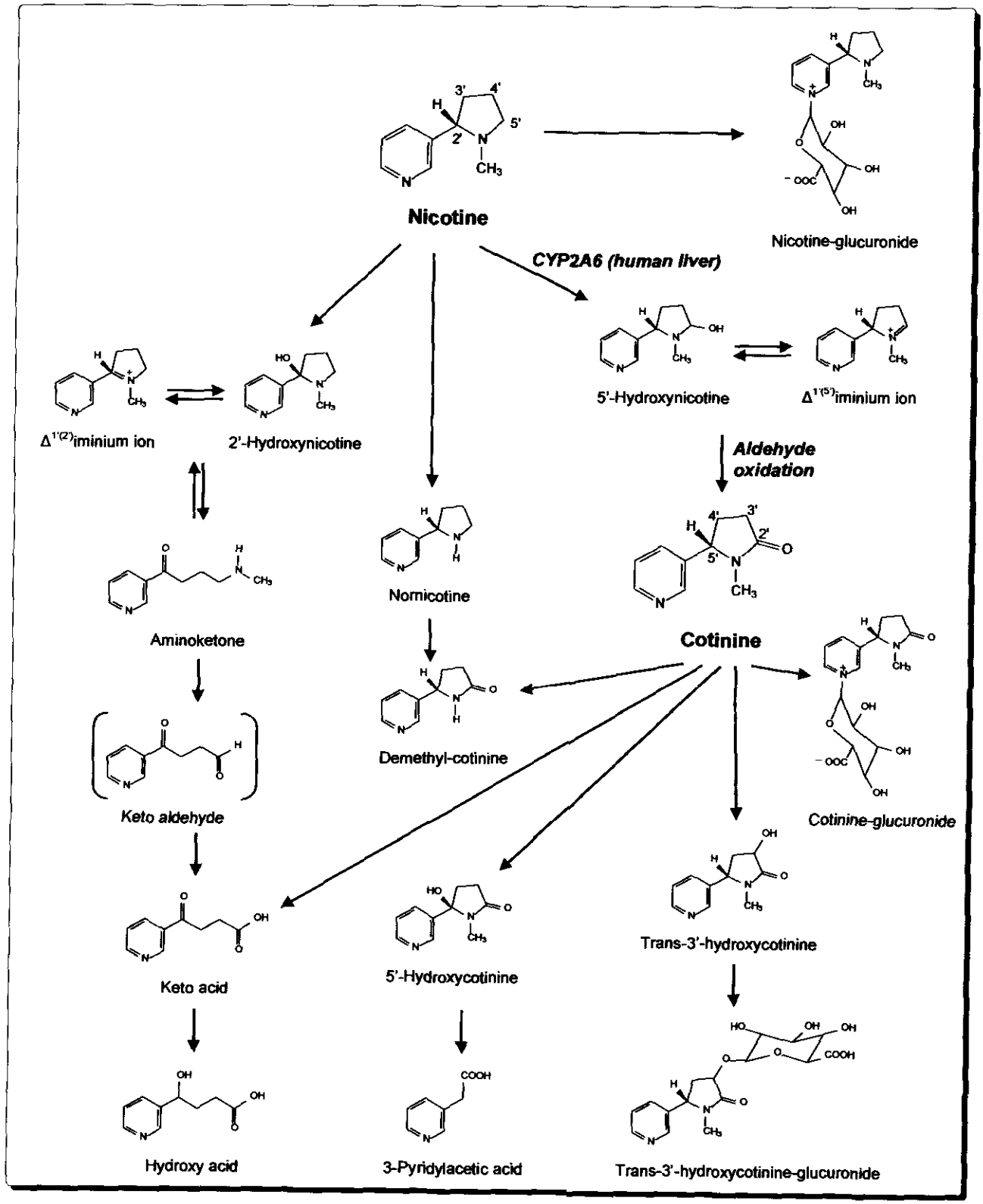

Figure 2.2: Pathways of nicotine metabolism initiated by 5'-hydroxylation and 2'-hydroxylation in humans. [Modified from Benowitz, *et al.* (1994); Hecht, *et al.* (2000); Yildiz (2004).[13,14,17]]

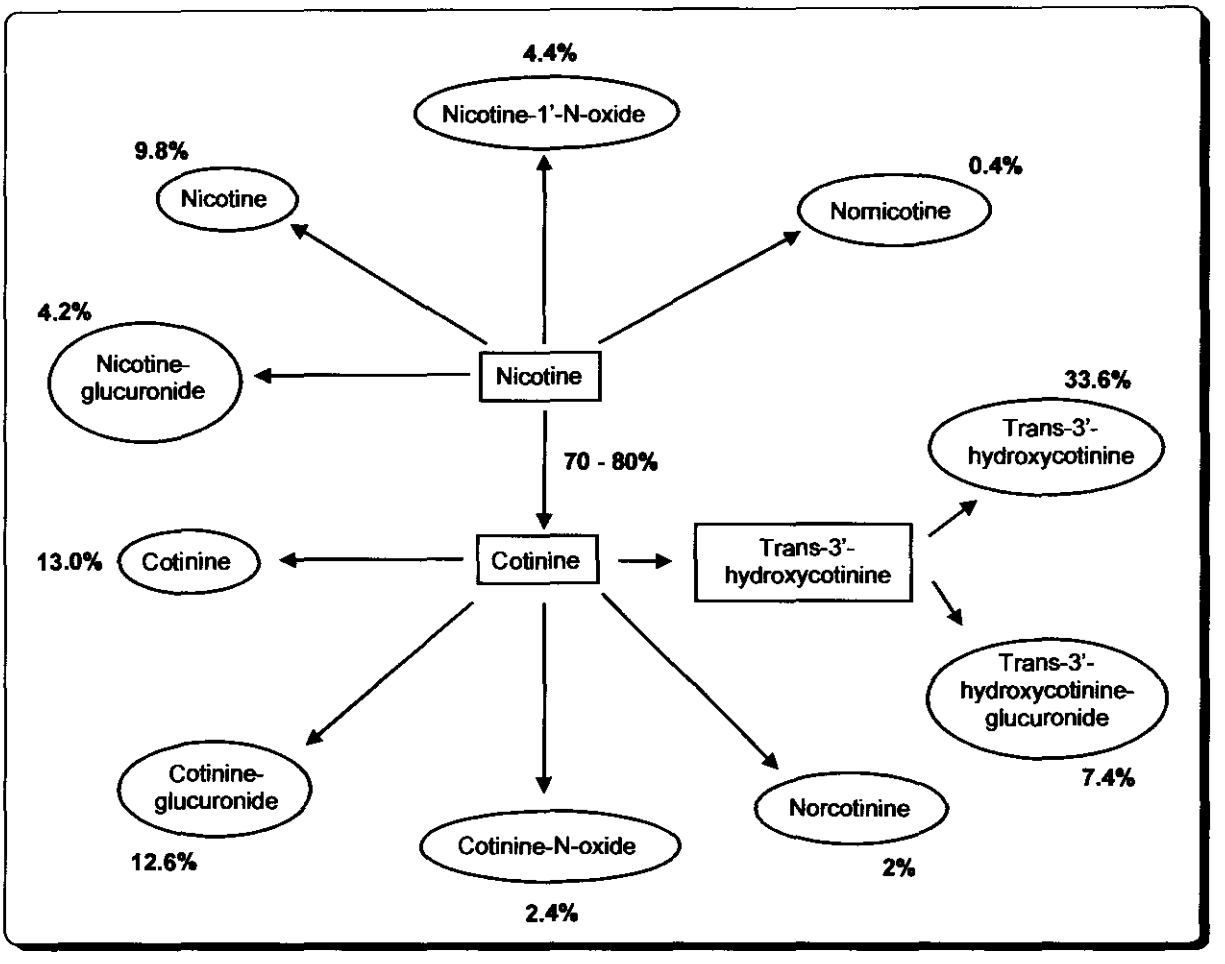

Figure 2.3: Quantitative scheme of nicotine metabolism, based on average excretion of metabolites as percent of systemic dose during transdermal nicotine application. The circled compounds indicate compounds excreted in urine. The estimate of norcotinine excretion is based on data of Byrd *et al.* (1992).[45] [Modified from Benowitz, *et al.* (1994).[17]]

2.2. Transport of nicotine and cotinine across blood-brain barrier

The blood-brain barrier (BBB) serves as a diffusion barrier which is essential for the normal functioning of the central nervous system.[66] The BBB is created by the tight apposition of endothelial cells lining blood vessels in the brain, forming a barrier between the circulation and the brain parenchyma (e.g. astrocytes, microglia)[67] (figure 2.4). General movement restrictions at the BBB are limited by endothelium connected by tight junctions (zonula occludens),[68] the absence of paracellular openings, a lack of pinocytosis activity, enzymatic restrictions and significant protein-mediated efflux.[69,70]

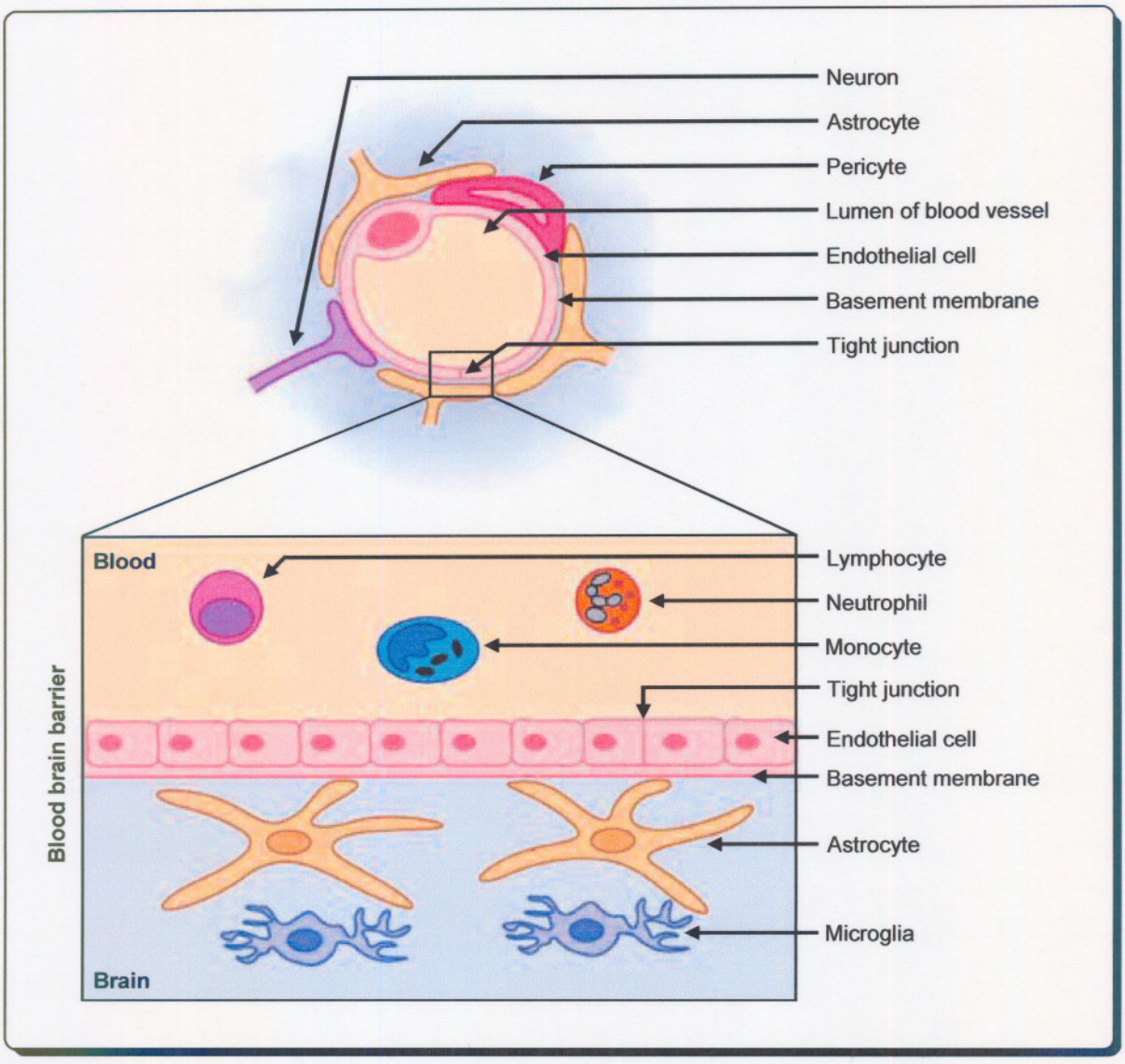

Figure 2.4: A schematic representation of the blood-brain barrier (BBB). A thin basement membrane, comprising of lamin, fibronectin and other proteins, surrounds the endothelial cells and associated pericytes and provides both mechanical support and a barrier function. [Modified from Francis *et al.* (2003).[67]]

Cigarette smoking delivers nicotine to the brain with drug levels peaking within 10 seconds after inhalation.[4] The rapid blood-brain transfer of nicotine in naive animals has been well documented due to the fact that it is a well-known cerebral blood flow marker.[71-74] However, limited data are present in the literature regarding BBB penetration of nicotine in animals subject to chronic nicotine exposure. Such studies are of significant importance because it has been reported that long-term

nicotine exposure changes both BBB function and morphology. Figure 2.5 represents a summary of the outcomes of nicotine's effects on the cerebral microcirculation.

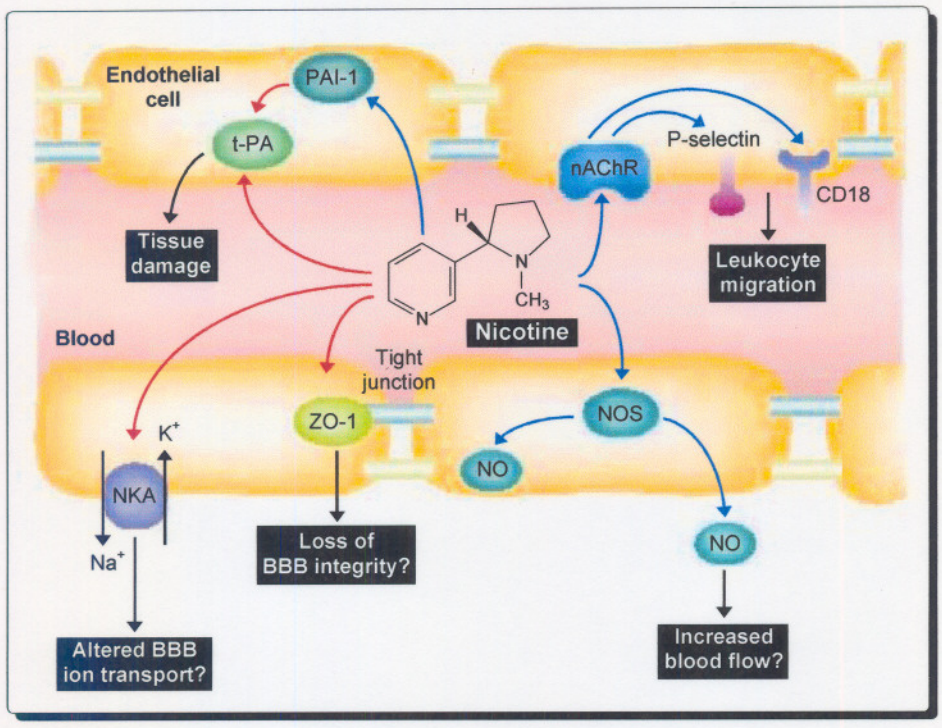

Figure 2.5: Effects of nicotine on the cerebral microcirculation. Nicotine might have adverse effects on the integrity and function of the BBB and interfere with the regulation of blood flow. Nicotine also affects the mediators of thrombosis [e.g. plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA)] and leukocyte migration (e.g. P-selectin and CD18). Blue arrows indicate stimulation or upregulation; red arrows indicate inhibition, downregulation or depletion. (NKA, Na^+-K^+-ATPase; ZO-1, zonula occludens-1). [Modified from Hawkins *et al.* (2002).[75]]

Specifically, nicotine has been shown to:

1. increase BBB endothelium microvilli formation,[76]
2. decrease *in vitro* zonula occludens-1 (ZO-1) expression.[77] ZO-1 is an important protein underlying the tight junctions in human and rat epithelial and endothelial cells.[78,79] ZO-1 plays a critical role in maintaining cell polarity as well as coupling the extracellular environment to intracellular signaling pathways and cytoskeleton,[78]

3. diminish levels or function of BBB nicotinic cholinergic receptors (nAChRs)[77] (refer to section 2.4.1), Na,K,2Cl-cotransporters,[80] and α2 Na,K-ATPase.[81] Both the Na,K,2Cl-cotransporter and Na,K-ATPase play significant roles in maintaining brain extracellular K^+ levels. In order to maintain proper neuronal conduction, it is of critical importance that brain extracellular K^+ concentrations are maintained efficiently constant and low in order to maintain the conduction of action potentials.[80]

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.[82,83] Cotinine has been detected in brain after nicotine exposure[83-85] but indirect data suggest that the presence of cotinine in the CNS may be the result of central nicotine metabolism by CYP2B1 in rats.[86]

2.3. Addiction and Parkinson's disease

The striatum, a key element of the basal ganglia, is divided into the dorsal and ventral striatum. In primates, the dorsal striatum comprises the caudate and putamen separated by the internal capsule, while in rodents, the dorsal striatum is either divided into medial and lateral parts or treated as one region. The ventral striatum includes the striatal part of the olfactory tubercle and the nucleus accumbens (NAc). The NAc is further divided into the medioventral shell and the dorsolateral core[87] (figure 2.6).

The striatum is densely innervated by **dopaminergic** fibers that originate in the substantia nigra (SN) and ventral tegmental area (VTA) and also receives excitatory input from the cortical and limbic regions. Two main pathways connect the striatum with the dopaminergic system. The **nigrostriatal** system, which plays an important role in control of motor activity, connects the SN pars compacta (SNc) and the striatum (dorsal and core of the NAc).[87] The nigrostriatal system plays a pivotal role in Parkinson's disease (PD). The second system is the **mesocorticolimbic** system, associated with reward and pleasure centers, where dopaminergic neurons originate in the VTA and project mainly to the shell of the NAc.[87,88]

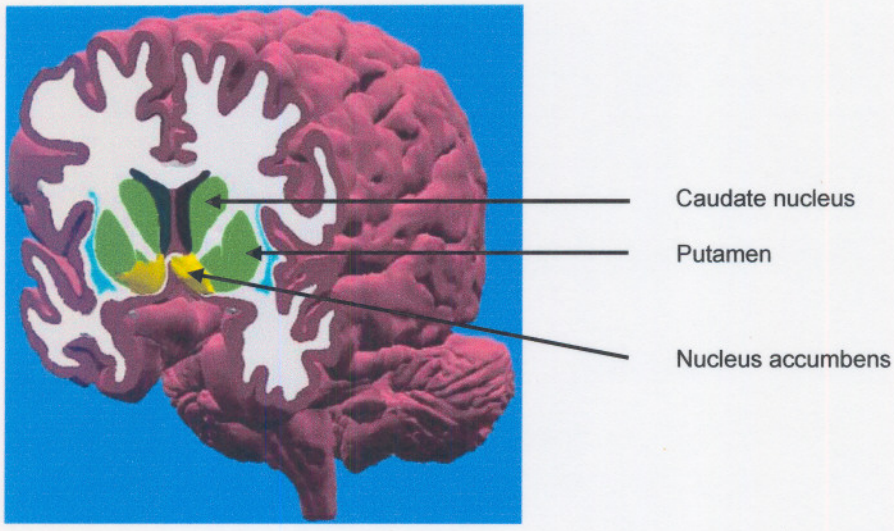

Figure 2.6 : An illustration of a coronal section of the brain indicating the position of the dorsal striatum (caudate nucleus and putamen) and the ventral striatum (NAc).[89]

Under normal circumstances the mesocorticolimbic system is crucial for the rewarding and reinforcing effects of positive natural stimuli associated with survival, including food and reproductive opportunities.[88] However, drugs can stimulate the reward circuitry with a strength, time course and reliability that exceeds almost any natural stimulus, powerfully consolidating responses to drug-associated stimuli.[90] Not only has the ventral striatum been implicated in the reward pathway, but the dorsal striatum has also been linked to reward-related activity.[91] In humans, dorsal striatum activation has been observed with reinforcers such as cocaine[92] and nicotine.[93] Drug-induced synaptic plasticity in the NAc and dorsal striatum therefore contribute to addiction by consolidating drug-wanting, drug-seeking and drug-taking behaviors.[88,91] Nevertheless, dopaminergic projections to the striatum play a critical role in the reinforcing properties of psychostimulants and possibly other drugs of abuse.[88,94]

After nicotine uptake into the brain, nicotine binds to the nigrostriatal and mesolimbic dopaminergic neurons at the terminal (presynaptic) nAChRs.[5,95] Most relevant to nicotine addiction is the nAChRs in the VTA which express for α4β2 and α3β2 subunits, as opposed to the dopaminergic terminal receptors [α4α5β2, α4α6β2(β3) and α6β2(β3)] found in rat striata, that may be important in PD.[96,97] For extensive literature on nAChRs see references [98-101]. Of the various neurotransmitters, dopamine (DA) plays a pivotal role in both cigarette smoke addiction and PD (for review of the effect of nicotine on brain neurotransmitters see reference [102]) Although various areas of the brain

take part, the mesocorticolimbic DA system plays a crucial and fundamental role in the acquirement of behaviors that are reinforced by addictive drugs, including nicotine.[103-105] For a review on reward pathways see reference [106]. Nevertheless, plasticity occurs particularly in the mesocorticolimbic DA system during the development of drug addiction.[107-110] In the case of nicotine addiction, the nAChRs are stimulated by nicotine in the VTA which evoke DA release in the NAc[95,105] (figure 2.7).

However, in the face of increased DA release in the VTA after a single dose of nicotine for more than one hour *in vivo,* nAChRs on dopaminergic neurons rapidly desensitize. This can be explained by data obtained by Mansvelder *et al.* (2002).[111] It is known that glutamate stimulation of dopaminergic neurons in the VTA results in increased activity of those neurons whereas gamma-aminobutyric acid (GABA) has the reverse effect of slowing down dopaminergic neuron activity and thus DA release.[112] Mansvelder *et al.* (2002) found that in glutamate producing cells, a single dose of nicotine induces long-term potentiation which promotes high-level activity for an extended time.[113] This occurs after an initial increase in GABA transmission which lasts a few minutes, GABA transmission decrease and do not recover fully for more than an hour after nicotine exposure.[111]

Nevertheless, released DA in the synaptic cleft binds to the postsynaptic dopaminergic receptors, is metabolized by catechol-O-methyl transferase (COMT), transported back into the presynaptic neuron by DA transporter (DAT) or is removed from the extracellular space by diffusion.[114]

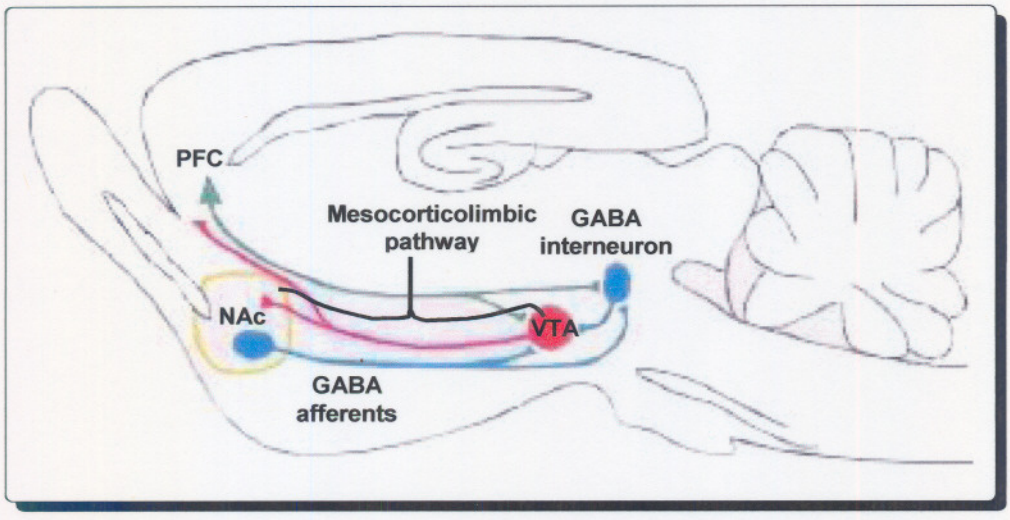

Figure 2.7: A simplified schematic representation of the VTA and afferent projections. Inhibitory GABAergic innervation of VTA DA neurons originates from the NAc and local interneurons. The most important structures and reward pathway is indicated in red. A reward stimulus activates the pathway where information travels from the VTA to the NAc. [Modified from Mansvelder *et al.* (2002).[111]]

Approximately 1% of Americans over the age of 50 are affected by PD.[115] Muscular rigidity, tremor and bradykinesia are the trademark symptoms of patients suffering from PD.[95] The cause of PD is largely primary (idiopathic) but can also be secondary to cerebral viral infections, exposure to manganese, carbon monoxide, organophosphates or the pethidine analog 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).[116] This neurodegenerative disorder is characterized pathologically by the selective loss of dopaminergic neurons in the SN of the midbrain resulting in low levels of DA in the striatum to which these neurons project.[117-119]

Of special interest to our group is the significant finding that smokers are less likely to develop PD as oppose to non-smokers.[120-124] A few proposals exist to explain the inverse relationship between cigarette smoking and PD.

One such proposal is that the "protective" action of cigarette smoking may be associated with the inhibition of monoamine oxidase (MAO), the mitochondrial membrane enzyme involved in the degradation of DA to 3,4-dihydroxy-phenyl-acetic acid (DOPAC) (for review articles on MAO see references [125,126]). It has been shown that cigarette smokers have lower blood platelet and peripheral organ MAO-B activities and lowered brain MAO-A and MAO-B activities when compared to non-smokers.[127-133] Inhibition of MAO will not only prevent the metabolism of DA, but it can also reduce oxidative stress which has been linked to neurodegeneration as in PD.[134] Hydrogen peroxide (H_2O_2) is produced when DA is metabolized by MAO which causes increased levels of H_2O_2 in the SN.[135] Through a ferrous iron-mediated catalysis known as the Fenton reaction, H_2O_2 gives rise to free radicals which causes neuronal injury and death[118] (figure 2.8). H_2O_2 is also subject to the Haber-Weiss reaction which leads to the formation of free radicals (figure 2.8).[136,137] Therefore, through inhibition of MAO, cigarette smoke may reduce free radical formation in the brain.

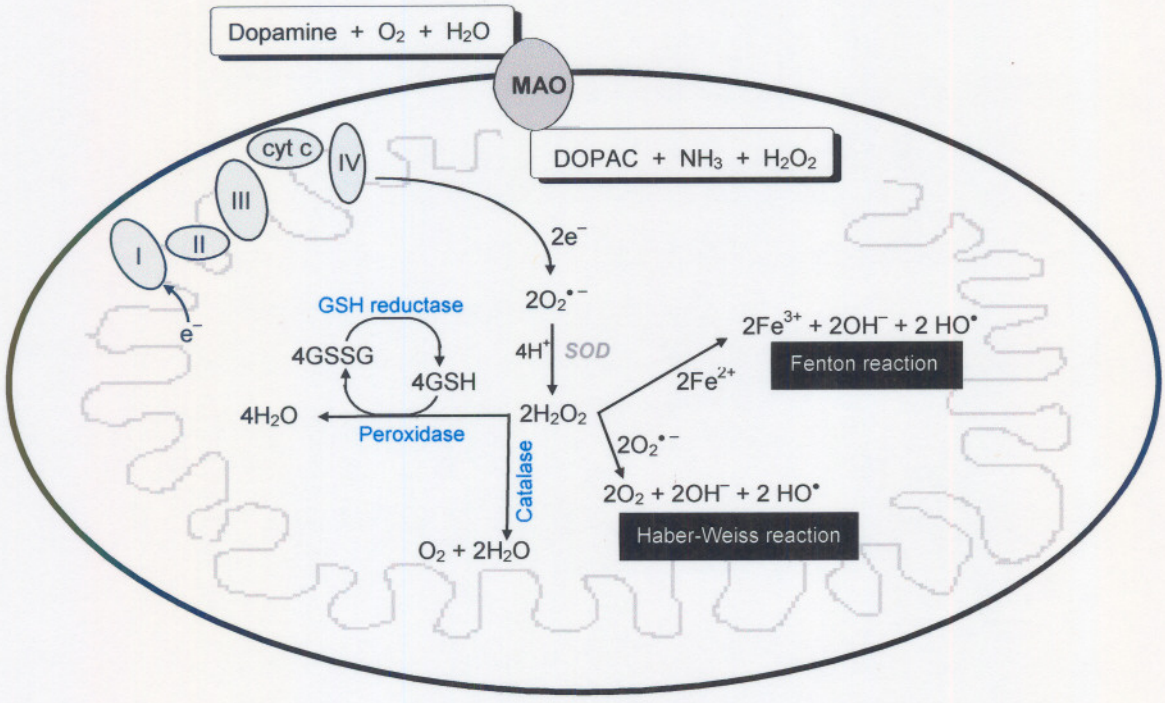

Figure 2.8: Simplistic representation of the Fenton reaction and the Haber-Weiss reaction.[136] (DOPAC, 3,4-dihydroxy-phenyl-acetic acid; GSH, glutathione; GSSG, glutathione disulfide; SOD, superoxide dismutase.)

Attempts to elucidate which compound(s) is responsible for MAO inhibition found that neither (*S*)-nicotine nor 4-phenylpyridine or hydrazine are responsible for the lowered MAO activity.[138] One compound however, 2,3,6-trimethyl-1,4-naphthoquinone (TMN, figure 2.9), was isolated from tobacco leaves and proven to reversibly inhibit both MAO-A and MAO-B *in vitro*.[139,140]

A **B**

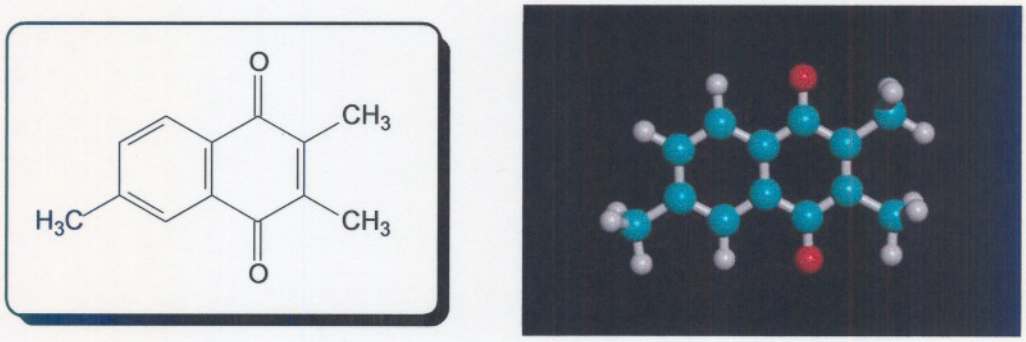

Figure 2.9: **A.** Molecular structure of TMN.

B. Ball and stick representation of TMN generated by Hyperchem version 7.[7]

Other proposals for the "protective" action of cigarette smoke focus on nicotine as the protective agent in cigarette smoke. Nicotine is believed to exert a neuroprotective role through the following mechanisms.

1. As mentioned above, oxidative stress is associated with neurodegenerative diseases, especially PD. Nicotine inhibits the Fenton reaction *in vitro*, probably by sequestration of Fe^{2+} and may therefore act as an antioxidant.[141-143]
2. Four effects have been shown to be mediated through nAChRs.[144]
    a. Nicotine pretreatment prevents glutamate-induced neurotoxicity in several neuronal cultures including striatal neurons.[145-150] *In vitro* nicotine has shown to attenuate Ca^{2+} overload which is triggered by glutamate. Nicotine also upregulates protein and mRNA expression of the anti-apoptotic molecule, bcl-2 and downregulates the pro-apoptotic factor bax.[151]
    b. Nicotine induces an increase in mRNA levels for both fibroblast growth factor and brain-derived neurotrophins in the striatum and ventral midbrain.[152,153] These growth factors have been shown to stimulate dopaminergic neuron survival *in vivo*.[152,154]
    c. Nicotine increases cerebral blood flow and increases cerebral glucose utilization in numerous brain regions in rat, including the SN.[155-159]
    d. PD, like most neurodegenerative diseases, is associated with chronic inflammation.[160,161] Activation of brain mononuclear phagocyte cells, called microglia, is a key step in the inflammation process. Under certain pathophysiological states microglia secrete various inflammatory factors[162] which can produce neuronal dysfunction and degeneration.[163,164] Nicotine inhibits the activation of microglia through α7 nAChRs.[165]

The treatment of PD, from a DA point of view, is focused on preventing DA metabolism through MAO inhibition, stimulating DA receptors (bromocriptine, pergolide, pramipexole and ropinirole) or increasing DA levels (L-DOPA co-administered with carbidopa).[117] To date only the MAO inhibitor, selegiline (deprenyl) has shown promise as a neuroprotective agent for PD.[118]

2.4. Effect of nicotine and cotinine on the dopaminergic system

There is little known about the effect of whole cigarette smoke on the dopaminergic system since the major focus of research has been on nicotine's effect (in isolation) on the dopaminergic system.

This section will cover, in brief, the reported effects of nicotine, in similar concentrations as obtained through cigarette smoke, as well as what is known surrounding the effect of cotinine on various targets in the dopaminergic system.[166-169]

2.4.1. Neuronal nicotinergic acetylcholine receptors

Little is known about the action of cotinine at neuronal structures. A recent study suggests that cotinine, a weak agonist for α7 nAChRs,[170] stimulates nAChRs to evoke DA release in a calcium-dependent manner from rat striatal slices.[171] However, no data are available of the effect of cotinine on the regulation of nAChRs.

Previous studies have indicated that chronic nicotine exposure causes an upregulation in nAChRs in humans, rats and mice.[167-169,172-176] This increase is specific to nicotinic AChRs, especially the α4β2 nAChR, but not the muscarinic AChRs.[177,178] However, chronic exposure to nicotine induces a rapid and long-lasting loss of nicotine-sensitive function of nAChRs in the brain which might explain the unexpected upregulation of nAChRs.[179] This process is distinct from classical desensitization observed with other agonists or psychostimulants such as cocaine, because of its slow reversibility.[172,179-182]

A simplistic hypothesis was put forward by Dani and Heinemann in 1996 for perpetuating nicotine use (figure 2.10). When smoking a cigarette, a small pulse of nicotine activates nAChRs that directly or indirectly induces DA release which provides a pleasurable feeling. With continued use, nicotine accumulates to a low steady-state concentration that causes significant nAChRs desensitization and over a period of time, longer-term inactivation.[104,183] As a result of decreased nAChRs turnover, nAChRs upregulate.[104]

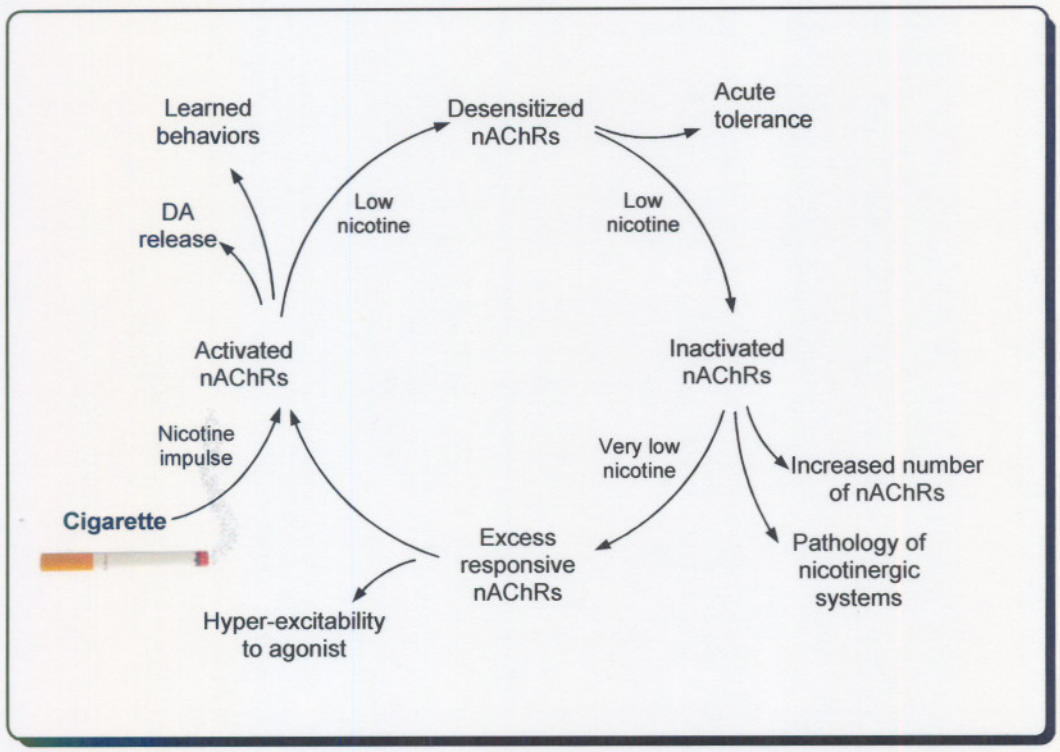

Figure 2.10: A hypothetical cycle proposed by Dani & Heinemann in 1996 for perpetuating nicotine use.[104]

2.4.2. Tyrosine hydroxylase, 6R-L-erythro-tetrahydrobiopterin and dopamine

Tyrosine hydroxylase (TH), a mixed-function monooxygenase, catalyzes the reaction from tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) in the biosynthesis pathway of the catecholamines; DA, norepinephrine and epinephrine (figure 2.11).[184,185]

It is therefore understandable that the regulation of the amount of TH enzyme and enzyme activity are the central means for controlling the biosynthesis of catecholamines. This has been shown in the adrenal gland, where nicotine not only triggers the release of catecholamines, but also promotes their biosynthesis by increasing the activity of TH.[186,187] However, in the brain, especially in the VTA, SN and the noradrenergic cell bodies of the locus coeruleus (LC), a much lower concentration of nicotine increases TH activity.[188,189] The increased TH activity is reflected by an increase in TH protein resulting from increased expression.[187,190,191] The increase in TH expression results in increased DA synthesis, which might be critical when correlating addiction and PD with the DA pathway (see reference [192] for additional information).[193] The effect of cotinine on TH activity and regulation has not been investigated.

6R-L-erythro-tetrahydrobiopterin (BH_4) is the common and natural cofactor for TH, phenylalanine hydroxylase, tryptophan hydroxylase and nitric oxide synthase (NOS).[194-199] BH_4 administration has proved to be a crucial entity in DA biosynthesis and release.[200,201] This finding is significant since patients with PD not only have reduced DA levels but also reduced BH_4 levels. However, the effect of cotinine on BH_4 activity and the effect of nicotine, cotinine and cigarette smoke on BH_4 biosynthesis have not been examined.

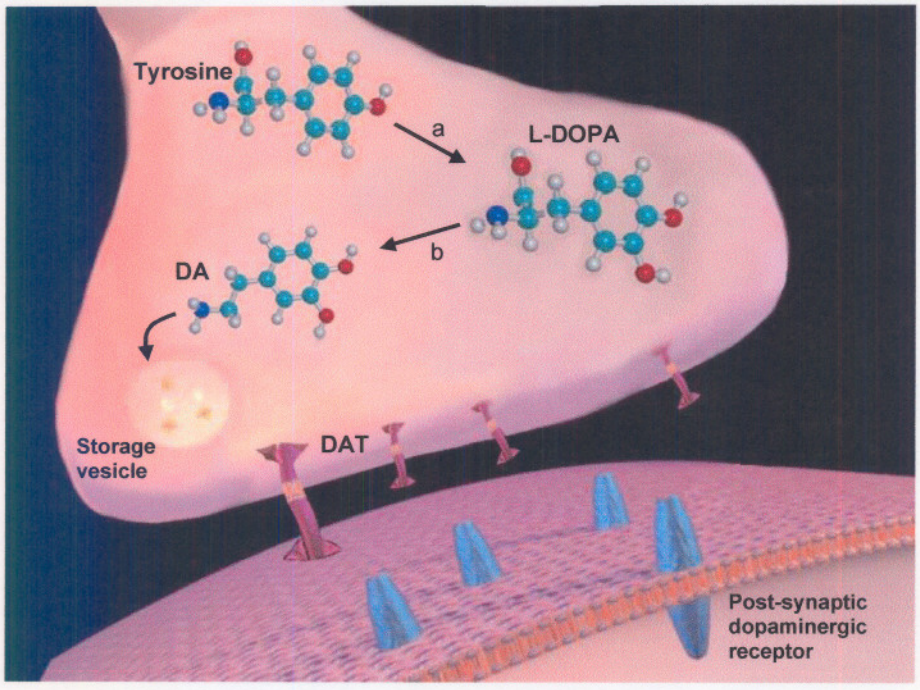

Figure 2.11: Biosynthesis pathway of DA. a) Tyrosine is converted to L-DOPA by TH with BH_4 and oxygen as its cofactors. This is the rate-limiting step in the biosynthetic pathway. b) L-DOPA is subsequently converted to DA by L-DOPA decarboxylase with pyridoxal phosphate (vitamin B6) as its cofactor. [Modified from National Institute on Drug Abuse. (2002).[202]]

2.4.3. Dopamine transporter

As part of the Na^+/Cl^--coupled neurotransmitter transporter family, which includes plasma membrane transporters for serotonin and norepinephrine, DAT terminates DA action in the synapse through rapid reuptake of DA into the presynaptic neuron.[203,204] DAT plays a key role in shaping neurotransmission mediated by the nigrostriatal and mesocorticolimbic DA pathways, since it is selectively expressed in dopaminergic neurons of the SN and the VTA.[205,206]

No data are available on the effect of cotinine on DAT expression or activity. On the other hand, nicotine has been reported to enhance DAT function and therefore to increase DA clearance in the NAc,[207] prefrontal cortex and striatum[114] of rats. This observation is not expected since DAT function is influenced by dopaminergic neuron membrane potential, where hyperpolarization increases the velocity of DA transport by DAT as opposed to depolarization which has the converse effect.[208] Stimulation of nAChRs by nicotine results in depolarization of the plasma membrane,[209] therefore, theoretically it would be expected that DAT function will be decreased with subsequent decreased DA clearance and increasing extracellular DA concentrations. However, the ability of nicotine to enhance DAT function seems to be nAChR mediated.[114] Li *et al.* demonstrated in June of 2004 that mRNA expression of DAT is upregulated with chronic nicotine and passive inhaled smoke in rat SN and VTA, including the dorsal part of the SNc.[210]

Moreover, DAT is not only a transporter of DA but also of 1-methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite of MPTP, which is known to cause parkinsonism in animals and humans by inhibiting complex I of the mitochondrial electron transfer chain.[211] However, there is no significant difference in DAT mRNA expression in surviving dopaminergic neurons of established PD patient brains compared to controls.[212]

2.5. Aims of this study

There is little known about the effect of whole cigarette smoke on the dopaminergic system since the major focus of research has been on nicotine's effect on the dopaminergic system as opposed to cigarette smoke itself. We therefore will be focusing on the following **hypotheses**:

1. **Cotinine is transported across the BBB. Nicotine as well as cotinine transport across the BBB differs from rats chronically treated with nicotine as compared to naive rats.** To test these hypotheses, our first aim was to determine brain uptake of cotinine. Our second aim was to determine cotinine and nicotine brain uptake in naive rats as well as rats treated chronically with nicotine. Both these aims were accomplished by utilizing our *in situ* brain perfusion model. Refer to chapter 3.
2. **(*S*)-Nicotine and nicotine containing smoke extract will upregulate TH and DAT *in vitro* but not (*S*)-cotinine, nicotine-free smoke extract or TMN.** Our aim was to determine TH and DAT regulation by Western Blot after acute treatment of PC12 cells with the various compounds or cigarette smoke extracts. Refer to chapter 4.

3. **(*S*)-Nicotine and nicotine containing smoke extract will upregulate TH and DAT *in vivo* but not (*S*)-cotinine or nicotine-free smoke extract.** To test this hypothesis we exposed rats chronically to either of the various compounds or cigarette smoke extracts by implanting the rats with ALZET® osmotic mini-pumps. We subsequently determined striatal TH and DAT regulation by Western blot. Refer to chapter 5.

4. **(*S*)-Nicotine and nicotine containing smoke extract release DA from rat striatal synaptosomes but not (*S*)-cotinine, nicotine-free smoke extract or TMN.** We used synaptosomes to test this hypothesis, by determining DA release after treatment with the various compounds. Refer to chapter 6.

Data gleaned from these studies will contribute towards our understanding of dopaminergic events after tobacco smoke exposure, measured *in vivo* as well as *in vitro*.

2.6. References

1. Kibiuk, L. 1998. Brain briefings: Nicotine and the brain. [Web:] http://www.sfn.org/content/Publications/BrainBriefings/nicotine.html#fullsize [Date of access: 12 October 2004].

2. National Center for chronic disease prevention and health promotion. 2004. Tobacco information and prevention source (TIPS). [Web:] http://www.cdc.gov/tobacco/issue.htm [Date of access: 8 September 2004].

3. World Health Organization. 1996. Tobacco or Health: First global status report. WHO tobacco or health programme. Geneva: WHO.

4. National Institute on Drug Abuse. 2002. Research Report Series - Nicotine Addiction. [Web:] http://www.nida.nih.gov/researchreports/nicotine/nicotine2.html#what [Date of access: 13 May 2004].

5. Le Houezec, J. & Benowitz, N.L. 1991. Basic and clinical psychopharmacology of nicotine. *Clin Chest Med*, 12: 681 - 699.

6. Wikipedia. 2004. Nicotine. [Web:] http://en.wikipedia.org/wiki/Nicotine [Date of access: 15 September 2004].

7. Hyperchem 7. 2004. Hypercube Inc. 1115 NW 4th St., Gainesville, Florida, 32601, USA.

8. Schievelbein, H. 1982. Nicotine, resorption and fate. *Pharmacol Ther*, 18: 233 - 248.

9. Schievelbein, H., Eberhardt, R., Loschenkohl, K. & others. 1973. Absorption of nicotine through the oral mucosa. I. Measurement of nicotine concentration in the blood after application of nicotine and total particulate matter. *Agents Actions*, 3: 254 - 258.

10. Dowshen, S. 2004. Smokeless tobacco. [Web:] http://kidshealth.org/teen/drug_alcohol/tobacco/smokeless.html [Date of access: 18 October 2004].

11. Heishman, S.J., Taylor, R.C. & Henningfield, J.E. 1994. Nicotine and Smoking: A Review of Effects on Human Performance. *Exp Clin Psychopharmacol*, 2: 345 - 395.

12. Snyder, M.J., Hsu, E. & Feyereisen, R. 1993. Induction of cytochrome P-450 activities by nicotine in the tobacco hornworm, manduca sexta. *J Chem Ecol*, 19: 2903 - 2910.

13. Yildiz, D. 2004. Nicotine, its metabolism and an overview of its biological effects. *Toxicon*, 43: 619 - 632.

14. Hecht, S.S., Hochalter, J.B., Villalta, P.W. & others. 2000. 2'-Hydroxylation of nicotine by cytochrome P450 2A6 and human liver microsomes: formation of a lung carcinogen precursor. *Proc Natl Acad Sci U S A*, 97: 12493 - 12497.

15. Messina, E.S., Tyndale, R.F. & Sellers, E.M. 1997. A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes. *J Pharmacol Exp Ther*, 282: 1608 - 1614.

16. Nakajima, M., Yamamoto, T., Nunoya, K. & others. 1996. Role of human cytochrome P4502A6 in C-oxidation of nicotine. *Drug Metab Dispos*, 24: 1212 - 1217.

17. Benowitz, N.L., Jacob, P., III, Fong, I. & others. 1994. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. *J Pharmacol Exp Ther*, 268: 296 - 303.

18. Schoedel, K.A., Sellers, E.M., Palmour, R. & others. 2003. Down-regulation of hepatic nicotine metabolism and a CYP2A6-like enzyme in African green monkeys after long-term nicotine administration. *Mol Pharmacol*, 63: 96 - 104.

19. Benowitz, N.L. & Jacob, P., III. 1993. Nicotine and cotinine elimination pharmacokinetics in smokers and nonsmokers. *Clin Pharmacol Ther*, 53: 316 - 323.

20. Benowitz, N.L. & Jacob, P., III. 2000. Effects of cigarette smoking and carbon monoxide on nicotine and cotinine metabolism. *Clin Pharmacol Ther*, 67: 653 - 659.

21. Yamazaki, H., Inoue, K., Hashimoto, M. & others. 1999. Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes. *Arch Toxicol*, 73: 65 - 70.

22. Miksys, S., Lerman, C., Shields, P.G. & others. 2003. Smoking, alcoholism and genetic polymorphisms alter CYP2B6 levels in human brain. *Neuropharmacology*, 45: 122 - 132.

23. Gervot, L., Rochat, B., Gautier, J.C. & others. 1999. Human CYP2B6: expression, inducibility and catalytic activities. *Pharmacogenetics*, 9: 295 - 306.

24. Hammond, D.K., Bjercke, R.J., Langone, J.J. & others. 1991. Metabolism of nicotine by rat liver cytochromes P-450. Assessment utilizing monoclonal antibodies to nicotine and cotinine. *Drug Metab Dispos*, 19: 804 - 808.

25. Nakayama, H., Okuda, H., Nakashima, T. & others. 1993. Nicotine metabolism by rat hepatic cytochrome P450s. *Biochem Pharmacol*, 45: 2554 - 2556.

26. Kyerematen, G.A., Owens, G.F., Chattopadhyay, B. & others. 1988. Sexual dimorphism of nicotine metabolism and distribution in the rat. Studies in vivo and in vitro. *Drug Metab Dispos*, 16: 823 - 828.

27. Mikus, G., Bochner, F., Eichelbaum, M. & others. 1994. Endogenous codeine and morphine in poor and extensive metabolisers of the CYP2D6 (debrisoquine/sparteine) polymorphism. *J Pharmacol Exp Ther*, 268: 546 - 551.

28. Hedenmalm, K., Sundgren, M., Granberg, K. & others. 1997. Urinary excretion of codeine, ethylmorphine, and their metabolites: relation to the CYP2D6 activity. *Ther Drug Monit*, 19: 643 - 649.

29. Zanger, U.M., Raimundo, S. & Eichelbaum, M. 2004. Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. *Naunyn Schmiedebergs Arch Pharmacol*, 369: 23 - 37.

30. Roberts, R.L., Luty, S.E., Mulder, R.T. & others. 2004. Association between cytochrome P450 2D6 genotype and harm avoidance. *Am J Med Genet*, 127B: 90 - 93.

31. Su, T., Bao, Z., Zhang, Q.Y. & others. 2000. Human cytochrome P450 CYP2A13: predominant expression in the respiratory tract and its high efficiency metabolic activation of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res*, 60: 5074 - 5079.

32. Maurice, M., Emiliani, S., Dalet-Beluche, I. & others. 1991. Isolation and characterization of a cytochrome P450 of the IIA subfamily from human liver microsomes. *Eur J Biochem*, 200: 511 - 517.

33. Getchell, M.L., Chen, Y., Ding, X. & others. 1993. Immunohistochemical localization of a cytochrome P-450 isozyme in human nasal mucosa: age-related trends. *Ann Otol Rhinol Laryngol*, 102: 368 - 374.

34. Su, T., Sheng, J.J., Lipinskas, T.W. & others. 1996. Expression of CYP2A genes in rodent and human nasal mucosa. *Drug Metab Dispos*, 24: 884 - 890.

35. Thornton-Manning, J.R., Nikula, K.J., Hotchkiss, J.A. & others. 1997. Nasal cytochrome P450 2A: identification, regional localization and metabolic activity toward hexamethylphosphoramide, a known nasal carcinogen. *Toxicol Appl Pharmacol*, 142: 22 - 30.

36. Koskela, S., Hakkola, J., Hukkanen, J. & others. 1999. Expression of CYP2A genes in human liver and extrahepatic tissues. *Biochem Pharmacol*, 57: 1407 - 1413.

37. Shimada, T., Yamazaki, H., Mimura, M. & others. 1996. Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. *Drug Metab Dispos*, 24: 515 - 522.

38. Crawford, E.L., Weaver, D.A., Demuth, J.P. & others. 1998. Measurement of cytochrome P450 2A6 and 2E1 gene expression in primary human bronchial epithelial cells. *Carcinogenesis (Lond)*, 19: 1867 - 1871.

39. Mace, K., Bowman, E.D., Vautravers, P. & others. 1998. Characterisation of xenobiotic-metabolising enzyme expression in human bronchial mucosa and peripheral lung tissues. *Eur J Cancer*, 34: 914 - 920.

40. Kimura, S., Kozak, C.A. & Gonzalez, F.J. 1989. Identification of a novel P450 expressed in rat lung: cDNA cloning and sequence, chromosome mapping, and induction by 3-methylcholanthrene. *Biochemistry*, 28: 3798 - 3803.

41. Hellmold, H., Magnusson, M., Pelto-Huikko, M. & others. 1998. Identification of CYP2A3 as a major cytochrome P450 enzyme in the female peripubertal rat breast. *Mol Pharmacol*, 53: 475 - 482.

42. Gopalakrishnan, R., Morse, M.A., Lu, J. & others. 1999. Expression of cytochrome P450 2A3 in rat esophagus: relevance to N-nitrosobenzylmethylamine. *Carcinogenesis*, 20: 885 - 891.

43. Benowitz, N.L. & Jacob, P. 1997. Individual differences in nicotine kinetics and metabolism in humans. *NIDA Res Monogr*, 173: 48 - 64.

44. Hucker, H.B., Gillette, J.R. & Brodie, B.B. 1960. Enzymatic pathway for the foramtion of cotinine, a major metabolite of nicotine in rabbit liver. *J Pharmacol Exp Ther*, 129: 94 - 100.

45. Byrd, G.D., Chang, K.M., Greene, J.M. & others. 1992. Evidence for urinary excretion of glucuronide conjugates of nicotine, cotinine, and trans-3'-hydroxycotinine in smokers. *Drug Metab Dispos*, 20: 192 - 197.

46. Hecht, S.S., Carmella, S.G. & Murphy, S.E. 1999. Effects of watercress consumption on urinary metabolites of nicotine in smokers. *Cancer Epidemiol Biomarkers Prev*, 8: 907 - 913.

47. Foundation for blood research. 2004. Cotinine testing. [Web:] http://www.fbr.org/publications/pamphlets/cotinine.html [Date of access: 29 October 2004].

48. Nakazawa, A., Shigeta, M. & Ozasa, K. 2004. Smoking cigarettes of low nicotine yield does not reduce nicotine intake as expected: a study of nicotine dependency in Japanese males. *BMC Public Health*, 4: 28 - .

49. Okayama, A. & Sato, T. 2004. Assessment of smoking status among workers using an improved colorimetric method. *Ind Health*, 42: 348 - 351.

50. Moriya, F. & Hashimoto, Y. 2004. Nicotine and cotinine levels in blood and urine from forensic autopsy cases. *Leg Med (Tokyo)*, 6: 164 - 169.

51. Zevin, S., Jacob, P., Geppetti, P. & others. 2000. Clinical pharmacology of oral cotinine. *Drug Alcohol Depend*, 60: 13 - 18.

52. Heinrich, J., Holscher, B., Seiwert, M. & others. 2004. Nicotine and Cotinine in Adults' Urine: The German Enviromental Survey 1998. *J Expo Anal Environ Epidemiol.* [Epub ahead of print].

53. Stevens, K.R. & Munoz, L.R. 2004. Cigarette smoking: Evidence to guide measurement. *Res Nurs Health*, 27: 281 - 292.

54. Benowitz, N.L. 1996. Pharmacology of nicotine: addiction and therapeutics. *Annu Rev Pharmacol Toxicol*, 36: 597 - 613.

55. Wormhoudt, L.W., Commandeur, J.N. & Vermeulen, N.P. 1999. Genetic polymorphisms of human N-acetyltransferase, cytochrome P450, glutathione-S-transferase, and epoxide hydrolase enzymes: relevance to xenobiotic metabolism and toxicity. *Crit Rev Toxicol*, 29: 59 - 124.

56. Kitagawa, K., Kunugita, N., Katoh, T. & others. 1999. The significance of the homozygous CYP2A6 deletion on nicotine metabolism: a new genotyping method of CYP2A6 using a single PCR-RFLP. *Biochem Biophys Res Commun*, 262: 146 - 151.

57. Oscarson, M., McLellan, R.A., Gullsten, H. & others. 1999. Characterisation and PCR-based detection of a CYP2A6 gene deletion found at a high frequency in a Chinese population. *FEBS Lett*, 448: 105 - 110.

58. Kawajiri, K., Watanabe, J., Eguchi, H. & others. 1995. Genetic polymorphisms of drug-metabolizing enzymes and lung cancer susceptibility. *Pharmacogenetics*, 5 Spec No: S70 - S73.

59. Daly, A.K., Brockmoller, J., Broly, F. & others. 1996. Nomenclature for human CYP2D6 alleles. *Pharmacogenetics*, 6: 193 - 201.

60. Watanabe, J., Hayashi, S. & Kawajiri, K. 1994. Different regulation and expression of the human CYP2E1 gene due to the RsaI polymorphism in the 5'-flanking region. *J Biochem (Tokyo)*, 116: 321 - 326.

61. Yang, M., Kunugita, N., Kitagawa, K. & others. 2001. Individual differences in urinary cotinine levels in Japanese smokers: relation to genetic polymorphism of drug-metabolizing enzymes. *Cancer Epidemiol Biomarkers Prev*, 10: 589 - 593.

62. Wewers, M.E., Ahijevych, K.L., Dhatt, R.K. & others. 2000. Cotinine levels in Southeast Asian smokers. *Nicotine Tob Res*, 2: 85 - 91.

63. Perez-Stable, E.J., Herrera, B., Jacob, P., III & others. 1998. Nicotine metabolism and intake in black and white smokers. *JAMA*, 280: 152 - 156.

64. Benowitz, N.L., Perez-Stable, E.J., Herrera, B. & others. 2002. Slower metabolism and reduced intake of nicotine from cigarette smoking in Chinese-Americans. *J Natl Cancer Inst*, 94: 108 - 115.

65. Wagenknecht, L.E., Cutter, G.R., Haley, N.J. & others. 1990. Racial differences in serum cotinine levels among smokers in the Coronary Artery Risk Development in (Young) Adults study. *Am J Public Health*, 80: 1053 - 1056.

66. Ballabh, P., Braun, A. & Nedergaard, M. 2004. The blood-brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiol Dis*, 16: 1 - 13.

67. Francis, K., van Beek, J., Canova, C., Neal, J.W. & others. 2003. The blood-brain barrier (BBB). [Web:] http://www-ermm.cbcu.cam.ac.uk/03006264h.htm [Date of access: 14 October 2004].

68. Butt, A.M., Jones, H.C. & Abbott, N.J. 1990. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *J Physiol*, 429: 47 - 62.

69. Neuwelt, E.A. 2004. Mechanisms of disease: the blood-brain barrier. *Neurosurgery*, 54: 131 - 140.

70. Begley, D.J. & Brightman, M.W. 2003. Structural and functional aspects of the blood-brain barrier. *Prog Drug Res*, 61: 39 - 78.

71. Ohno, K., Pettigrew, K.D. & Rapoport, S.I. 1979. Local cerebral blood flow in the conscious rat as measured with 14C-antipyrine, 14C-iodoantipyrine and 3H-nicotine. *Stroke*, 10: 62 - 67.

72. Suzuki, R., Nitsch, C., Fujiwara, K. & others. 1984. Regional changes in cerebral blood flow and blood-brain barrier permeability during epileptiform seizures and in acute hypertension in rabbits. *J Cereb Blood Flow Metab*, 4: 96 - 102.

73. Todd, M.M. & Weeks, J. 1996. Comparative effects of propofol, pentobarbital, and isoflurane on cerebral blood flow and blood volume. *J Neurosurg Anesthesiol*, 8: 296 - 303.

74. Tomiyama, Y., Brian, J.E., Jr. & Todd, M.M. 1999. Cerebral blood flow during hemodilution and hypoxia in rats : role of ATP-sensitive potassium channels. *Stroke*, 30: 1942 - 1947.

75. Hawkins, B.T., Brown, R.C. & Davis, T.P. 2002. Smoking and ischemic stroke: a role for nicotine? *Trends Pharmacol Sci*, 23: 78 - 82.

76. Booyse, F.M., Osikowicz, G. & Quarfoot, A.J. 1981. Effects of chronic oral consumption of nicotine on the rabbit aortic endothelium. *Am J Pathol*, 102: 229 - 238.

77. Abbruscato, T.J., Lopez, S.P., Mark, K.S. & others. 2002. Nicotine and cotinine modulate cerebral microvascular permeability and protein expression of ZO-1 through nicotinic acetylcholine receptors expressed on brain endothelial cells. *J Pharm Sci*, 91: 2525 - 2538.

78. Itoh, M., Nagafuchi, A., Yonemura, S. & others. 1993. The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J Cell Biol*, 121: 491 - 502.

79. Gao, P. & Shivers, R.R. 2004. Correlation of the presence of blood-brain barrier tight junctions and expression of zonula occludens protein ZO-1 in vitro: a freeze-fracture and immunofluorescence study. *J Submicrosc Cytol Pathol*, 36: 7 - 15.

80. Abbruscato, T.J., Lopez, S.P., Roder, K. & others. 2004. Regulation of Blood-Brain Barrier Na,K,2Cl-Cotransporter through Phosphorylation during in Vitro Stroke Conditions and Nicotine Exposure. *J Pharmacol Exp Ther*, 310: 459 - 468.

81. Wang, L., McComb, J.G., Weiss, M.H. & others. 1994. Nicotine downregulates alpha 2 isoform of Na,K-ATPase at the blood-brain barrier and brain in rats. *Biochem Biophys Res Commun*, 199: 1422 - 1427.

82. Halldin, C., Nagren, K., Swahn, C.G. & others. 1992. (S)- and (R)-[11C]nicotine and the metabolite (R/S)-[11C]cotinine. Preparation, metabolite studies and in vivo distribution in the human brain using PET. *Int J Rad Appl Instrum B*, 19: 871 - 880.

83. Riah, O., Courriere, P., Dousset, J.C. & others. 1998. Nicotine is more efficient than cotinine at passing the blood-brain barrier in rats. *Cell Mol Neurobiol*, 18: 311 - 318.

84. Crooks, P.A. & Dwoskin, L.P. 1997. Contribution of CNS nicotine metabolites to the neuropharmacological effects of nicotine and tobacco smoking. *Biochem Pharmacol*, 54: 743 - 753.

85. Crooks, P.A., Li, M. & Dwoskin, L.P. 1997. Metabolites of nicotine in rat brain after peripheral nicotine administration. Cotinine, nornicotine, and norcotinine. *Drug Metab Dispos*, 25: 47 - 54.

86. Miksys, S., Hoffmann, E. & Tyndale, R.F. 2000. Regional and cellular induction of nicotine-metabolizing CYP2B1 in rat brain by chronic nicotine treatment. *Biochem Pharmacol*, 59: 1501 - 1511.

87. Fasano, S. & Brambilla, R. 2002. Cellular mechanisms of striatum-dependent behavioral plasticity and drug addiction. *Curr Mol Med*, 2: 649 - 665.

88. Hyman, S.E. & Malenka, R.C. 2001. Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat Rev Neurosci*, 2: 695 - 703.

89. Gobbi, P. & Di Motta, D. 1998. Atlante anatomica del sistema nervoso centrale. [Web:] http://mail.biocfarm.unibo.it/aunsnc/3dobjl22.html [Date of access: 9 October 2004].

90. Berke, J.D. & Hyman, S.E. 2000. Addiction, dopamine, and the molecular mechanisms of memory. *Neuron*, 25: 515 - 532.

91. Delgado, M.R., Locke, H.M., Stenger, V.A. & others. 2003. Dorsal striatum responses to reward and punishment: effects of valence and magnitude manipulations. *Cogn Affect Behav Neurosci*, 3: 27 - 38.

92. Breiter, H.C., Gollub, R.L., Weisskoff, R.M. & others. 1997. Acute effects of cocaine on human brain activity and emotion. *Neuron*, 19: 591 - 611.

93. Stein, E.A., Pankiewicz, J., Harsch, H.H. & others. 1998. Nicotine-induced limbic cortical activation in the human brain: a functional MRI study. *Am J Psychiatry*, 155: 1009 - 1015.

94. Nestler, E.J. 2001. Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci*, 2: 119 - 128.

95. Mihailescu, S. & Drucker-Colin, R. 2000. Nicotine, brain nicotinic receptors, and neuropsychiatric disorders. *Arch Med Res*, 31: 131 - 144.

96. Salin-Pascual, R.J., Alcocer-Castillejos, N.V. & Alejo-Galarza, G. 2003. Nicotine dependence and psychiatric disorders. *Rev Invest Clin*, 55: 677 - 693.

97. Stone, C. 2003. Chemically correct: Nicotine. [Web:] http://www.bodybuilding.com/fun/par16.htm [Date of access: 13 September 2004].

98. McGehee, D.S. & Role, L.W. 1995. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol*, 57: 521 - 546.

99. Role, L.W. & Berg, D.K. 1996. Nicotinic receptors in the development and modulation of CNS synapses. *Neuron*, 16: 1077 - 1085.

100. Jones, S., Sudweeks, S. & Yakel, J.L. 1999. Nicotinic receptors in the brain: correlating physiology with function. *Trends Neurosci*, 22: 555 - 561.

101. Gotti, C., Fornasari, D. & Clementi, F. 1997. Human neuronal nicotinic receptors. *Prog Neurobiol*, 53: 199 - 237.

102. Balfour, D.J. 1982. The effects of nicotine on brain neurotransmitter systems. *Pharmacol Ther*, 16: 269 - 282.

103. Balfour, D.J., Wright, A.E., Benwell, M.E. & others. 2000. The putative role of extra-synaptic mesolimbic dopamine in the neurobiology of nicotine dependence. *Behav Brain Res*, 113: 73 - 83.

104. Dani, J.A. & Heinemann, S. 1996. Molecular and cellular aspects of nicotine abuse. *Neuron*, 16: 905 - 908.

105. Di Chiara, G. 2000. Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur J Pharmacol*, 393: 295 - 314.

106. Picciotto, M.R. & Corrigall, W.A. 2002. Neuronal systems underlying behaviors related to nicotine addiction: neural circuits and molecular genetics. *J Neurosci*, 22: 3338 - 3341.

107. Koob, G.F. & Le Moal, M. 1997. Drug abuse: hedonic homeostatic dysregulation. *Science*, 278: 52 - 58.

108. Koob, G.F. 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci*, 13: 177 - 184.

109. Wise, R.A. 1996. Neurobiology of addiction. *Curr Opin Neurobiol*, 6: 243 - 251.

110. Kreek, M.J. 1996. Opiates, opioids and addiction. *Mol Psychiatry*, 1: 232 - 254.

111. Mansvelder, H.D., Keath, J.R. & McGehee, D.S. 2002. Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron*, 33: 905 - 919.

112. Zickler, P. 2003. Nicotine's multiple effects on the brain's reward system drive addiction. [Web:] http://www.drugabuse.gov/NIDA_notes/NNVol17N6/Nicotine.html [Date of access: 12 November 2004].

113. Mansvelder, H.D. & McGehee, D.S. 2000. Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron*, 27: 349 - 357.

114. Middleton, L.S., Cass, W.A. & Dwoskin, L.P. 2004. Nicotinic receptor modulation of dopamine transporter function in rat striatum and medial prefrontal cortex. *J Pharmacol Exp Ther*, 308: 367 - 377.

115. Mitchell, S.L., Kiely, D.K., Kiel, D.P. & others. 1996. The epidemiology, clinical characteristics, and natural history of older nursing home residents with a diagnosis of Parkinson's disease. *J Am Geriatr Soc*, 44: 394 - 399.

116. Calne, D.B. & Langston, J.W. 1983. Aetiology of Parkinson's disease. *Lancet*, 2: 1457 - 1459.

117. Young, R. 1999. American Academy of Family Physicians: Update on Parkinson's Disease. [Web:] http://www.aafp.org/afp/990415ap/2155.html [Date of access: 8 October 2004].

118. Ross, G.W. & Petrovitch, H. 2001. Current evidence for neuroprotective effects of nicotine and caffeine against Parkinson's disease. *Drugs Aging*, 18: 797 - 806.

119. Lotharius, J. & Brundin, P. 2002. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci*, 3: 932 - 942.

120. Baumann, R.J., Jameson, H.D., McKean, H.E. & others. 1980. Cigarette smoking and Parkinson disease: 1. Comparison of cases with matched neighbors. *Neurology*, 30: 839 - 843.

121. Godwin-Austen, R.B., Lee, P.N., Marmot, M.G. & others. 1982. Smoking and Parkinson's disease. *J Neurol Neurosurg Psychiatry*, 45: 577 - 581.

122. Kessler, I.I. 1972. Epidemiologic studies of Parkinson's disease. 3. A community-based survey. *Am J Epidemiol*, 96: 242 - 254.

123. Kessler, I.I. & Diamond, E.L. 1971. Epidemiologic studies of Parkinson's disease. I. Smoking and Parkinson's disease: a survey and explanatory hypothesis. *Am J Epidemiol*, 94: 16 - 25.

124. Nefzger, M.D., Quadfasel, F.A. & Karl, V.C. 1968. A retrospective study of smoking in Parkinson's disease. *Am J Epidemiol*, 88: 149 - 158.

125. Shih, J.C., Chen, K. & Ridd, M.J. 1999. Monoamine oxidase: from genes to behavior. *Annu Rev Neurosci*, 22: 197 - 217.

126. Shih, J.C. & Thompson, R.F. 1999. Monoamine oxidase in neuropsychiatry and behavior. *Am J Hum Genet*, 65: 593 - 598.

127. Oreland, L., Fowler, C.J. & Schalling, D. 1981. Low platelet monoamine oxidase activity in cigarette smokers. *Life Sci*, 29: 2511 - 2518.

128. Yong, V.W. & Perry, T.L. 1986. Monoamine oxidase B, smoking, and Parkinson's disease. *J Neurol Sci*, 72: 265 - 272.

129. Norman, T.R., Chamberlain, K.G. & French, M.A. 1987. Platelet monoamine oxidase: low activity in cigarette smokers. *Psychiatry Res*, 20: 199 - 205.

130. Berlin, I., Said, S., Spreux-Varoquaux, O. & others. 1995. Monoamine oxidase A and B activities in heavy smokers. *Biol Psychiatry*, 38: 756 - 761.

131. Fowler, J.S., Volkow, N.D., Wang, G.J. & others. 1996. Brain monoamine oxidase A inhibition in cigarette smokers. *Proc Natl Acad Sci U S A*, 93: 14065 - 14069.

132. Fowler, J.S., Volkow, N.D., Wang, G.J. & others. 1996. Inhibition of monoamine oxidase B in the brains of smokers. *Nature*, 379: 733 - 736.

133. Fowler, J.S., Logan, J., Wang, G.J. & others. 2003. Low monoamine oxidase B in peripheral organs in smokers. *Proc Natl Acad Sci U S A*, 100: 11600 - 11605.

134. Foley, P. & Riederer, P. 2000. Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson's disease. *J Neurol*, 247 Suppl 2: II82 - II94.

135. Alexi, T., Borlongan, C.V., Faull, R.L. & others. 2000. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. *Prog Neurobiol*, 60: 409 - 470.

136. Olanow, C.W. 1990. Oxidation reactions in Parkinson's disease. *Neurology*, 40: suppl - 7.

137. Beal, M.F. 1995. Mitochondrial dysfunction and oxidative damage in neurodegenerative diseases. ed. New York: Springer-Verlag. 128p.

138. Fowler, J.S., Volkow, N.D., Logan, J. & others. 1998. An acute dose of nicotine does not inhibit MAO B in baboon brain in vivo. *Life Sci*, 63: L19 - L23.

139. Castagnoli, K.P., Steyn, S.J., Petzer, J.P. & others. 2001. Neuroprotection in the MPTP Parkinsonian C57BL/6 mouse model by a compound isolated from tobacco. *Chem Res Toxicol*, 14: 523 - 527.

140. Khalil, A.A., Steyn, S. & Castagnoli, N., Jr. 2000. Isolation and characterization of a monoamine oxidase inhibitor from tobacco leaves. *Chem Res Toxicol*, 13: 31 - 35.

141. Ferger, B., Spratt, C., Earl, C.D. & others. 1998. Effects of nicotine on hydroxyl free radical formation in vitro and on MPTP-induced neurotoxicity in vivo. *Naunyn Schmiedebergs Arch Pharmacol*, 358: 351 - 359.

142. Linert, W., Bridge, M.H., Huber, M. & others. 1999. In vitro and in vivo studies investigating possible antioxidant actions of nicotine: relevance to Parkinson's and Alzheimer's diseases. *Biochim Biophys Acta*, 1454: 143 - 152.

143. Soto-Otero, R., Mendez-Alvarez, E., Hermida-Ameijeiras, A. & others. 2002. Effects of (-)-nicotine and (-)-cotinine on 6-hydroxydopamine-induced oxidative stress and neurotoxicity: relevance for Parkinson's disease. *Biochem Pharmacol*, 64: 125 - 135.

144. Quik, M. & Jeyarasasingam, G. 2000. Nicotinic receptors and Parkinson's disease. *Eur J Pharmacol*, 393: 223 - 230.

145. Akaike, A., Tamura, Y., Yokota, T. & others. 1994. Nicotine-induced protection of cultured cortical neurons against N-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. *Brain Res*, 644: 181 - 187.

146. Shimohama, S., Akaike, A. & Kimura, J. 1996. Nicotine-induced protection against glutamate cytotoxicity. Nicotinic cholinergic receptor-mediated inhibition of nitric oxide formation. *Ann N Y Acad Sci*, 777: 356 - 361.

147. Kaneko, S., Maeda, T., Kume, T. & others. 1997. Nicotine protects cultured cortical neurons against glutamate-induced cytotoxicity via alpha7-neuronal receptors and neuronal CNS receptors. *Brain Res*, 765: 135 - 140.

148. Zamani, M.R., Allen, Y.S., Owen, G.P. & others. 1997. Nicotine modulates the neurotoxic effect of beta-amyloid protein(25-35)) in hippocampal cultures. *Neuroreport*, 8: 513 - 517.

149. Minana, M.D., Montoliu, C., Llansola, M. & others. 1998. Nicotine prevents glutamate-induced proteolysis of the microtubule-associated protein MAP-2 and glutamate neurotoxicity in primary cultures of cerebellar neurons. *Neuropharmacology*, 37: 847 - 857.

150. Marin, P., Maus, M., Desagher, S. & others. 1994. Nicotine protects cultured striatal neurones against N-methyl-D-aspartate receptor-mediated neurotoxicity. *Neuroreport*, 5: 1977 - 1980.

151. Sun, X., Liu, Y., Hu, G. & others. 2004. Protective effects of nicotine against glutamate-induced neurotoxicity in PC12 cells. *Cell Mol Biol Lett*, 9: 409 - 422.

152. Maggio, R., Riva, M., Vaglini, F. & others. 1998. Nicotine prevents experimental parkinsonism in rodents and induces striatal increase of neurotrophic factors. *J Neurochem*, 71: 2439 - 2446.

153. Belluardo, N., Blum, M., Mudo, G. & others. 1998. Acute intermittent nicotine treatment produces regional increases of basic fibroblast growth factor messenger RNA and protein in the tel- and diencephalon of the rat. *Neuroscience*, 83: 723 - 740.

154. Bean, A.J., Elde, R., Cao, Y.H. & others. 1991. Expression of acidic and basic fibroblast growth factors in the substantia nigra of rat, monkey, and human. *Proc Natl Acad Sci U S A*, 88: 10237 - 10241.

155. Linville, D.G. & Arneric, S.P. 1991. Cortical cerebral blood flow governed by the basal forebrain: age-related impairments. *Neurobiol Aging*, 12: 503 - 510.

156. Linville, D.G., Williams, S., Raszkiewicz, J.L. & others. 1993. Nicotinic agonists modulate basal forebrain control of cortical cerebral blood flow in anesthetized rats. *J Pharmacol Exp Ther*, 267: 440 - 448.

157. Uchida, S., Kagitani, F., Nakayama, H. & others. 1997. Effect of stimulation of nicotinic cholinergic receptors on cortical cerebral blood flow and changes in the effect during aging in anesthetized rats. *Neurosci Lett*, 228: 203 - 206.

158. Marenco, T., Bernstein, S., Cumming, P. & others. 2000. Effects of nicotine and chlorisondamine on cerebral glucose utilization in immobilized and freely-moving rats. *Br J Pharmacol*, 129: 147 - 155.

159. Grunwald, F., Schrock, H. & Kuschinsky, W. 1988. The effect of an acute nicotine infusion on the local cerebral glucose utilization of the awake rat. *Klin Wochenschr*, 66 Suppl 11: 37 - 41.

160. McGeer, P.L., Itagaki, S., Boyes, B.E. & others. 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology*, 38: 1285 - 1291.

161. Barcia, C., Fernandez, B.A., Poza, M. & others. 2003. Parkinson's disease and inflammatory changes. *Neurotox Res*, 5: 411 - 418.

162. Streit, W.J. 2000. Microglial response to brain injury: a brief synopsis. *Toxicol Pathol*, 28: 28 - 30.

163. Tan, J., Town, T. & Mullan, M. 2002. CD40-CD40L interaction in Alzheimer's disease. *Curr Opin Pharmacol*, 2: 445 - 451.

164. Liu, B. & Hong, J.S. 2003. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther*, 304: 1 - 7.

165. Shytle, R.D., Mori, T., Townsend, K. & others. 2004. Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. *J Neurochem*, 89: 337 - 343.

166. Benwell, M.E., Balfour, D.J. & Anderson, J.M. 1988. Evidence that tobacco smoking increases the density of (-)-[3H]nicotine binding sites in human brain. *J Neurochem*, 50: 1243 - 1247.

167. Marks, M.J., Stitzel, J.A. & Collins, A.C. 1985. Time course study of the effects of chronic nicotine infusion on drug response and brain receptors. *J Pharmacol Exp Ther*, 235: 619 - 628.

168. Marks, M.J., Pauly, J.R., Gross, S.D. & others. 1992. Nicotine binding and nicotinic receptor subunit RNA after chronic nicotine treatment. *J Neurosci*, 12: 2765 - 2784.

169. Schwartz, R.D. & Kellar, K.J. 1985. In vivo regulation of [3H]acetylcholine recognition sites in brain by nicotinic cholinergic drugs. *J Neurochem*, 45: 427 - 433.

170. Briggs, C.A. & McKenna, D.G. 1998. Activation and inhibition of the human alpha7 nicotinic acetylcholine receptor by agonists. *Neuropharmacology*, 37: 1095 - 1102.

171. Dwoskin, L.P., Teng, L., Buxton, S.T. & others. 1999. (S)-(-)-Cotinine, the major brain metabolite of nicotine, stimulates nicotinic receptors to evoke [3H]dopamine release from rat striatal slices in a calcium-dependent manner. *J Pharmacol Exp Ther*, 288: 905 - 911.

172. Wonnacott, S. 1990. The paradox of nicotinic acetylcholine receptor upregulation by nicotine. *Trends Pharmacol Sci*, 11: 216 - 219.

173. Marks, M.J., Burch, J.B. & Collins, A.C. 1983. Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. *J Pharmacol Exp Ther*, 226: 817 - 825.

174. Schwartz, R.D. & Kellar, K.J. 1983. Nicotinic cholinergic receptor binding sites in the brain: regulation in vivo. *Science*, 220: 214 - 216.

175. Breese, C.R., Marks, M.J., Logel, J. & others. 1997. Effect of smoking history on [3H]nicotine binding in human postmortem brain. *J Pharmacol Exp Ther*, 282: 7 - 13.

176. Benwell, M.E., Balfour, D.J. & Anderson, J.M. 1988. Evidence that tobacco smoking increases the density of (-)-[3H]nicotine binding sites in human brain. *J Neurochem*, 50: 1243 - 1247.

177. Dani, J.A. & Heinemann, S. 1996. Molecular and cellular aspects of nicotine abuse. *Neuron*, 16: 905 - 908.

178. Flores, C.M., Rogers, S.W., Pabreza, L.A. & others. 1992. A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. *Mol Pharmacol*, 41: 31 - 37.

179. Lukas, R.J., Ke, L., Bencherif, M. & others. 1996. Regulation by nicotine of its own receptors. *Drug Dev Res*, 38: 136 - 148.

180. Peng, X., Gerzanich, V., Anand, R. & others. 1994. Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol*, 46: 523 - 530.

181. Lester, R.A. & Dani, J.A. 1994. Time-dependent changes in central nicotinic acetylcholine channel kinetics in excised patches. *Neuropharmacology*, 33: 27 - 34.

182. Ferrari, R., Le Novere, N., Picciotto, M.R. & others. 2002. Acute and long-term changes in the mesolimbic dopamine pathway after systemic or local single nicotine injections. *Eur J Neurosci*, 15: 1810 - 1818.

183. Pidoplichko, V.I., Noguchi, J., Areola, O.O. & others. 2004. Nicotinic cholinergic synaptic mechanisms in the ventral tegmental area contribute to nicotine addiction. *Learn Mem*, 11: 60 - 69.

184. Levitt, M., Spector, S., Sjoerdsma, A. & others. 1965. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J Pharmacol Exp Ther*, 148: 1 - 8.

185. Kumer, S.C. & Vrana, K.E. 1996. Intricate regulation of tyrosine hydroxylase activity and gene expression. *J Neurochem*, 67: 443 - 462.

186. Slotkin, T.A., Seidler, F.J., Lau, C. & others. 1976. Effects of chronic chlorisondamine administration on the sympatho-adrenal axis. *Biochem Pharmacol*, 25: 1311 - 1315.

187. Fossom, L.H., Sterling, C. & Tank, A.W. 1991. Activation of tyrosine hydroxylase by nicotine in rat adrenal gland. *J Neurochem*, 57: 2070 - 2077.

188. Mitchell, S.N., Smith, K.M., Joseph, M.H. & others. 1993. Increases in tyrosine hydroxylase messenger RNA in the locus coeruleus after a single dose of nicotine are followed by time-dependent increases in enzyme activity and noradrenaline release. *Neuroscience*, 56: 989 - 997.

189. Smith, K.M., Mitchell, S.N. & Joseph, M.H. 1991. Effects of chronic and subchronic nicotine on tyrosine hydroxylase activity in noradrenergic and dopaminergic neurones in the rat brain. *J Neurochem*, 57: 1750 - 1756.

190. Hofle, G., Weiler, R., Fischer-Colbrie, R. & others. 1991. Stimulation of rat adrenal medulla can induce differential changes in the peptide and mRNA levels of chromogranins, neuropeptides and other constituents of chromaffin granules. *Regul Pept*, 32: 321 - 331.

191. Hiremagalur, B. & Sabban, E.L. 1995. Nicotine elicits changes in expression of adrenal catecholamine biosynthetic enzymes, neuropeptide Y and immediate early genes by injection but not continuous administration. *Brain Res Mol Brain Res*, 32: 109 - 115.

192. Serova, L. & Sabban, E.L. 2002. Involvement of alpha 7 nicotinic acetylcholine receptors in gene expression of dopamine biosynthetic enzymes in rat brain. *J Pharmacol Exp Ther*, 303: 896 - 903.

193. Armando, I., Terron, J.A., Falcon-Neri, A. & others. 2002. Increased angiotensin II AT(1) receptor expression in paraventricular nucleus and hypothalamic-pituitary-adrenal axis stimulation in AT(2) receptor gene disrupted mice. *Neuroendocrinology*, 76: 137 - 147.

194. Kaufman, S. 1958. A new cofactor required for the enzymatic conversion of phenylalanine to tyrosine. *J Biol Chem*, 230: 931 - 939.

195. Lovenberg, W., Jequier, E. & SJOERDSMA, A. 1967. Tryptophan hydroxylation: measurement in pineal gland, brainstem, and carcinoid tumor. *Science*, 155: 217 - 219.

196. Kwon, N.S., Nathan, C.F. & Stuehr, D.J. 1989. Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J Biol Chem*, 264: 20496 - 20501.

197. Mayer, B., John, M., Heinzel, B. & others. 1991. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. *FEBS Lett*, 288: 187 - 191.

198. Mayer, B., John, M. & Bohme, E. 1990. Purification of a Ca2+/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. *FEBS Lett*, 277: 215 - 219.

199. Nagatsu, T., Levitt, M. & Udenfriend, S. 1964. Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. *J Biol Chem*, 239: 2910 - 2917.

200. Kettler, R., Bartholini, G. & Pletscher, A. 1974. In vivo enhancement of tyrosine hydroxylation in rat striatum by tetrahydrobiopterin. *Nature*, 249: 476 - 478.

201. Koshimura, K., Miwa, S., Lee, K. & others. 1990. Enhancement of dopamine release in vivo from the rat striatum by dialytic perfusion of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin. *J Neurochem*, 54: 1391 - 1397.

202. National Institute on Drug Abuse. 2002. The Brain & the Actions of Cocaine, Opiates, and Marijuana. [Web:] http://www.nida.nih.gov/pubs/teaching/Teaching2.html [Date of access: 23 July 2004].

203. Gillette, J.S. & Bloomquist, J.R. 2003. Differential up-regulation of striatal dopamine transporter and alpha-synuclein by the pyrethroid insecticide permethrin. *Toxicol Appl Pharmacol*, 192: 287 - 293.

204. Miller, G.W., Gainetdinov, R.R., Levey, A.I. & others. 1999. Dopamine transporters and neuronal injury. *Trends Pharmacol Sci*, 20: 424 - 429.

205. Freed, C., Revay, R., Vaughan, R.A. & others. 1995. Dopamine transporter immunoreactivity in rat brain. *J Comp Neurol*, 359: 340 - 349.

206. Ciliax, B.J., Heilman, C., Demchyshyn, L.L. & others. 1995. The dopamine transporter: immunochemical characterization and localization in brain. *J Neurosci*, 15: 1714 - 1723.

207. Hart, C. & Ksir, C. 1996. Nicotine effects on dopamine clearance in rat nucleus accumbens. *J Neurochem*, 66: 216 - 221.

208. Sonders, M.S., Zhu, S.J., Zahniser, N.R. & others. 1997. Multiple ionic conductances of the human dopamine transporter: the actions of dopamine and psychostimulants. *J Neurosci*, 17: 960 - 974.

209. Calabresi, P., Lacey, M.G. & North, R.A. 1989. Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording. *Br J Pharmacol*, 98: 135 - 140.

210. Li, S., Kim, K.Y., Kim, J.H. & others. 2004. Chronic nicotine and smoking treatment increases dopamine transporter mRNA expression in the rat midbrain. *Neurosci Lett*, 363: 29 - 32.

211. Tipton, K.F. & Singer, T.P. 1993. Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *J Neurochem*, 61: 1191 - 1206.

212. Counihan, T.J. & Penney, J.B., Jr. 1998. Regional dopamine transporter gene expression in the substantia nigra from control and Parkinson's disease brains. *J Neurol Neurosurg Psychiatry*, 65: 164 - 169.

CHAPTER 3:

IN SITU BRAIN PERFUSION OF COTININE AND NICOTINE

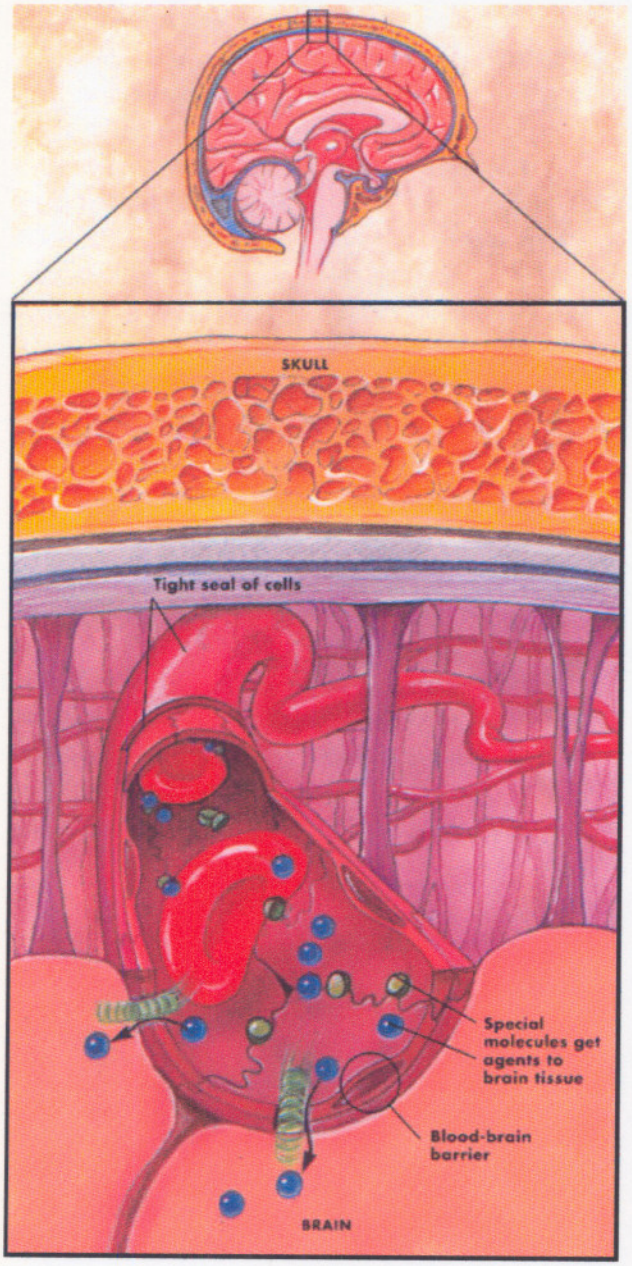

Illustration by Kibiuk, 1998.[1]

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LIST OF ABBREVIATIONS

C	concentration of tracer in perfusion fluid (dpm/ml)
CNS	central nervous system
F	cerebral perfusion flow rate
K_{in}	unidirectional uptake transfer constants
PA	cerebrovascular permeability-surface area products
Q	quantity of tracer in brain (dpm/g) at the end of perfusion
SEM	standard error of mean
T	perfusion time (s)
Vo	"vascular volume" in ml/g at T = 0 s

3.1. Introduction

The blood to brain transfer kinetics of nicotine and its major metabolite cotinine (figure 3.1) are determinant factors for central nervous system (CNS) concentrations of these major tobacco alkaloids. For CNS access, nicotine and/or cotinine must traverse the specialized neurovascular endothelium, which theoretically can limit plasma to brain distribution.

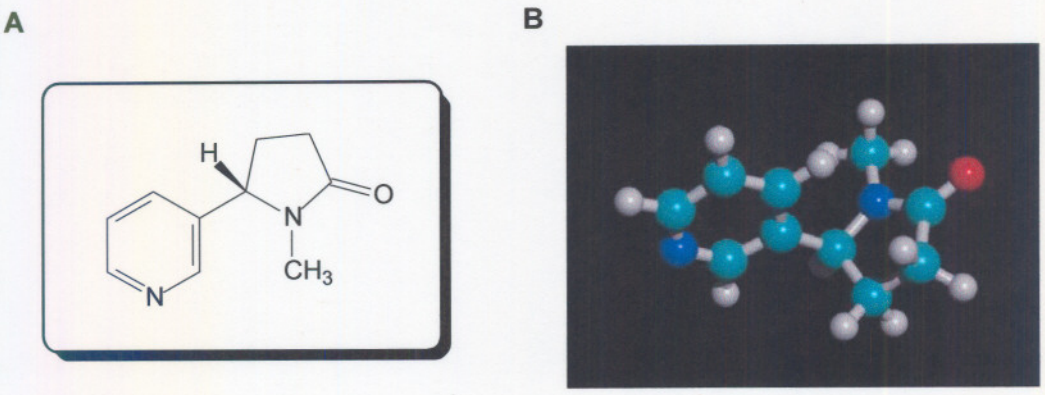


Figure 3.1: **A.** Molecular structure of (*S*)-cotinine.

B. Ball and stick representation of (*S*)-cotinine generated by Hyperchem version 7.[4]

In the two decades since the late sixties, six different techniques were developed to study transport at the BBB: the indicator-diffusion technique, single injection external registration technique, brain uptake index technique, isolated perfused brain technique and intravenous injection technique.[17-22] Each of these techniques has several limitations.[23] In 1984 Takasato *et al.* published an *in situ* brain perfusion technique in rats.[24] This method has various unique characteristics and several advantages when compared to other techniques that measure cerebrovascular transport.

1. The **kinetics** of brain uptake via perfusion are **relatively simple** since the compounds of interest are delivered to the brain with a known concentration which does not vary with time.[25]
2. Transport across the BBB can be measured over a **broad range of intervals** (anywhere from 5 s to >30 min), which allows for accurate determination of permeability over a 10^5 range. This is made possible through the control of the investigator over the perfusion uptake time.[24]

3. *In situ* brain perfusion is ideal for the characterization of the kinetics of saturable transport across the BBB and the effects of plasma protein binding.[26-28]
4. It is possible to study solute efflux from brain after preloading by switching the perfusate to solute-free saline with the *in situ* brain perfusion technique.[26,29]
5. The *in situ* brain perfusion technique is **one hundred times more sensitive** than the indicator-diffusion technique, single injection external registration technique and brain uptake index technique. Only the intravenous injection technique compares with the *in situ* technique.[18-22]
6. Almost absolute **control** is permitted **over the perfusate composition** when performing *in situ* brain perfusion. This advantage is shared partially by the indicator dilution and brain uptake index techniques.[24]
7. In all arterial injection techniques, the carotid bolus is assumed not to mix with blood from the point of injection to the brain capillaries.[20,23] However, this has never been proven. Conversely, in the case of the *in situ* model, the **contribution of the systemic blood flow** to the right cerebral hemisphere is known to be less than 5% of total flow during perfusion.
8. Radiotracer compounds can undergo metabolism by tissues other than the brain. These radiotracer metabolites can readily enter the brain and may contribute to brain radioactivity, providing a false representation of transport. The *in situ* model **avoids errors due to radiotracer metabolism**.[24]

As previously mentioned, nicotine affects BBB function and morphology, and therefore it is essential that studies be performed on the uptake of nicotine in animals that are treated chronically with nicotine. However, limited and conflicting data[2,12] are present in the literature regarding cotinine BBB penetration in animals subject to chronic nicotine exposure. Although cotinine has been detected in brain after nicotine exposure,[10-12] indirect data from literature suggest that the presence of cotinine in brain may be the result of central nicotine metabolism by CYP2B1 in rats.[3]

Our objectives were to determine the blood-brain transfer of cotinine in our robust *in situ* brain perfusion model and to compare the kinetics with that of nicotine. Further, the degree and rate of cotinine (as well as nicotine) brain uptake were evaluated in naive and chronically nicotine treated rats using the same model.

3.2. Experimental procedures

3.2.1. Materials

High specific activity [^{3}H]nicotine (10 Ci/mmol, >98% purity) was obtained from Tocris Cookson Inc. (MO, USA), [^{3}H]cotinine from American Radiolabeled Chemicals, Inc. (MO, USA) and [^{14}C]sucrose (4.75 mCi/mmol) from Dupont-New England Nuclear (MA, USA). [^{3}H]Nicotine and [^{3}H]cotinine were dried prior to being dissolved in perfusion buffer to remove volatile tritium contaminants including $^3H_20$.

3.2.2. Animals

Male Fisher-344 rats, 220 – 330 g were purchased from Charles River (NC, USA). Animals were housed in pairs in standard polypropylene cages in a vivarium maintained at approximately 20 °C – 22 °C and 50% humidity with a 12 h light/dark cycle. Filtered water and rodent chow (Laboratory Animal Resource Center, Lubbock, TX, USA) were available *ad libitum* throughout the study. All studies were approved by the Animal Care and Use Committee of Texas Tech University Health Sciences Center (TX, USA; protocol number 04-010-04) and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

3.2.3. Nicotine administration through osmotic mini-pump

Osmotic mini-pumps (2ML4, 28 day, capacity: 2.098 ml; Durect Corporation, CA, USA) were loaded with (*S*)-nicotine (4.8 mg/kg/day; dissolved in sterile saline) or 0.9% saline as control. Chronic nicotine administration was comparable to previous methodology that provided nicotine and cotinine blood levels similar to those found in heavy smokers.[30] Prior to implantation, osmotic mini-pumps were primed in sterile saline at 37 °C for 24 h according to the manufacturer's specifications. The osmotic mini-pumps were then implanted interscapularly[31] into the rats.

In brief, the surgical procedure entailed the following: the animals were sedated with sodium pentobarbital (50 mg/kg) after which a small thoracolumbar subcutaneous incision (~3.5 cm) was made and a pocket projecting caudally, large enough to accommodate the osmotic mini-pump, was opened using a blunt hemostat. Manual insertion of the osmotic mini-pump was completed under sterile conditions and the wound closed with surgical staples. Rats were monitored during recovery after which they were eating and grooming normally with *ad libitum* access to food and water. The contents of the osmotic mini-pumps were released over a period of 28 days at a rate of 2.5 µL/hr. Nicotine levels (~72 ng/ml) were verified by HPLC in a subset of animals on day 28.

3.2.4. Perfusion procedure

After 28 days of nicotine exposure, animals were anesthetized with sodium pentobarbital (50 mg/kg; intraperitoneal). The brain uptake of [^{3}H]nicotine and [^{3}H]cotinine was assessed using the *in situ* rat brain perfusion technique of Takasato *et al.* (1984) and Smith & Allen (2003)[24,25] with modifications described by Smith (1996) and Allen & Smith (2001).[26,29] A polyethylene-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[26] (figure 3.2). 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 7.4 (osmolarity ~290 mOsm; verified) and contained (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.

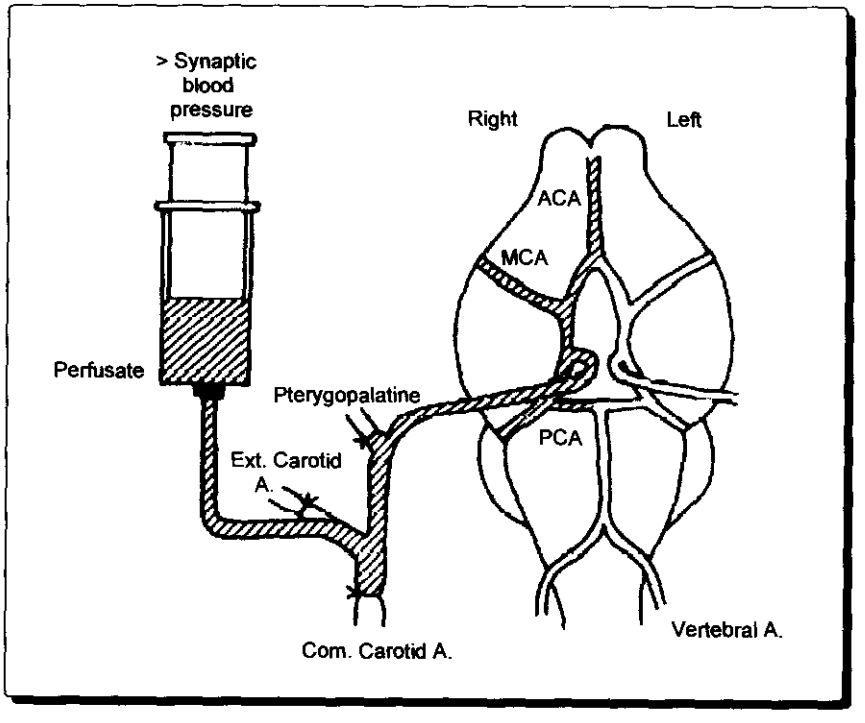


Figure 3.2: Diagram of i*n situ* brain perfusion technique of right cerebral hemisphere in the rat.[24]

The perfusion fluid was infused into the left carotid artery via infusion pump for 60 s at 10 ml/min (Harvard Apparatus, MA, USA). 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, CA, USA).

3.2.5. 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[25] from the following relationship:

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

Where, Q^* = *quantity of tracer in brain (dpm/g) at the end of perfusion*

C^* = *concentration of tracer in perfusion fluid (dpm/ml)*

Unidirectional uptake transfer constants, or K_{in}, were then calculated from brain distribution volume versus time, using linear regression analysis,[25] by equation 2:

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

Where, K_{in} = *unidirectional uptake transfer*

T = *perfusion time (s)*

Vo = *extrapolated intercept (T = 0 s; "vascular volume" in ml/g)*

Tracer trapped in the vascular space was accounted for by subtracting the vascular volume (concurrently measured with [^{14}C]sucrose).

Cerebral perfusion flow rate (F) was determined and was in agreement with previously published values (data not shown).[32] K_{in} values were then converted to apparent cerebrovascular permeability-surface area products (PA) using the Crone-Renkin equation below:[25]

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

Where, PA = permeability-surface area products

F = cerebral perfusion flow rate

3.2.6. 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 (95% confidence level), and regional data were evaluated by one-way ANOVA analysis followed by Bonferroni's multiple comparison test. *A priori,* differences were considered statistically significant at $p < 0.05$. Errors are reported as standard error of mean (SEM) (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA).

3.3. Results

Brain uptake of [^{3}H]nicotine was evaluated with short perfusions of 15 s (figure 3.3A and 3.2) to prevent significant brain to blood efflux (i.e., unilateral uptake) and metabolism of nicotine to cotinine. No significant difference in total brain [^{3}H]nicotine uptake (cerebral perfusion flow rates) were noted between naive ($3.11 \pm 0.42 \times 10^{-2}$ ml/s/g) and nicotine treated ($3.31 \pm 1.1 \times 10^{-2}$ ml/s/g) rats (figure 3.3A). To further verify that chronic nicotine exposure did not alter flow, we also evaluated [^{3}H]diazepam perfusions.[32] As seen in figure 3.3B, no apparent flow alterations were noted between control ($5.11 \pm 0.98 \times 10^{-2}$ ml/s/g) and nicotine exposure ($4.61 \pm 0.81 \times 10^{-2}$ 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.[25,32]

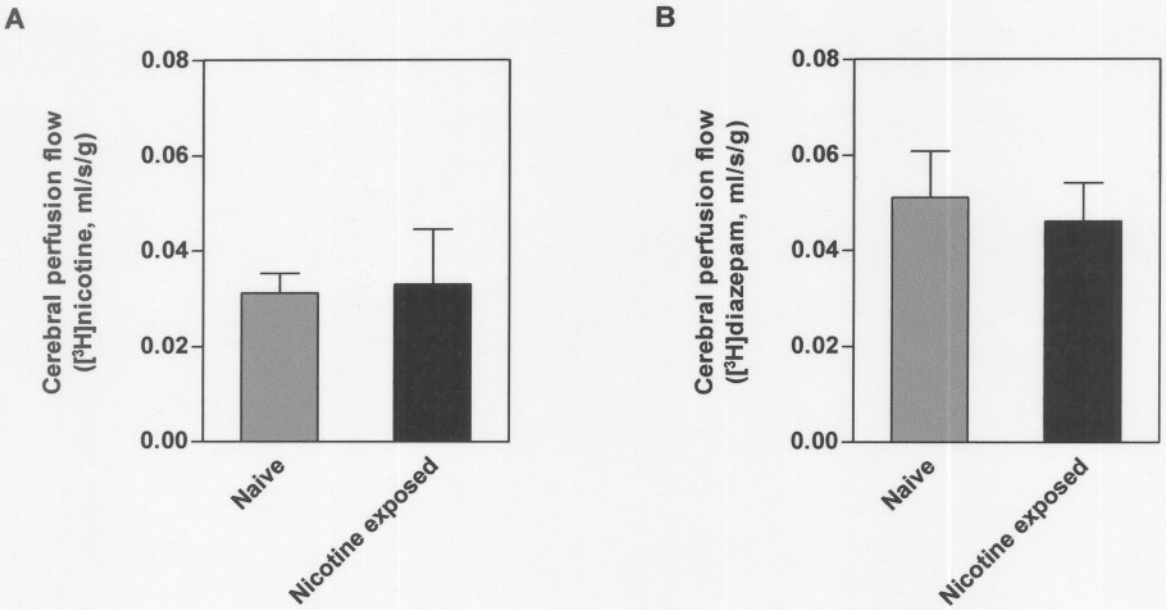


Figure 3.3: Cerebral perfusion flow as measured by the brain uptake of [^{3}H]diazepam (**A**) and [^{3}H]nicotine (**B**) in naive and nicotine treated rats (28 days x 4.8 mg/kg/day). No significant differences are noted between groups. Data suggest that chronic nicotine exposure does not result in increased cerebral perfusion flow or movement of [^{3}H]nicotine across the BBB. All data represent mean + SEM for total brain; n = 6 for both points.

Regional brain analyses confirmed the lack of significant differences in [^{3}H]nicotine uptake into brain between naive and nicotine treated animals (figure 3.4). [^{3}H]Nicotine uptake in this study showed a similar pattern of cerebral perfusion flow commonly found in *in situ* perfused brain, where flow rates are found to be highest in the cortical regions ($4.02 \pm 0.59 \times 10^{-2}$ ml/s/g), and lower in the cerebellum ($1.39 \pm 0.40 \times 10^{-2}$ ml/s/g) and pons medulla ($0.94 \pm 0.21 \times 10^{-2}$ ml/s/g).[25]

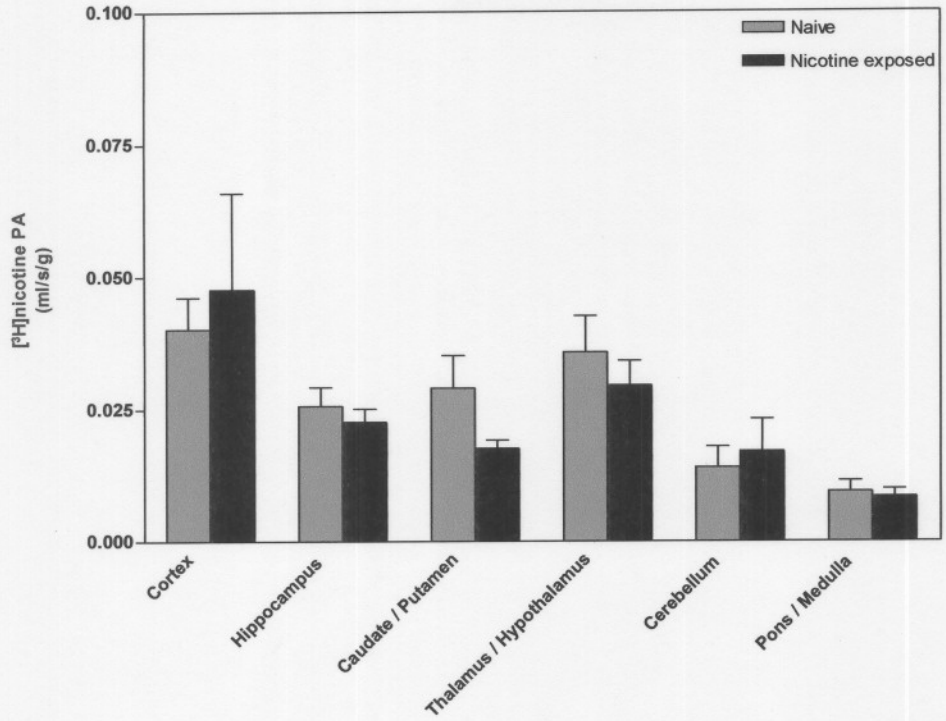


Figure 3.4: Regional brain uptake of [^{3}H]nicotine in naive rats and in nicotine treated rats (28 days x 4.8 mg/kg/day). No significant changes were noted throughout all brain regions. These data support those shown in figure 3.3 in that chronic nicotine exposure does not appear to increase [^{3}H]nicotine uptake across the BBB. All data represent mean + SEM; n = 6 for all points.

Considering that [^{3}H]cotinine brain uptake is approximately 10 fold less than that of [^{3}H]nicotine, the 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 figure 3.5A and B, the total brain uptake of [^{3}H]cotinine in animals subjected to chronic nicotine exposure ($2.07 \pm 0.25 \times 10^{-3}$ ml/s/g) was found not to be significantly altered from control in whole brain ($2.03 \pm 0.17 \times 10^{-3}$ ml/s/g) or regionally (figure 3.6).

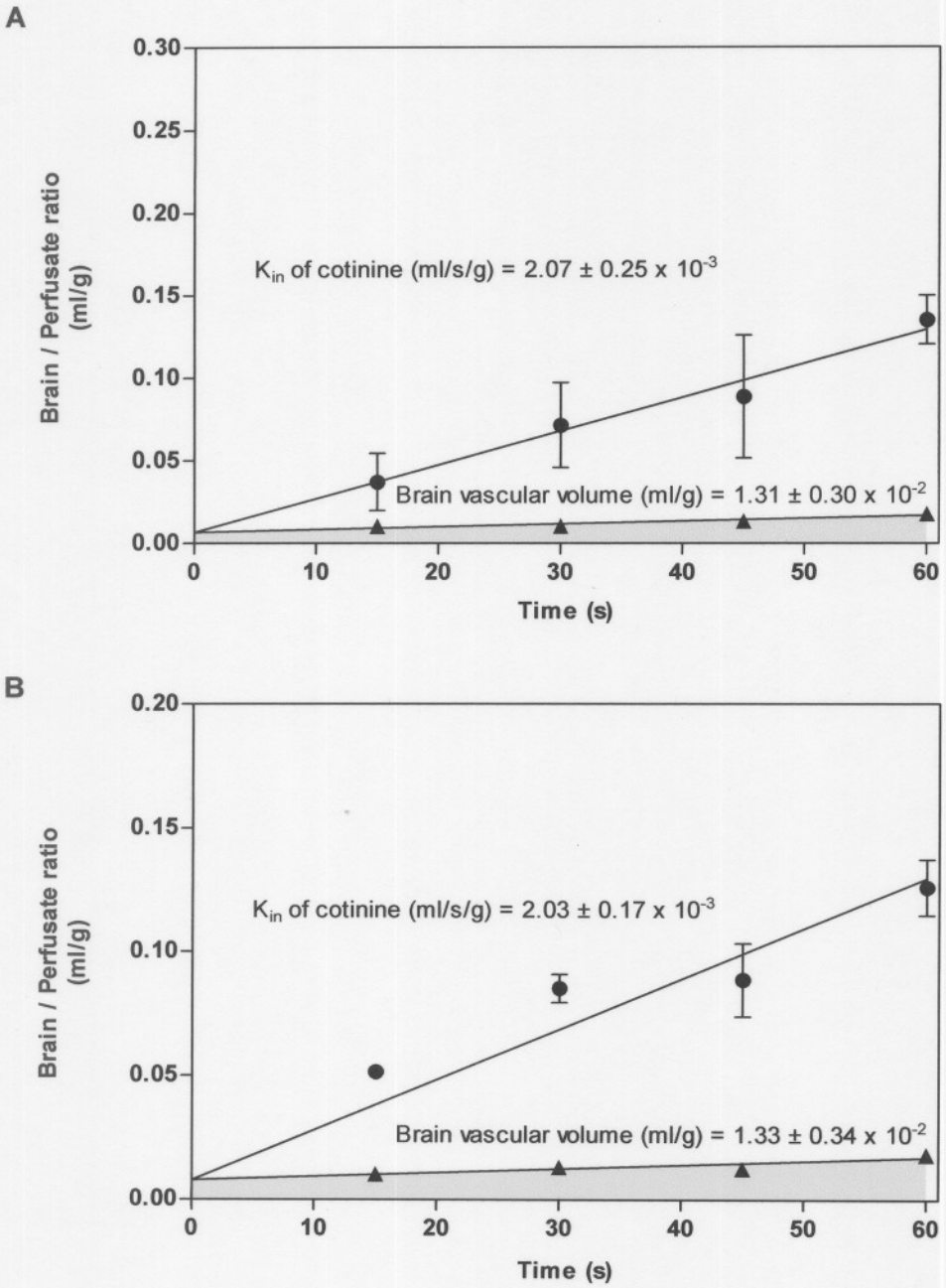


Figure 3.5: Time course of [^{3}H]cotinine brain uptake and cerebrovascular volume in naive rats (**A**) and in nicotine treated rats (**B**) (28 days x 4.8 mg/kg/day). Calculation of [^{3}H]cotinine K_{in} is based upon linear regression of brain distribution volume per time. No significant differences are noted between groups. Data suggest that chronic nicotine exposure does not result in increased movement of [^{3}H]cotinine across the BBB. All data represent mean ± SEM for total brain; n = 3-5 for all points (where SEM is not visible, the SEM is contained within the symbol).

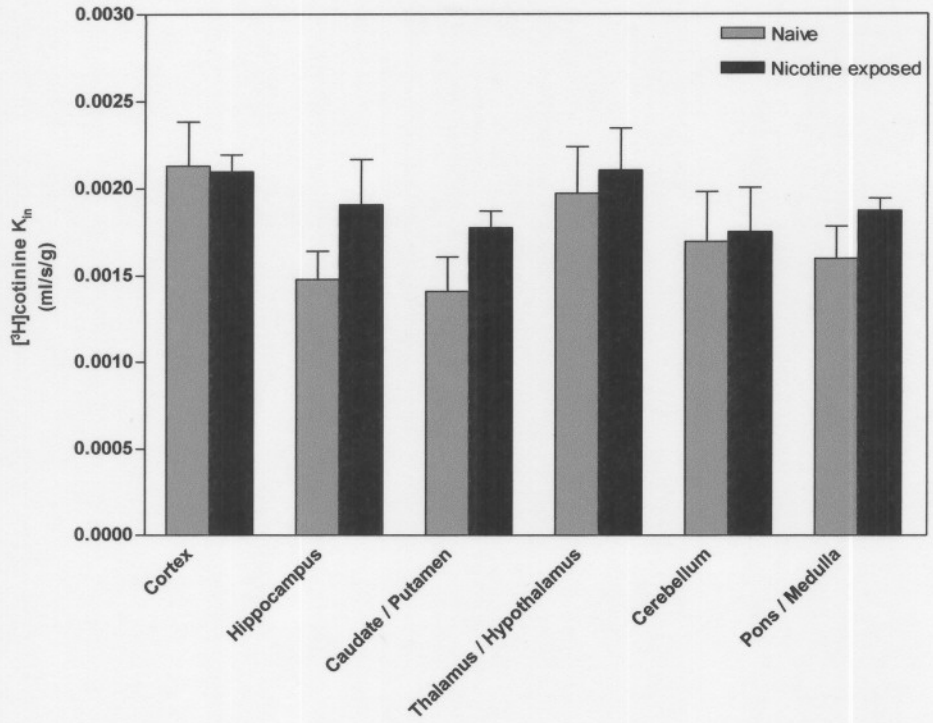


Figure 3.6: Regional brain uptake of [^{3}H]cotinine in naive rats and in nicotine treated rats (28 days x 4.8 mg/kg/day). Similar to whole brain studies, no significant changes were noted. All data represent mean + SEM; n = 3-5 for all points.

In contrast to [^{3}H]nicotine regional data where flow differences result in changes of uptake between cortical and subcortical regions, [^{3}H]cotinine is a permeability limited compound and regional alterations of [^{3}H]cotinine brain uptake are minimized (cortex: 2.13 $\pm$ 0.26 x 10^{-3} ml/s/g; pons medulla: 1.60 $\pm$ 0.19 x 10^{-3} ml/s/g).

Vascular volume measurements after chronic nicotine exposure (using the impermeable marker [^{14}C]sucrose) were also assessed concurrently in all experiments to determine BBB integrity (i.e., increased vascular volumes would indicate BBB disruption). Preliminary experiments in our laboratory demonstrated that control osmotic mini-pump (containing 0.9% saline) implantation procedures had no effect on baseline vascular volume (data not shown). 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 treated groups (1.31 $\pm$ 0.30 x 10^{-2} ml/g) (figure 3.5A and B). Also, no regional alterations in vascular volume were noted (figure 3.7).

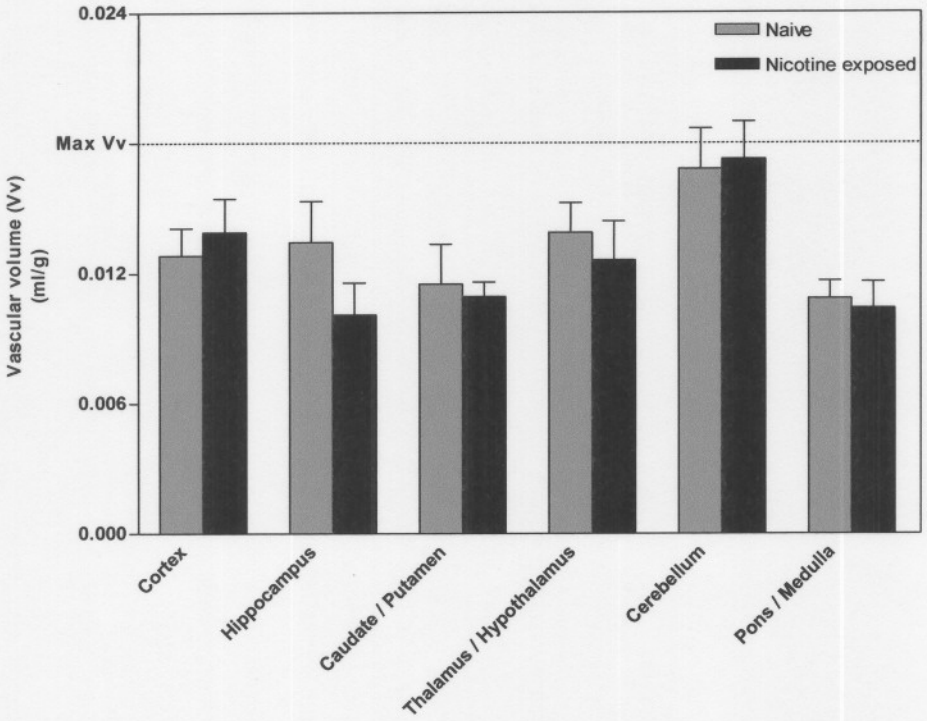


Figure 3.7: Regional vascular volume (measured by [^{14}C]sucrose) in naive rats and in nicotine treated rats (28 days x 4.8 mg/kg/day). [^{14}C]sucrose does not penetrate the BBB in the time frames evaluated and therefore accurately measures BBB integrity. No significant changes were noted between naive rats and in nicotine treated groups. All data represent mean + SEM; n = 3-5 for all points.

3.4. Discussion

Data presented in the current study demonstrate that, in accordance with our hypotheses: 1) [^{3}H]cotinine penetrates the BBB, 2) [^{3}H]nicotine BBB uptake into brain is not altered after chronic nicotine exposure as measured in the *in situ* perfusion model, and 3) similar to the data found for [^{3}H]nicotine, [^{3}H]cotinine BBB transfer is not altered by chronic nicotine exposure. To our knowledge this is the first report detailing brain uptake of nicotine and cotinine following chronic *in vivo* nicotine exposure and also a thorough rate determination study of cotinine BBB penetration (figure 3.8).

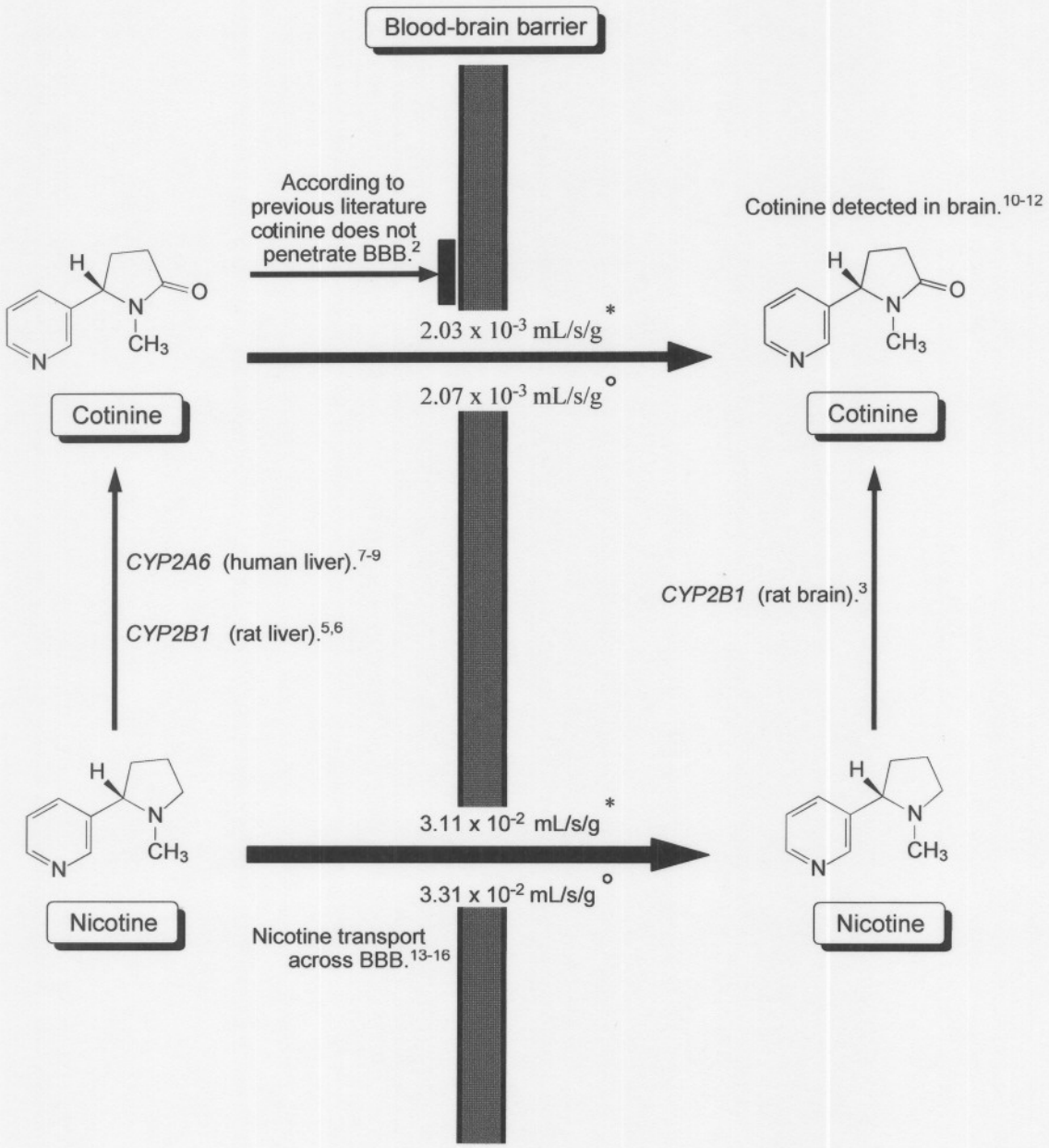


Figure 3.8: An illustration depicting previous work as well as our results from this study which found that cotinine is transferred across the BBB. (A * indicates results of this study in naive rats and ^{O} for nicotine treated rats.)

Chronic tobacco exposure decreases cerebral blood flow[33] and accordingly diminishes BBB transfer of compounds that are flow dependent (i.e., extraction approximately ≥ 80%). Therefore, to determine if nicotine alone has a similar effect, we evaluated the uptake of [^{3}H]diazepam in both

naive and chronic nicotine treated animals. It appears from our data that 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.[34] The authors suggested several rationales for the increased nicotine brain distribution including alterations in BBB transfer rates, increased nAChR binding, and sequestering of nicotine in glial cytosol through its protonation (pKa =9.13, glial cytosol pH ~ 6.5-7.0).[34] 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% of flow ([^{3}H]diazepam uptake) and consequently should only be minimally influenced by flow alterations. Therefore, if chronic nicotine exposure resulted in BBB alterations that may influence blood-brain transfer of [^{3}H]nicotine, such influences should be apparent in our model. However, our findings suggest that there is no alteration of [^{3}H]nicotine brain uptake after chronic nicotine exposure either in whole brain or in any measured brain region. Therefore, we suggest that the increased CNS distribution of nicotine seen in previous studies may be from the result of either increased neuronal binding or glial sequestering.

Nicotine primarily undergoes liver aldehyde oxidation via CYP2A6 in humans or CYP2B1 in rats to form cotinine,[8,9,35,36] the major metabolite of nicotine. There is controversy as to whether cotinine penetrates the BBB from plasma[2,12] or whether the detection of cotinine in brain is solely the result of central nicotine metabolism. Recently, nicotine has been shown to be metabolized via CYP2B1 in rat brain and this process is upregulated after chronic nicotine exposure[3] (though the amount of nicotine metabolism in brain has not been elucidated). However, if cotinine can transfer into brain in any reasonable amounts, the CYP2B1 mediated pathway may be of lesser importance. Therefore, in order to determine whether brain cotinine concentrations are also altered by BBB transfer in naive and nicotine treated animals, we evaluated the brain uptake of [^{3}H]cotinine.

Surprisingly, and contrary to previous reports our data demonstrate that there is significant BBB transfer of cotinine that is generally homogeneous among brain regions. The PA of [^{3}H]cotinine is consistent for BBB passive diffusion of a molecule with a logP of 0.04.[34,37] This rate of [^{3}H]cotinine BBB penetration is significant considering that it is comparable to other neuroactive molecules including theophylline, adenosine and choline.[29,37]

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.[38,39] Calculation of influx (influx = C x PA) reveals that cotinine enters brain at a rate of 0.5-0.7 ng/s/g or ~43-61 µg/g/day. On the other hand, nicotine plasma levels vary significantly, between 10-50 ng/ml in smokers,[40,41] following increment peaks of 5-30 ng/ml per cigarette (t½ ~ 2 h).[42,43] Therefore, assuming that the highest average nicotine plasma level is 40 – 50 ng/ml over a 24 h period, the BBB influx of nicotine should approximately be 1.32 -1.65 ng/s/g or 114 – 143 µg/g/day. Comparison of influx data measured for the two compounds suggests that cotinine enters the brain at amounts approximately 40% of 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 activity.

Data presented in the current study also demonstrate that vascular volumes are not altered after chronic nicotine exposure. These data are consistent with other reports, demonstrating that chronic physiologically relevant nicotine exposure does not alter BBB integrity of epithelium[44] or endothelium *in vivo*.[45-48] Taken together, these data suggest that chronic nicotine treated animals retain an intact BBB. In summary, our data demonstrate that both nicotine and cotinine significantly penetrate the BBB, and that transfer rates are not affected by chronic heavy nicotine exposure.

Our next aim was to determine the effect of nicotine, cotinine, TMN, nicotine-containing smoke extract and nicotine-free smoke extract on biomarkers of the dopaminergic system *in vitro*.

3.5. References

1. Kibiuk, L. 1998. Brain briefings: Nicotine and the brain. [Web:] http://www.sfn.org/content/Publications/BrainBriefings/nicotine.html#fullsize [Date of access: 12 October 2004].

2. Halldin, C., Nagren, K., Swahn, C.G. & others. 1992. (S)- and (R)-[11C]nicotine and the metabolite (R/S)-[11C]cotinine. Preparation, metabolite studies and in vivo distribution in the human brain using PET. *Int J Rad Appl Instrum B*, 19: 871 - 880.

3. Miksys, S., Hoffmann, E. & Tyndale, R.F. 2000. Regional and cellular induction of nicotine-metabolizing CYP2B1 in rat brain by chronic nicotine treatment. *Biochem Pharmacol*, 59: 1501 - 1511.

4. Hyperchem 7. 2004. Hypercube Inc. 1115 NW 4th St., Gainesville, Florida, 32601, USA.

5. Hammond, D.K., Bjercke, R.J., Langone, J.J. & others. 1991. Metabolism of nicotine by rat liver cytochromes P-450. Assessment utilizing monoclonal antibodies to nicotine and cotinine. *Drug Metab Dispos*, 19: 804 - 808.

6. Nakayama, H., Okuda, H., Nakashima, T. & others. 1993. Nicotine metabolism by rat hepatic cytochrome P450s. *Biochem Pharmacol*, 45: 2554 - 2556.

7. Benowitz, N.L., Jacob, P., III, Fong, I. & others. 1994. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. *J Pharmacol Exp Ther*, 268: 296 - 303.

8. Messina, E.S., Tyndale, R.F. & Sellers, E.M. 1997. A major role for CYP2A6 in nicotine C-oxidation by human liver microsomes. *J Pharmacol Exp Ther*, 282: 1608 - 1614.

9. Nakajima, M., Yamamoto, T., Nunoya, K. & others. 1996. Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. *J Pharmacol Exp Ther*, 277: 1010 - 1015.

10. Crooks, P.A. & Dwoskin, L.P. 1997. Contribution of CNS nicotine metabolites to the neuropharmacological effects of nicotine and tobacco smoking. *Biochem Pharmacol*, 54: 743 - 753.

11. Crooks, P.A., Li, M. & Dwoskin, L.P. 1997. Metabolites of nicotine in rat brain after peripheral nicotine administration. Cotinine, nornicotine, and norcotinine. *Drug Metab Dispos*, 25: 47 - 54.

12. Riah, O., Courriere, P., Dousset, J.C. & others. 1998. Nicotine is more efficient than cotinine at passing the blood-brain barrier in rats. *Cell Mol Neurobiol*, 18: 311 - 318.

13. Ohno, K., Pettigrew, K.D. & Rapoport, S.I. 1979. Local cerebral blood flow in the conscious rat as measured with 14C-antipyrine, 14C-iodoantipyrine and 3H-nicotine. *Stroke*, 10: 62 - 67.

14. Suzuki, R., Nitsch, C., Fujiwara, K. & others. 1984. Regional changes in cerebral blood flow and blood-brain barrier permeability during epileptiform seizures and in acute hypertension in rabbits. *J Cereb Blood Flow Metab*, 4: 96 - 102.

15. Todd, M.M. & Weeks, J. 1996. Comparative effects of propofol, pentobarbital, and isoflurane on cerebral blood flow and blood volume. *J Neurosurg Anesthesiol*, 8: 296 - 303.

16. Tomiyama, Y., Brian, J.E., Jr. & Todd, M.M. 1999. Cerebral blood flow during hemodilution and hypoxia in rats : role of ATP-sensitive potassium channels. *Stroke*, 30: 1942 - 1947.

17. Andjus, R.K., Suhara, K. & Sloviter, H.A. 1967. An isolated, perfused rat brain preparation, its spontaneous and stimulated activity. *J Appl Physiol*, 22: 1033 - 1039.

18. Crone, C. 1965. The permeability of brain capillaries to non-electrolytes. *Acta Physiol Scand*, 64: 407 - 417.

19. Ohno, K., Pettigrew, K.D. & Rapoport, S.I. 1978. Lower limits of cerebrovascular permeability to nonelectrolytes in the conscious rat. *Am J Physiol*, 235: H299 - H307.

20. Oldendorf, W.H. 1971. Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am J Physiol*, 221: 1629 - 1639.

21. Patlak, C.S. & Fenstermacher, J.D. 1975. Measurements of dog blood-brain transfer constants by ventriculocisternal perfusion. *Am J Physiol*, 229: 877 - 884.

22. Raichle, M.E., Eichling, J.O., Straatmann, M.G. & others. 1976. Blood-brain barrier permeability of 11C-labeled alcohols and 15O-labeled water. *Am J Physiol*, 230: 543 - 552.

23. Fenstermacher, J.D., Blasberg, R.G. & Patlak, C.S. 1981. Methods for quantifying the transport of drugs across brain barrier systems. *Pharmacol Ther*, 14: 217 - 248.

24. Takasato, Y., Rapoport, S.I. & Smith, Q.R. 1984. An in situ brain perfusion technique to study cerebrovascular transport in the rat. *Am J Physiol*, 247: H484 - H493.

25. Smith, Q.R. & Allen, D.D. 2003. In situ brain perfusion technique. *Methods Mol Med*, 89: 209 - 218.

26. Allen, D.D. & Smith, Q.R. 2001. Characterization of the blood-brain barrier choline transporter using the in situ rat brain perfusion technique. *J Neurochem*, 76: 1032 - 1041.

27. Momma, S., Aoyagi, M., Rapoport, S.I. & others. 1987. Phenylalanine transport across the blood-brain barrier as studied with the in situ brain perfusion technique. *J Neurochem*, 48: 1291 - 1300.

28. Smith, Q.R. & Nagura, H. 2001. Fatty acid uptake and incorporation in brain: studies with the perfusion model. *J Mol Neurosci*, 16: 167 - 172.

29. Smith, Q.R. 1996. Brain perfusion systems for studies of drug uptake and metabolism in the central nervous system. *Pharm Biotechnol*, 8: 285 - 307.

30. Hawkins, B.T., Abbruscato, T.J., Egleton, R.D. & others. 2004. Nicotine increases in vivo blood-brain barrier permeability and alters cerebral microvascular tight junction protein distribution. *Brain Res*, 1027: 48 - 58.

31. Castagnoli, K., Steyn, S.J., Magnin, G. & others. 2002. Studies on the interactions of tobacco leaf and tobacco smoke constituents and monoamine oxidase. *Neurotox Res*, 4: 151 - 160.

32. Lockman, P.R., Koziara, J., Roder, K.E. & others. 2003. In vivo and in vitro assessment of baseline blood-brain barrier parameters in the presence of novel nanoparticles. *Pharm Res*, 20: 705 - 713.

33. Rogers, R.L., Meyer, J.S., Shaw, T.G. & others. 1983. Cigarette smoking decreases cerebral blood flow suggesting increased risk for stroke. *JAMA*, 250: 2796 - 2800.

34. Ghosheh, O.A., Dwoskin, L.P., Miller, D.K. & others. 2001. Accumulation of nicotine and its metabolites in rat brain after intermittent or continuous peripheral administration of [2'-(14)C]nicotine. *Drug Metab Dispos*, 29: 645 - 651.

35. Dempsey, D., Tutka, P., Jacob, P., III & others. 2004. Nicotine metabolite ratio as an index of cytochrome P450 2A6 metabolic activity. *Clin Pharmacol Ther*, 76: 64 - 72.

36. Yamanaka, H., Nakajima, M., Nishimura, K. & others. 2004. Metabolic profile of nicotine in subjects whose CYP2A6 gene is deleted. *Eur J Pharm Sci*, 22: 419 - 425.

37. Smith, Q.R. 2003. A review of blood-brain barrier transport techniques. *Methods Mol Med*, 89: 193 - 208.

38. Benowitz, N.L., Hall, S.M., Herning, R.I. & others. 1983. Smokers of low-yield cigarettes do not consume less nicotine. *N Engl J Med*, 309: 139 - 142.

39. Paoletti, P., Fornai, E., Maggiorelli, F. & others. 1996. Importance of baseline cotinine plasma values in smoking cessation: results from a double-blind study with nicotine patch. *Eur Respir J*, 9: 643 - 651.

40. Russell, M.A. & Feyerabend, C. 1978. Cigarette smoking: a dependence on high-nicotine boli. *Drug Metab Rev*, 8: 29 - 57.

41. Benowitz, N.L., Kuyt, F. & Jacob, P., III. 1982. Circadian blood nicotine concentrations during cigarette smoking. *Clin Pharmacol Ther*, 32: 758 - 764.

42. Armitage, A.K., Dollery, C.T., George, C.F. & others. 1975. Absorption and metabolism of nicotine from cigarettes. *Br Med J*, 4: 313 - 316.

43. Isaac, P.F. & Rand, M.J. 1972. Cigarette smoking and plasma levels of nicotine. *Nature*, 236: 308 - 310.

44. Minty, B.D., Royston, D., Jones, J.G. & others. 1984. The effect of nicotine on pulmonary epithelial permeability in man. *Chest*, 86: 72 - 74.

45. Booyse, F.M., Osikowicz, G. & Quarfoot, A.J. 1981. Effects of chronic oral consumption of nicotine on the rabbit aortic endothelium. *Am J Pathol*, 102: 229 - 238.

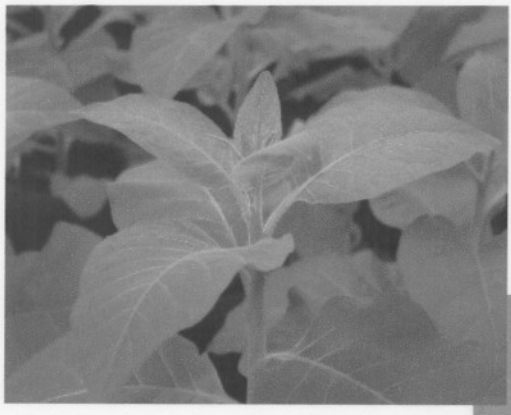
46. Allen, D.R., Browse, N.L., Rutt, D.L. & others. 1988. The effect of cigarette smoke, nicotine, and carbon monoxide on the permeability of the arterial wall. *J Vasc Surg*, 7: 139 - 152.

47. Myers, T.O., Joyner, W.L. & Gilmore, J.P. 1988. Extravasation of macromolecules and vascular reactivity of microvessels in response to nicotine in the hamster. *Int J Microcirc Clin Exp*, 7: 139 - 153.

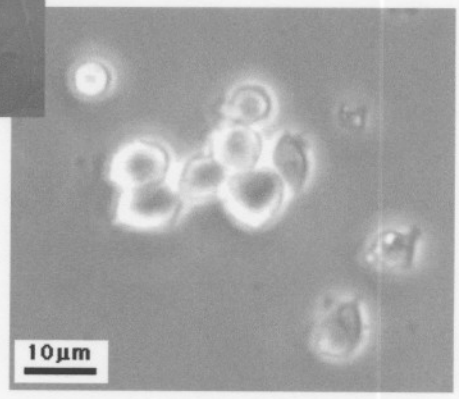
48. Mayhan, W.G. & Sharpe, G.M. 1998. Nicotine impairs histamine-induced increases in macromolecular efflux: role of oxygen radicals. *J Appl Physiol*, 84: 1589 - 1595.

CHAPTER 4:

IN VITRO EFFECTS OF TOBACCO SMOKE CONSTITUENTS ON THE REGULATION OF TYROSINE HYDROXYLASE AND DOPAMINE TRANSPORTER IN PC12 CELLS



Tobacco plant[1]



Undifferentiated pheochromocytoma cells.[2]

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LIST OF ABBREVIATIONS

ATR/FT-IR	Attenuated total reflectance Fourier transform infrared spectroscopy
CSE	cigarette smoke extract
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
N-CSE	nicotine containing cigarette smoke extract (Marlboro®)
NF-CSE	nicotine-free cigarette smoke extract (Quest®)
PC12	pheochromocytoma
PG	propylene glycol
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulphate
TTBS	Tween Tris buffer saline

4.1. Introduction

In our previous experiments we found that blood to brain transfer of cotinine and nicotine is not affected after chronic nicotine exposure. Subsequently we wanted to investigate the effect of the readily available nicotine and cotinine in brain, as well as cigarette smoke extracts (CSEs) on TH and DAT regulation, important factors in the dopaminergic system.

Our *in vitro* and subsequently our *in vivo* (chapter 5) experiments were conducted, with CSEs from Marlboro® filter and Quest® 3 cigarettes, among other compounds. Marlboro® filter cigarettes, a well known nicotine containing cigarette has been on the market for 50 years[3] as opposed to Quest® 3 that has only been on the market for less than 2 years and is only available in 8 of the 50 states in the USA.[4] Vector Tobacco Inc., an innovative tobacco research company, developed Quest® cigarettes through genetic engineering. The basic technology of reduced-nicotine tobacco was developed in the mid 1990s by Dr. Mark Conkling of North Carolina State University. Through genetic engineering, tobacco plants are created that produce dramatically reduced levels (approaching zero content) of nicotine and nitrosamines.[5]

Vector Tobacco Inc. launched three Quest® cigarettes that contain different levels of nicotine, ranging from 0.6 mg (Quest® 1), 0.3 mg (Quest® 2) and no more than 0.05 mg (Quest® 3) nicotine. Quest® 1 has 17% less nicotine than an average "light" cigarette whereas Quest® 2 has 58% less nicotine and Quest® 3 is regarded as nicotine-free. The Quest® range of cigarettes are not marketed as a smoking-cessation product, but rather is "aimed at smokers who seek to reduce their nicotine exposure". Through this marketing maneuver, Quest® is allowed on the market without the extensive testing required by the Food and Drug Administration (FDA) for approved smoking cessation products.

Tobacco itself consists of over 4,000 chemical constituents. When a cigarette is lit, a burning zone is created at the end of the cigarette. The chemical constituents are then subject to temperatures ranging from ambient to approximately 950 °C in the presence of varying concentrations of oxygen.[6] Puffing and the smoldering between puffs are classified as the two types of burning that occur within a cigarette. Mainstream smoke is formed during puffing when air is drawn into the cigarette through the burning zone. There are several mechanisms that occur in the burning zone which contribute to the generation of smoke components, including combustion, pyrolysis, distillation and aerosol particle formation.[6]

The interior of the burning zone can be divided into the exothermic combustion zone and the endothermic pyrolysis/distillation zone. Figure 4.1 provides a schematic representation of the major smoke formation mechanisms that occur inside the cigarette.

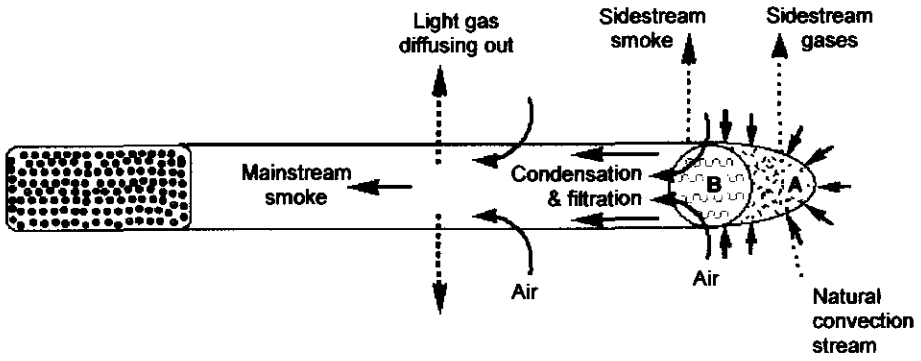

Figure 4.1: A schematic representation of processes involved in the burning cigarette, where A depicts the combustion zone and B the pyrolysis and distillation zone. [Modified from Baker (1987).[7]]

(*S*)-Nicotine is the major stereoisomer of nicotine found in tobacco products that binds stereoselectively to nAChRs.[8,9] (*R*)-Nicotine is present in small quantities in cigarette smoke, due to racemization during the pyrolysis process and is a weak nAChR agonist.[9]

The potential contribution of cigarette smoke components including the major nicotine metabolite, cotinine is of particular importance to the neuropharmacological effects resulting from tobacco exposure. Therefore, to reiterate, our hypothesis states that nicotine and nicotine-containing CSE (N-CSE, Marlboro® filter cigarettes), but not cotinine, TMN or nicotine-free CSE (NF-CSE, Quest® 3 cigarettes) upregulate TH and DAT *in vitro* in PC12 cells (chapter 2, section 2.5). PC12 cells were chosen for this study since this cell line exhibits several important characteristics including the presence of nAChRs[10-13] and the synthesis and release of DA.[14,15] This is of particular importance since TH and DAT[16] are plentiful in dopaminergic terminals of the rat striatum where we will be able to correlate our *in vitro* data with our *in vivo* data. The PC12 cell line, therefore serves as an excellent model to examine the physiological regulation of TH and DAT.

4.2. Experimental procedures

4.2.1. Materials

PC12 cells were obtained from the American Type Culture Collection (ATCC, MD, USA). RPMI 1640 media and streptomycin/penicillin were purchased from Gibco (MD, USA). Horse serum and fetal bovine serum were obtained from JHR Biosciences (KS, USA). Poly-DL-Lysine, (*S*)-nicotine,

(*S*)-cotinine and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, USA). Propylene glycol (PG) was obtained from Fisher (PA, USA). Marlboro® filter (Philip Morris, Inc., VA, USA) and Quest® 3 cigarettes (Vector Tobacco Inc., NC, USA) were obtained from commercial sources.

4.2.2. Preparation of smoke extracts

Commercial cigarettes (Marlboro® filter cigarettes, Philip Morris Inc., VA, USA and Quest® 3, Vector Tobacco Inc., NC, USA) were smoked continuously by the apparatus shown in figure 4.2. Marlboro® filter cigarettes contain 1.1 mg nicotine per cigarette and Quest® 3 contains no more than 0.05 mg nicotine per cigarette and is therefore claimed to be a "nicotine-free" product (verified in our laboratory, data not shown).

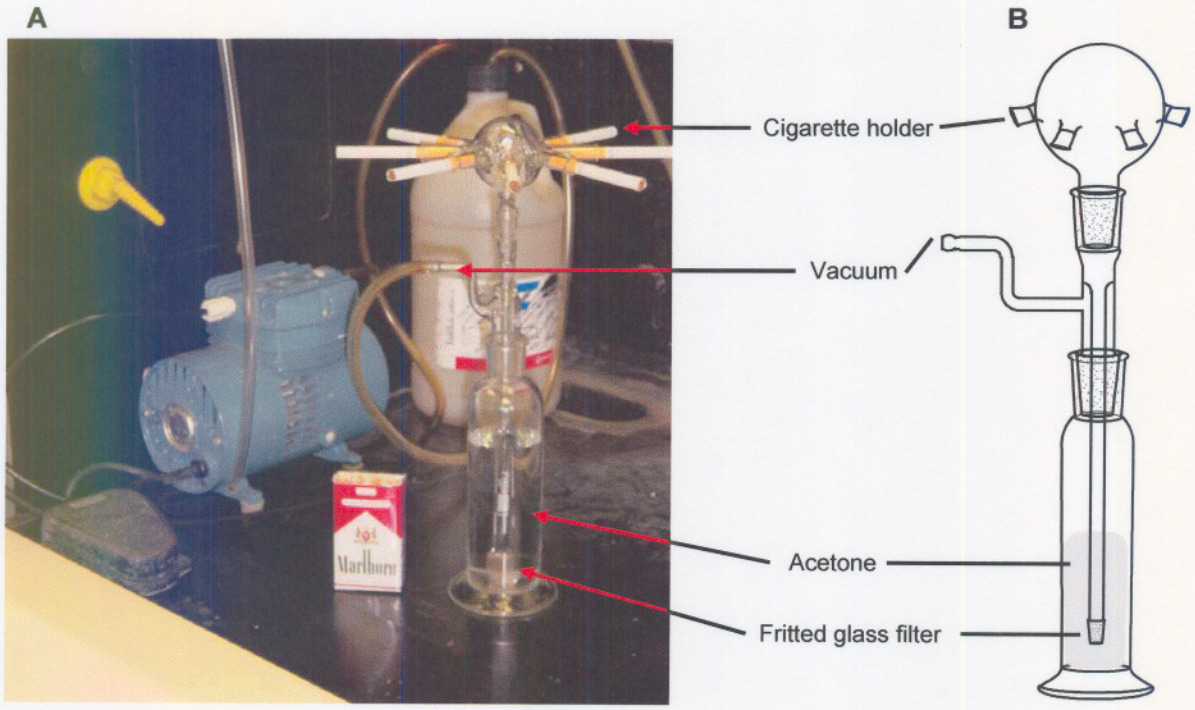

Figure 4.2: Photograph (**A**) and schematic representation (**B**) of the apparatus used in the preparation of the CSEs.

Mainstream smoke was drawn through 250 ml acetone by application of a vacuum to the acetone containing vessel (see figure 4.2). Initially, vacuum generated by the vacuum pump was too strong when directly connected to the smoke machine. Introducing a second chamber between the vacuum pump and smoke machine to attenuate the vacuum's intensity solved this problem. The

apparatus can hold up to eight cigarettes simultaneously. To simulate a smoker, each cigarette was smoked for a total of 5 min with alternating increments of puffing (3 s) and rest (30 s). A CSE-acetone solution was generated which was evaporated under vacuum to yield the smoke condensate residue. The residue was subsequently re-dissolved in PG/dimethyl sulfoxide (PG/DMSO, 1:1) to ensure a "high-dose" smoke condensate (120 mg/ml).[17] The products of the Marlboro® and Quest® cigarette extraction were designated as nicotine containing CSE (N-CSE) and nicotine-free CSE (NF-CSE) respectively.

4.2.3. Synthesis of 2,3,6-trimethyl-1,4-naphthoquinone (TMN)

The method of Khalil *et al.* (2000) was used with minor modifications.[18] Briefly, 2,3-dimethyl-1,4-benzoquinone (1.0 g, 7 mmol) was prepared as described elsewhere[19] and subsequently heated in a sealed vial at ~80 ℃ for 3 h with isoprene (0.57 g, 8.4 mmol) in 2 ml ethanol. This mixture was evaporated under reduced pressure to yield 2,3,6-trimethyl-4a,5,8,8a-tetrahydro-1,4-naphtaquinone (figure 4.3). The crude tetrahydronaphthoquinone (0.8 g) was heated under reflux in xylene (30 ml) containing Pd/C (800 mg), for 7 h. The mixture was then cooled, filtered and the solvent removed. The residue was subsequently filtered through a column of neutral Al_2O_3 (50 g) using $CHCl_3$. The fractions containing TMN were crystallized from absolute ethanol to yield 0.3 g (37%) of pure TMN. The melting point (uncorrected) was determined to be between 97 – 103 ℃. Literature reports the melting point of TMN as 103 °C.[20] Attenuated total reflectance Fourier transform infrared spectroscopy (ATR/FT-IR) was used for identification and the spectrum was in agreement with previous reports (see figure 4.4).[20]

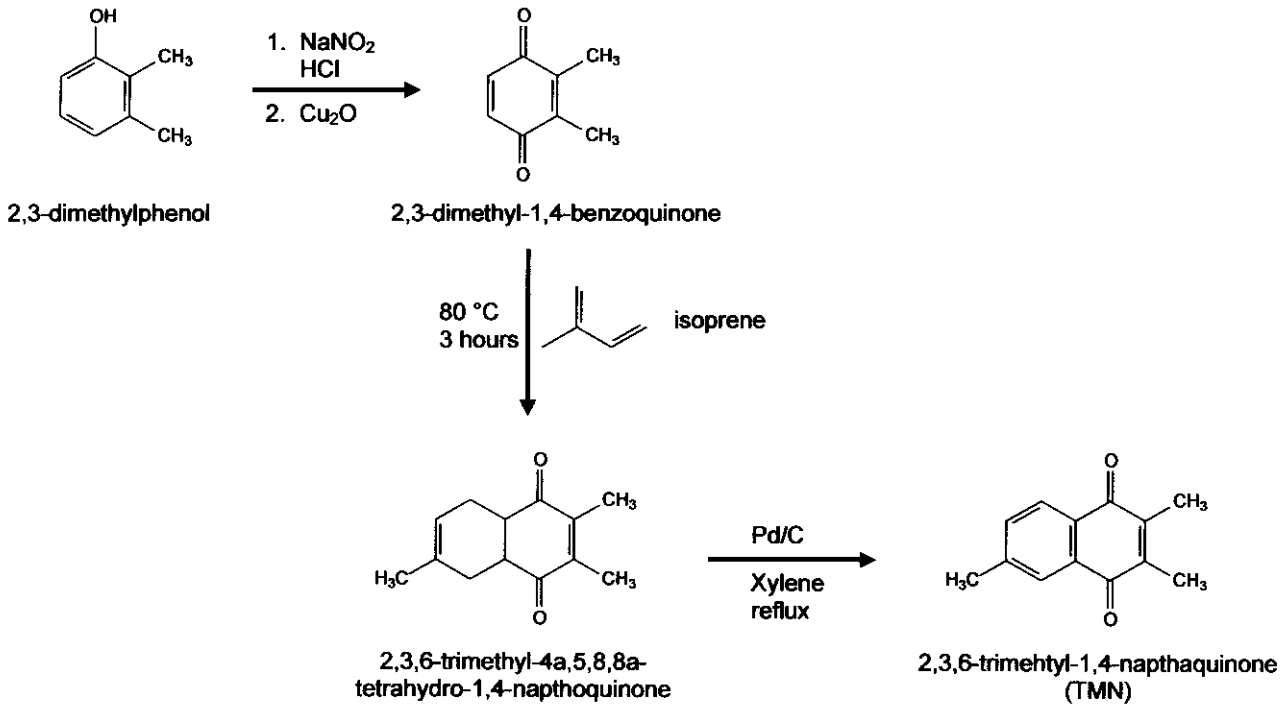

Figure 4.3: Synthesis of TMN. [Modified from Khalil *et al.* (2000).[18]]

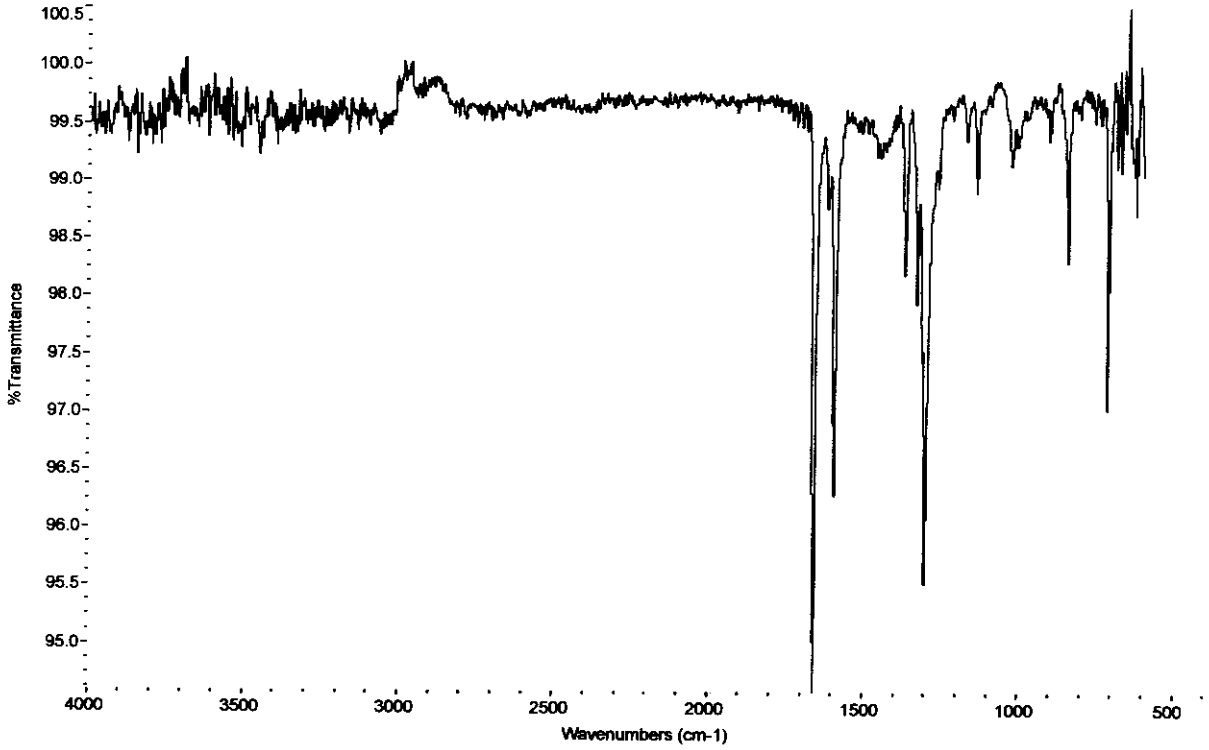

Figure 4.4: ATR/FT-IR spectrum of TMN.

4.2.4. PC12 cell culture procedure

The procedure of Hiremagalur (1993) was used with modifications.[21] PC12 cells were seeded into 35 mm Petri dishes coated with poly-DL-lysine at a density of 3 x 10^5 cells/ml. The cells were grown in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, 25 U/ml penicillin and 25 µg/ml streptomycin. The culture medium was replaced every 2 – 3 days. The cells were maintained in an incubator at 37 °C, 95% relative humidity and 5% CO_2.

On the day of treatment, the culture media was exchanged with fresh media, and the compounds were added to each culture dish, with the control dish receiving PG/DMSO alone. The final concentration of (*S*)-nicotine, (*S*)-cotinine and TMN was 100 µM, whereas the concentration for N-CSE was calculated to ensure that the nicotine content was approximately 100 µM, assuming 50% nicotine extraction. Accordingly, the same weight/volume was used for the NF-CSE which was used for the N-CSE.

The cells were incubated for 24 h with the respective compounds after which the cells were prepared for Western Blot analysis by trypsinizing (0.25%), adding 1 ml of a mixture of culture media and serum followed by aspiration. The total volume of the samples were transferred to their respective microcentrifuge tubes and centrifuged at 10,000 x g for 10 min. The pellet was resuspended in 500 µl ice-cold solution of 0.1 M sodium phosphate buffer (pH 7.4), after which the samples were sonicated for 2 x 10 s with 10 s intervals between the sonications. The samples were then centrifuged at 10,000 x g for 10 min whereafter the supernatant was stored at –75 °C until the day of analysis.

4.2.5. Western blot analysis

The protein concentration of each sample was determined according to the method of Bradford.[22] An aliquot of thawed supernatant equivalent to 50 µg protein was lyophilized. Distilled water (20 µl) and 5 µl sample buffer [sodium dodecyl sulphate (SDS) 5%; glycerol 40%; TRIS-HCl 0.5 M pH 6.8; β-mercaptoethanol 10%; bromophenol blue 0.04%] were added to each sample, boiled for 5 min to denature the protein and subsequently centrifuged at 10,000 x g for 10 min.

Twenty five microlitres of each sample was subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide/0.27% *N,N'*-methylenebisacrylamide resolving gel) for 120 min at 120 volts. Samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) for 210 min at 60 mA. The membranes were rinsed in TTBS (Tween Tris buffer saline, Tris 10 mM; NaCl 145 mM; Tween-20 0.25%) twice for 5 min each and incubated overnight at 4 °C in blocking buffer (4.5% non-fat dry milk in TTBS) followed by incubation overnight at 4 °C with primary antibody

(monoclonal anti-TH, 1:10,000 or polyclonal anti-DAT 1:100). After the membranes were rinsed twice with TTBS for 5 min, they were incubated for 120 min with horseradish peroxidase-conjugated secondary antibody in blocking buffer [1:4,000 anti-mouse IgG (H+L)] at room temperature (20 °C) for 120 min.

Finally, the membranes were washed twice with TTBS for 5 min each and developed on X-ray films for 3 min using standard enhanced chemiluminescence (ECL) solution.

4.2.6. Statistical analysis

The level of luminescence emitted from each band within a set area was detected using the VersaDoc Model 1000 System (Biorad). Quantity One, Version 4.4.0 software (Biorad, CA, USA) was used to quantify the luminescence and density of bands. The optical density obtained from the blot of the control rats' samples were compared to that of the blots of the treated rats. The optical density data were analyzed with Prism 3 (GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA) using Student's t-test with the confidence level set at 95%. Errors are reported as SEM.

4.3. Results and discussion

Previous reports have documented that nicotine not only stimulates nAChRs in the VTA and SN which evokes DA release in the dorsal (caudate and putamen) and ventral striatum (NAc),[23,24] but also promotes DA biosynthesis by increasing TH activity.[25,26] Increased TH activity is subsequently reflected by an increase in TH protein which results from increased expression.[27-29] Studies performed with nicotine and other nAChRs agonists indicate that TH mRNA is induced in cultured adrenal medullary cell model systems.[21,30,31] Other studies, performed in PC12 cells, indicated that nicotine treatment increase TH gene expression (mRNA).[21,32]

Although nicotine is the major component found in cigarette smoke, there are more than 4,000 chemicals in tobacco products.[33] Therefore, to elucidate whether there is any contribution from nicotine's main metabolite, cotinine, or other compounds found in CSE (other than nicotine) to the changing TH protein levels, we investigated the effect of 24 h exposure of (*S*)-nicotine, (*S*)-cotinine, TMN or CSEs obtained from Marlboro® and Quest® in PC12 cells, on TH regulation. These data support our hypothesis in that (*S*)-nicotine ($p < 0.05$) and Marlboro® (N-CSE, $p < 0.05$), but not (*S*)-cotinine and Quest® (NF-CSE) upregulate TH (figure 4.5).

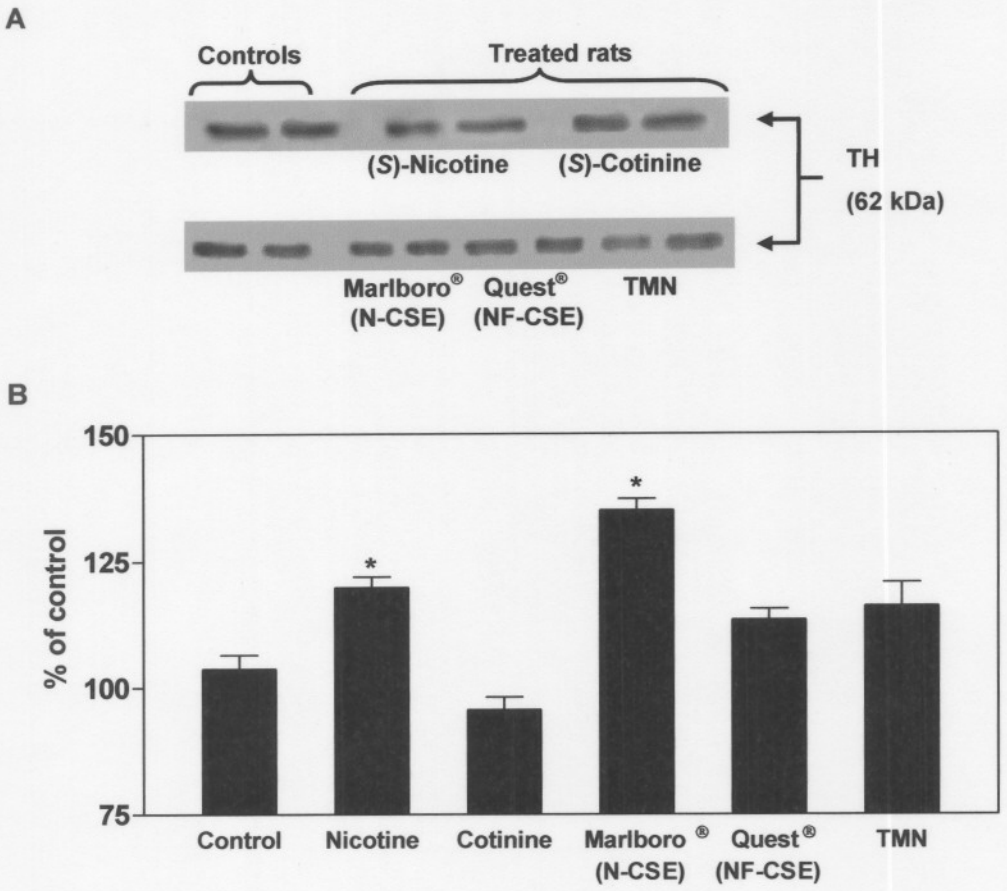

Figure 4.5: Changes in TH protein levels (**B**) assayed by western blot of PC12 cells treatment with (*S*)-nicotine, (*S*)-cotinine, Marlboro® (N-CSE), Quest® (NF-CSE) or TMN. Representative TH western blots (**A**) and summary data (mean ± SEM, n = 2 for each group) are shown. (Images enhanced in Adobe Photoshop.)

Our data support previous findings[21,32] of increased TH protein levels after nicotine treatment, since an increase in TH protein is a result of increased TH mRNA.[21] Cotinine, however, had no effect on TH protein levels after 24 h treatment in PC12 cells. As opposed to Marlboro®, Quest® 3 (a nicotine-free cigarette) used in this study, is claimed to have all the other ingredients of cigarette smoke except nicotine and nitrosamines, the cancer causing agents in cigarette smoke. Therefore, apart from nicotine and the nitrosamines, we propose that other compounds in cigarette smoke do not contribute to altered TH protein levels as seen with Marlboro® treatment of the PC12 cells.

There is however a statistical significance between TH upregulation caused by nicotine and Marlboro®. This can be explained by keeping the following in mind: for the current *in vitro* study, as well as our *in vivo* experiments (see chapter 5), we extracted cigarette smoke through acetone.

This organic solvent was chosen since the acetone extract was found to exhibit the greatest MAO-B inhibition activity (one measure of pharmacological activity elicited by extracted compounds) *in vitro*, as opposed to the hexane or 0.1 M sodium phosphate buffer.[34] When the *in vitro* experiments were performed, extraction rates were assumed to be 50%. The greater upregulation of TH by Marlboro® can be explained by assuming that the extraction procedure was more successful (> 50 %), with a higher nicotine content than what was calculated for.

In conclusion, since TH regulation is nAChR mediated,[35] we conclude that TH regulation is dependent on full nAChR agonism, since cotinine, a weak nAChR agonist did not affect TH regulation. To our knowledge, this is the first report on the effect of cotinine, TMN and CSEs on TH regulation performed in PC12 cells.

In 1996 Hart and Ksir reported that nicotine increases DA clearance (enhance DAT function) in the NAc in anesthetized rats.[36] A more recent study which supports these data, performed by Middleton *et al.* in 2004[37] found that DAT function in the striatum and medial prefrontal cortex of rat is enhanced by nicotine after subcutaneous treatment. However, *in vitro* data obtained from various studies did not support these findings. *In vitro* studies showed that nicotine causes a decrease in DA uptake into PC12 cells.[38] Findings from our laboratory pertaining to DAT expression in PC12 cells treated with (*S*)-nicotine, (*S*)-cotinine, N-CSE, NF-CSE or TMN for 24 h, was inconclusive in this regard (data not shown).

In vitro experiments do not take the complexity of the biological system into account. Therefore, our next aim was to determine the effects on TH and DAT regulation in rats after chronic treatment with (*S*)-nicotine, (*S*)-cotinine, N-CSE and NF-CSE.

4.4. References

1. Knez, M. 2003. Chemical nanostructuring and self-assembly. [Web:] http://www.fkf.mpg.de/kern/Res_act/solid_liquid/tob_plant_infect.jpg [Date of access: 7 December 2004].

2. Hill, M. 1999. Tissue culture: PC12 cells. [Web:] http://anatomy.med.unsw.edu.au/cbl/research/methods/cellspc12.htm [Date of access: 23 November 2004].

3. Philip Morris USA. Personal communication with consumer response center. 30 November 2004.

4. Vector Tobacco Inc. Personal communication with consumer response center. 2 December 2004.

5. Shanahan, J. 2004. Nicotine-free cigarettes? [Web:] http://www.geo-pie.cornell.edu/crops/tobacco.html [Date of access: 30 November 2004].

6. Baker, R.R. 1981. Product formation mechanisms inside a burning cigarette. *Prog Energ Comb Sci*, 135 - 153.

7. Baker, R.R. 1987. A review of pyrolysis studies to unravel reaction steps in burning tobacco. *J Anal Appl Pyrolysis*, 11: 555 - 573.

8. Salin-Pascual, R.J., Alcocer-Castillejos, N.V. & Alejo-Galarza, G. 2003. Nicotine dependence and psychiatric disorders. *Rev Invest Clin*, 55: 677 - 693.

9. Benowitz, N.L. 1996. Pharmacology of nicotine: addiction and therapeutics. *Annu Rev Pharmacol Toxicol*, 36: 597 - 613.

10. Greene, L.A. & Rein, G. 1977. Release of (3H)norepinephrine from a clonal line of pheochromocytoma cells (PC12) by nicotinic cholinergic stimulation. *Brain Res*, 138: 521 - 528.

11. Messing, R.O., Stevens, A.M., Kiyasu, E. & others. 1989. Nicotinic and muscarinic agonists stimulate rapid protein kinase C translocation in PC12 cells. *J Neurosci*, 9: 507 - 512.

12. Lai, H.L., Yang, T.H., Messing, R.O. & others. 1997. Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the A2a-adenosine receptor-mediated cAMP response. *J Biol Chem*, 272: 4970 - 4977.

13. Park, T.J., Song, S.K. & Kim, K.T. 1997. A2A adenosine receptors inhibit ATP-induced Ca2+ influx in PC12 cells by involving protein kinase A. *J Neurochem*, 68: 2177 - 2185.

14. Greene, L.A. & Tischler, A.S. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A*, 73: 2424 - 2428.

15. Margioris, A.N., Markogiannakis, E., Makrigiannakis, A. & others. 1992. PC12 rat pheochromocytoma cells synthesize dynorphin. Its secretion is modulated by nicotine and nerve growth factor. *Endocrinology*, 131: 703 - 709.

16. Javitch, J.A., Strittmatter, S.M. & Snyder, S.H. 1985. Differential visualization of dopamine and norepinephrine uptake sites in rat brain using [3H]mazindol autoradiography. *J Neurosci*, 5: 1513 - 1521.

17. Sherratt, A.J., Culpepper, B.T. & Lubawy, W.C. 1988. Prostacyclin and thromboxane formation following chronic exposure to cigarette smoke condensate administered via osmotic pumps in rats. A method for chronic administration of particulates of whole smoke. *J Pharmacol Methods*, 20: 47 - 56.

18. Khalil, A.A., Steyn, S. & Castagnoli, N., Jr. 2000. Isolation and characterization of a monoamine oxidase inhibitor from tobacco leaves. *Chem Res Toxicol*, 13: 31 - 35.

19. Norris, R.K. & Sternhell, S. 1966. Long-range spin-spin coupling in 1,4-benzoquinones and some related compounds. *Aust J Chem*, 19: 617 - 627.

20. Thomson, R.H. 1987. Naturally occurring quinones III: recent advances. 3rd ed. London: Chapman & Hall. 732p.

21. Hiremagalur, B., Nankova, B., Nitahara, J. & others. 1993. Nicotine increases expression of tyrosine hydroxylase gene. Involvement of protein kinase A-mediated pathway. *J Biol Chem*, 268: 23704 - 23711.

22. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72: 248 - 254.

23. Mihailescu, S. & Drucker-Colin, R. 2000. Nicotine, brain nicotinic receptors, and neuropsychiatric disorders. *Arch Med Res*, 31: 131 - 144.

24. Di Chiara, G. 2000. Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur J Pharmacol*, 393: 295 - 314.

25. Mitchell, S.N., Smith, K.M., Joseph, M.H. & others. 1993. Increases in tyrosine hydroxylase messenger RNA in the locus coeruleus after a single dose of nicotine are followed by time-dependent increases in enzyme activity and noradrenaline release. *Neuroscience*, 56: 989 - 997.

26. Smith, K.M., Mitchell, S.N. & Joseph, M.H. 1991. Effects of chronic and subchronic nicotine on tyrosine hydroxylase activity in noradrenergic and dopaminergic neurones in the rat brain. *J Neurochem*, 57: 1750 - 1756.

27. Fossom, L.H., Sterling, C. & Tank, A.W. 1991. Activation of tyrosine hydroxylase by nicotine in rat adrenal gland. *J Neurochem*, 57: 2070 - 2077.

28. Hofle, G., Weiler, R., Fischer-Colbrie, R. & others. 1991. Stimulation of rat adrenal medulla can induce differential changes in the peptide and mRNA levels of chromogranins, neuropeptides and other constituents of chromaffin granules. *Regul Pept*, 32: 321 - 331.

29. Hiremagalur, B. & Sabban, E.L. 1995. Nicotine elicits changes in expression of adrenal catecholamine biosynthetic enzymes, neuropeptide Y and immediate early genes by injection but not continuous administration. *Brain Res Mol Brain Res*, 32: 109 - 115.

30. Craviso, G.L., Hemelt, V.B. & Waymire, J.C. 1992. Nicotinic cholinergic regulation of tyrosine hydroxylase gene expression and catecholamine synthesis in isolated bovine adrenal chromaffin cells. *J Neurochem*, 59: 2285 - 2296.

31. Gueorguiev, V.D., Zeman, R.J., Meyer, E.M. & others. 2000. Involvement of alpha7 nicotinic acetylcholine receptors in activation of tyrosine hydroxylase and dopamine beta-hydroxylase gene expression in PC12 cells. *J Neurochem*, 75: 1997 - 2005.

32. Sabban, E.L. & Gueorguiev, V.D. 2002. Effects of short- and long-term nicotine treatment on intracellular calcium and tyrosine hydroxylase gene expression. *Ann N Y Acad Sci*, 971: 39 - 44.

33. National Institute on Drug Abuse. 2002. Research Report Series - Nicotine Addiction. [Web:] http://www.nida.nih.gov/researchreports/nicotine/nicotine2.html#what [Date of access: 13 May 2004].

34. Fourie Sorensen, I. 2004. Influence of tobacco smoke constituents on monoamine oxidase activity *in vitro* and on human placental monoamine oxidase A activity *in vivo*. Potchefstroom : North-West University. (Thesis-PhD). 167 p.

35. Li, S., Kim, K.Y., Kim, J.H. & others. 2004. Chronic nicotine and smoking treatment increases dopamine transporter mRNA expression in the rat midbrain. *Neurosci Lett*, 363: 29 - 32.

36. Hart, C. & Ksir, C. 1996. Nicotine effects on dopamine clearance in rat nucleus accumbens. *J Neurochem*, 66: 216 - 221.

37. Middleton, L.S., Cass, W.A. & Dwoskin, L.P. 2004. Nicotinic receptor modulation of dopamine transporter function in rat striatum and medial prefrontal cortex. *J Pharmacol Exp Ther*, 308: 367 - 377.

38. Huang, C.L., Chen, H.C., Huang, N.K. & others. 1999. Modulation of dopamine transporter activity by nicotinic acetylcholine receptors and membrane depolarization in rat pheochromocytoma PC12 cells. *J Neurochem*, 72: 2437 - 2444.

CHAPTER 5:

IN VIVO EFFECTS OF TOBACCO SMOKE CONSTITUENTS ON THE REGULATION OF RAT STRIATAL TYROSINE HYDROXYLASE AND DOPAMINE TRANSPORTER

ALZET osmotic mini-pumps

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LIST OF ABBREVIATIONS

N-CSE	nicotine containing cigarette smoke extract (Marlboro®)
NF-CSE	nicotine-free cigarette smoke extract (Quest®)

5.1. Introduction

The neurotransmitter, DA plays a crucial role in both PD and addiction. The dopaminergic pathways of importance are the mesocorticolimbic and nigrostriatal systems in PD and addiction respectively. In PD, the loss of dopaminergic neurons is responsible for the symptoms and signs of muscular rigidity, tremor and bradykinesia.[1] However, in the case of addiction, the reinforcing properties of nicotine lie in the release of DA from the presynaptic neuron.[2,3]

An enzyme that plays a pivotal role in DA synthesis is the cystolic enzyme, TH, which catalyzes the conversion of tyrosine to L-DOPA. DAT, as a transmembrane transporter, is responsible for the recycling of DA back into the presynaptic neuron from the synaptic cleft.

Since smokers are both addicted to smoking as well as showing a lower incidence of PD, the two biomarkers of the dopaminergic system, TH and DAT, which accounts for both PD and addiction, were assessed *in vivo* in this study.

5.2. Experimental procedures

5.2.1. Materials

(*S*)-Nicotine, (*S*)-cotinine and monoclonal anti-TH were purchased from Sigma (MO, USA). Polyclonal anti-DAT was purchased from Santa Cruz (CA, USA). Anti-mouse IgG (H+L), horseradish peroxidase-conjugate was purchased from Promega (WI, USA). ECL solution was purchased from Amersham Pharmacia Biotech (NJ, USA). All other solvents were purchased from commercial sources.

5.2.2. Animals

Male Sprague-Dawley rats, 200 – 250 g were obtained from Charles River (NC, USA). Animals were housed in pairs in standard polypropylene cages in a vivarium maintained at approximately 20 °C – 22 °C and 50% humidity with a 12 h light/dark cycle. Filtered water and rodent chow (Laboratory Animal Resource Center, Lubbock, TX, USA) were available *ad libitum* throughout the study. All studies were approved by the Animal Care and Use Committee of Texas Tech University Health Sciences Center (TX, USA; protocol number 03-015-06) and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

5.2.3. Preparation of smoke extracts

The preparation of the CSEs was conducted according to the method in chapter 4, section 4.2.2. In short, the CSEs were obtained by drawing either Marlboro® or Quest® cigarette smoke through 250 ml acetone with a vacuum pump. The CSE-acetone solution was removed under reduced pressure and the residue re-dissolved into a mixture of PG/DMSO (1:1) to ensure a "high-dose" CSE of 120 mg/ml.[4]

5.2.4. Preparation and implantation of osmotic mini-pumps

The preparation and implantation of the osmotic mini-pumps were conducted according to the method in chapter 3, section 3.2.3 with modifications. Osmotic mini-pumps (2ML4, 28 day, capacity 2.098 ml; Durect Corporation, CA, USA) were loaded with solutions containing N-CSE (120 mg/ml Marlboro®-CSE), NF-CSE (120 mg/ml Quest®-CSE), (*S*)-nicotine (4.8 mg/kg/day) or (*S*)-cotinine (5.2 mg/kg/day). Both (*S*)-nicotine and (*S*)-cotinine were dissolved in PG whereas the CSEs were dissolved in PG/DMSO (1:1). The animals were sedated with 0.7 ml/kg of a mixture of ketamine (100 mg/ml), xylazine (20 mg/ml) and acepromazine (10 mg/ml).

5.2.5. Western blot analysis

The western blot analysis was conducted according to chapter 4, section 4.2.5 with modifications. Striatal homogenates of treated Sprague-Dawley rats were prepared as follows: rats were euthanized by carbon dioxide and decapitation on day 28. The striata were rapidly removed on ice, weighed and homogenized with a disposable Kontes Pellet Pestle in an ice-cold solution of 0.1 M sodium phosphate buffer (pH 7.4, 10 µl/mg tissue weight).[5] Protein concentration was determined according to the method of Bradford.[6] The homogenates were stored at –75 °C until western blot assay.

Each sample was prepared where an aliquot of thawed homogenate, equivalent to 50 µg protein, was added to 2.5 µl NaCl (500 mM) and 2.5 µl 10% SDS. Distilled water was added to the preparations to ensure a final volume of 25 µl. The samples were aspirated and 4x sample buffer (SDS 5%; glycerol 40%; TRIS-HCl 0.5 M pH 6.8; β-mercaptoethanol 10%; bromophenol blue 0.04%) was added, boiled for 5 min to denaturate the protein, followed by centrifugation at 10,000 x g for 10 min.

A 15 µl aliquot of each sample was subjected to SDS–polyacrylamide gel electrophoresis (10% acrylamide/0.27% *N,N'*-methylenebisacrylamide resolving gel) for 120 min at 120 volts. Samples were transferred to PVDF membranes for 210 min at 60 mA. The membranes were rinsed twice for 5 min each in TTBS and incubated overnight at 4 °C in blocking buffer (4.5% non-fat dry milk in TTBS) followed by incubation overnight at 4 °C with primary anitbody (monoclonal anti-TH, 1:10,000 or polyclonal anti-DAT, 1:100). After the membranes were rinsed twice with TTBS for 5 min, the membranes were incubated for 120 min with horseradish peroxidase-conjugated secondary antibody in blocking buffer [1:4,000 anti-mouse IgG (H+L)] at room temperature (20 °C) for 120 min.

Finally the membranes were washed twice with TTBS for 5 min each and developed on X-ray films for 5 s using ECL solution.

5.2.6. Statistical analysis

The level of luminescence emitted from each band within a set area was detected using the VersaDoc Model 1000 System (Biorad). Quantity One, Version 4.4.0 software (Biorad, CA, USA) was used to quantify the luminescence and density of bands. The optical density obtained from the blot of the control rats' samples were compared to that of the blots obtained from treated rats. The optical density data were analyzed with Prism 3 (GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA) using Student's t-test with the confidence level set at 95%. Errors are reported as SEM.

5.3. Results and discussion

The biomarkers, TH and DAT investigated in our previous chapter, are of central importance to both PD and addiction. Our *in vitro* findings suggested that (*S*)-nicotine and N-CSE led to upregulation of TH. We concluded that this effect is mainly due to (*S*)-nicotine's effect as opposed to other compounds in CSE.

To determine the effect of CSE *in vivo* we treated male Sprague-Dawley rats for a period of 28 days to simulate chronic nicotine exposure as opposed to acute exposure *in vitro* investigated in the previous chapter.

It is known from literature that chronic nicotine treatment produce long-lasting effects on the catecholaminergic neurons in the brain, many of which are due to the ability of nicotine to modulate gene expression.[7,8] One of the genes that is upregulated by nicotine encodes for the rate-limiting enzyme in the DA biosynthesis pathway, TH. Studies performed in rats indicated that TH mRNA levels increased in the adrenal medulla, a TH rich organ, after chronic nicotine treatment, an effect that is nAChRs mediated.[9]

Our results support previous findings[10] that chronic nicotine exposure causes an increase in TH regulation in the rat striatum *in vivo*. The results of the TH western blot assays are shown in figure 5.1. Our data support our hypothesis (chapter 2, section 2.5) in that striatal TH was upregulated in rats after 28 days of treatment with (*S*)-nicotine ($p < 0.005$) and N-CSE ($p < 0.005$). Conversely, there was no statistically significant difference in the regulation of striatal TH when rats were treated with (*S*)-cotinine or NF-CSE. These findings suggest that the major compound in

CSE which was responsible for TH upregulation was nicotine with little contribution by other compounds found in CSE.

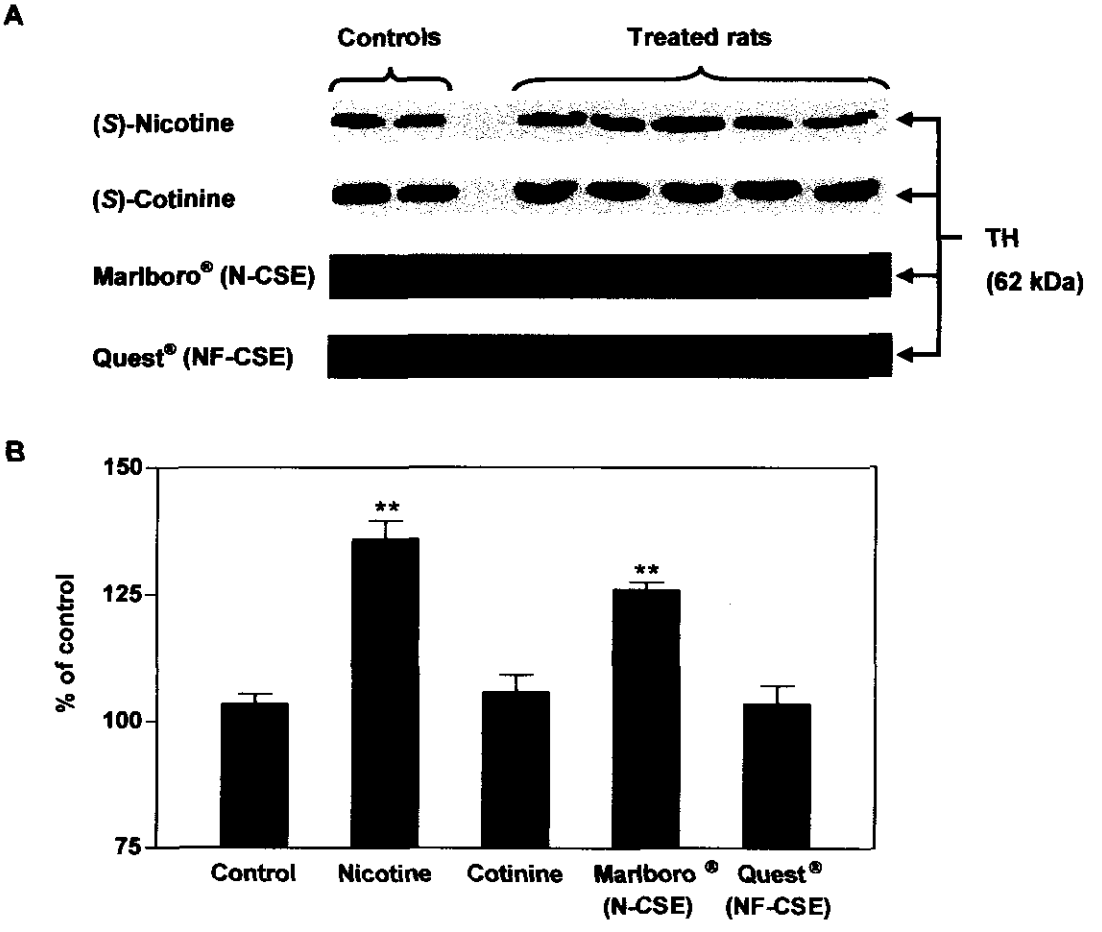


Figure 5.1: Changes in TH protein levels (**B**) assayed by western blot of rat striata after chronic treatment with (*S*)-nicotine, (*S*)-cotinine, Marlboro® (N-CSE) or Quest® (NF-CSE). Representative TH western blots (**A**) and summary data (mean ± SEM, n = 5 for each treatment group and n = 2 for controls) are shown. (Images enhanced in Adobe Photoshop.)

A study performed in 2004 by Li *et al.* showed that chronic nicotine administration to rats and passively inhaled smoke by rats greatly upregulated DAT mRNA in the VTA and SN.[10] This study by Li *et al.* (2004)[10] was first in examining the effect of chronic nicotine treatment on DAT mRNA levels. Our data support our hypothesis and previous findings from Li *et al.* (2004)[10] in demonstrating that (*S*)-nicotine ($p < 0.005$) and N-CSE ($p < 0.05$) upregulated DAT in a statistically significant manner. There was no statistically significant difference in the regulation of striatal DAT

after chronic (*S*)-cotinine and NF-CSE administration (figure 5.2). As with our *in vivo* TH regulation, these findings suggest that the major compound in CSE which was responsible for DAT upregulation was nicotine little contribution by other compounds found in CSE.

In conclusion, since both TH and DAT regulation are nAChR mediated,[9,10] we conclude that TH and DAT regulation is dependent on full nAChRs agonism, since cotinine, a weak nAChR agonist did not affect TH or DAT regulation *in vivo*. To our knowledge, this is the first report on the effect of cotinine, N-CSE and NF-CSE on TH and DAT regulation in rats.

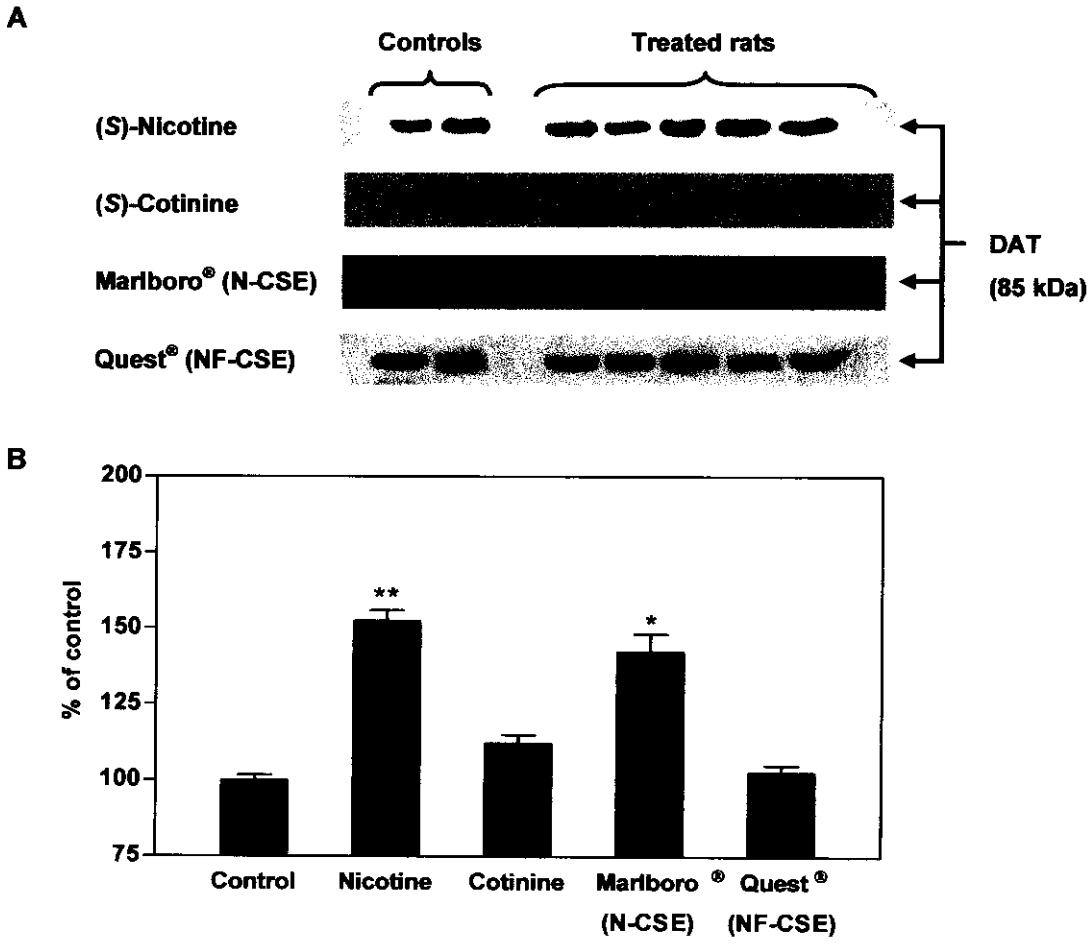


Figure 5.2: Changes in DAT protein levels (**B**) assayed by western blot of rat striata after chronic treatment with (*S*)-nicotine, (*S*)-cotinine, Marlboro® (N-CSE) or Quest® (NF-CSE). Representative DAT western blots (**A**) and summary data (mean ± SEM, n = 5 for each treatment group and n = 2 for controls) are shown. There was no statistical significance in striatal DAT regulation of the Quest® and (*S*)-cotinine treated rats. (Images enhanced in Adobe Photoshop.)

Our next aim, the effect of various compounds and CSEs on DA release from rat striatal synaptosomes, ties in with our *in vitro* and *in vivo* studies on the dopaminergic pathway biomarkers.

5.4. References

1. Mihailescu, S. & Drucker-Colin, R. 2000. Nicotine, brain nicotinic receptors, and neuropsychiatric disorders. *Arch Med Res*, 31: 131 - 144.

2. Leshner, A.I. & Koob, G.F. 1999. Drugs of abuse and the brain. *Proc Assoc Am Physicians*, 111: 99 - 108.

3. Koob, G.F. 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci*, 13: 177 - 184.

4. Sherratt, A.J., Culpepper, B.T. & Lubawy, W.C. 1988. Prostacyclin and thromboxane formation following chronic exposure to cigarette smoke condensate administered via osmotic pumps in rats. A method for chronic administration of particulates of whole smoke. *J Pharmacol Methods*, 20: 47 - 56.

5. Riederer, F., Luborzewski, A., God, R. & others. 2002. Modification of tyrosine hydroxylase activity by chloral derived beta-carbolines in vitro. *J Neurochem*, 81: 814 - 819.

6. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72: 248 - 254.

7. Dani, J.A. & De Biasi, M. 2001. Cellular mechanisms of nicotine addiction. *Pharmacol Biochem Behav*, 70: 439 - 446.

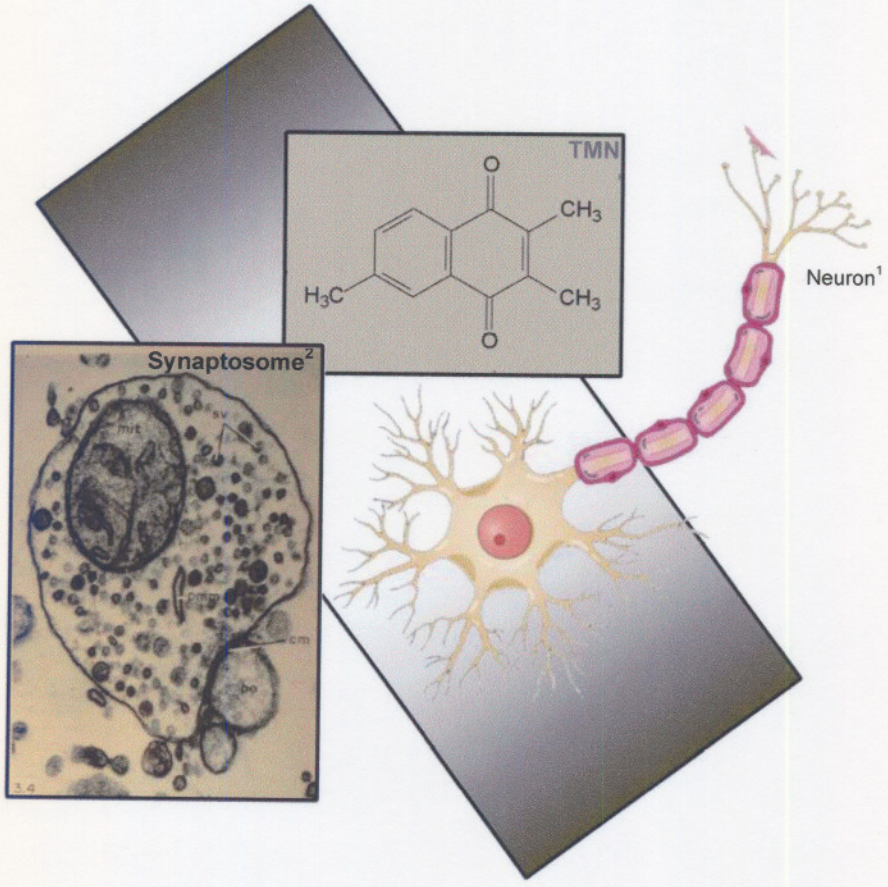
8. Harlan, R.E. & Garcia, M.M. 1998. Drugs of abuse and immediate-early genes in the forebrain. *Mol Neurobiol*, 16: 221 - 267.

9. Yoshimura, R., Xu, L., Sun, B. & others. 2004. Nicotinic and muscarinic acetylcholine receptors are essential for the long-term response of tyrosine hydroxylase gene expression to chronic nicotine treatment in rat adrenal medulla. *Brain Res Mol Brain Res*, 126: 188 - 197.

10. Li, S., Kim, K.Y., Kim, J.H. & others. 2004. Chronic nicotine and smoking treatment increases dopamine transporter mRNA expression in the rat midbrain. *Neurosci Lett*, 363: 29 - 32.

CHAPTER 6:

IN VITRO RELEASE OF DOPAMINE FROM RAT STRIATAL SYNAPTOSOMES TREATED WITH (*S*)-NICOTINE, (*S*)-COTININE, TMN AND CSEs



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LIST OF ABBREVIATIONS

[^{3}H]DA	[^{3}H]Dopamine
r^2	correlation coefficient

6.1. Introduction

In our preceding experiments (reported in chapter 4 and 5), we studied the effects of nicotine, cotinine and CSEs on the dopaminergic system in terms of DA synthesis (TH regulation) and uptake from the synaptic cleft (DAT regulation).

The dopaminergic system is an important entity in the pathophysiology of PD. Epidemiological studies have indicated that smokers are less likely to develop PD as opposed to non-smokers.[3-7] DA does not only play a critical role in PD, but also in cigarette smoke addiction. Substantial evidence from various animal studies demonstrated that DA release in the ventral striatum results in the reinforcing properties of nicotine.[8,9] Several of these studies have used nicotine doses that simulate human cigarette smoking.[10-12]

Since we have already established the effect of nicotine, cotinine and CSEs on the dopaminergic biomarkers, TH and DAT, we aimed to also determine the effect on DA release from striatal synaptosomes. Our hypothesis (chapter 2, section 2.5) is that (*S*)-nicotine and N-CSE but not (*S*)-cotinine, NF-CSE or TMN release DA from rat striatal synaptosomes. From these studies we hope to determine whether nicotine is the main mediator of DA release and whether there are other additive or antagonistic compounds in CSE.

6.2. Experimental procedures

6.2.1. Materials

[^{3}H]Dopamine (20.3 Ci/mmol) was obtained from New England Nuclear (DE, USA). (*S*)-Nicotine and (*S*)-cotinine were purchased from Sigma (MO, USA). Buffer constituents were obtained from other commercial sources.

6.2.2. Animals

Male Sprague-Dawley rats, 200 – 250 g were obtained from Charles River (NC, USA). All studies were approved by the Animal Care and Use Committee of Texas Tech University Health Sciences Center (TX, USA; protocol number 04-003-02) and were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

6.2.3. Preparations of synaptosomes and dopamine release studies

Rats were euthanized by gas anesthesia (isoflurane) and decapitated, whereafter the striata were rapidly removed on ice. The striatal tissue was homogenized in 4 ml sucrose buffer (0.32 M sucrose, 2 mM HEPES, pH 7.4) and subsequently centrifuged for 10 min at 1,000 x g. The supernatant was removed and centrifuged for 15 min at 10,000 x g. The resulting pellets (P2) were washed once and resuspended in incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, $CaCl_2.2H_2O$, 10 mM sucrose, 50 mM Tris-HCl, 0.05 µM pargyline, 0.1 µM ascorbic acid, pH 7.4), containing 100 nM [^{3}H]dopamine ([^{3}H]DA) and incubated for 5 min at 37 C. The synaptosomes were subsequently re-centrifuged at 10,000 x g for 10 min.

The supernatant was discarded and the pellets washed once with cold incubation buffer before being resuspended. The synaptosomes were aliquoted into tubes, to which the drug was added and subsequently incubated for 1 min [(*S*)-nicotine, (*S*)-cotinine, NF-CSE or N-CSE] or 10 min

(TMN) at 37 C. Test compounds were dissolved in DMSO. The final DMSO concentration in the incubations did not exceed 0.1%, where the controls received 0.1% DMSO/buffer solution alone. Synaptosomes subsequently received 3 ml of wash buffer at 37 C, were filtered through 1 micron glass microfiber filters (Whatman GF/B) under vacuum and washed an additional 3 times with 37 C buffer. Scintillation cocktail was added to each sample, followed by liquid scintillation counting to measure the radioactivity of [^{3}H]DA within the synaptosomes.

For each compound screened, fresh striatal tissue was used. A dose-response curve was determined for (*S*)-nicotine, (*S*)-cotinine and TMN whereas for N-CSE and NF-CSE, the extracts were screened at a concentration of 120 mg/ml (ensuring a final concentration of 300 µM nicotine assuming 50% extraction) (see chapter 4, section 4.2.4). For the NF-CSE, the same mg/ml ratio was used, assuming that the rest of the CSE composition was the same for both brands of cigarettes.

6.2.4. Statistical analysis

DA release data were evaluated by Student's t-test and concentration-response data were evaluated by non-linear least squares fit to a four parameter logistic equation. The resulting EC_{50} values and the correlation coefficient (r^2) of the fit were calculated using Prism (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA). Errors are reported as SEM.

6.3. Results

The results of the [^{3}H]DA release studies are shown in figures 6.1 to 6.4. From our data it is evident that nicotine mediated DA release from striatal synaptosomes in a dose-dependent manner (see figure 6.1). Under our laboratory conditions, the EC_{50} value of nicotine was 54 µM which was similar to an EC_{50} value between 1 and 4 µM as seen from previous studies.[13-16] The r^2 of the sigmoidal fit was 0.909, suggesting a good fit of the experimental data.

Figure 6.2 indicates that (*S*)-cotinine evoked DA release from striatal synaptosomes with an EC_{50} value of 97 µM, which was similar to a previous reported EC_{50} (30 µM) value for (*S*)-cotinine. The r^2 of the sigmoidal fit was statistically significant ($r^2 = 0.702$).

In addition, we proceeded to determine N-CSE and NF-CSE mediated DA release from striatal synaptosomes. The results of these experiments are shown in figure 6.3. N-CSE mediated DA release in a statistical significant ($p < 0.05$) manner. Conversely, NF-CSE only released a small amount of DA, which was not statistically significant from the control.

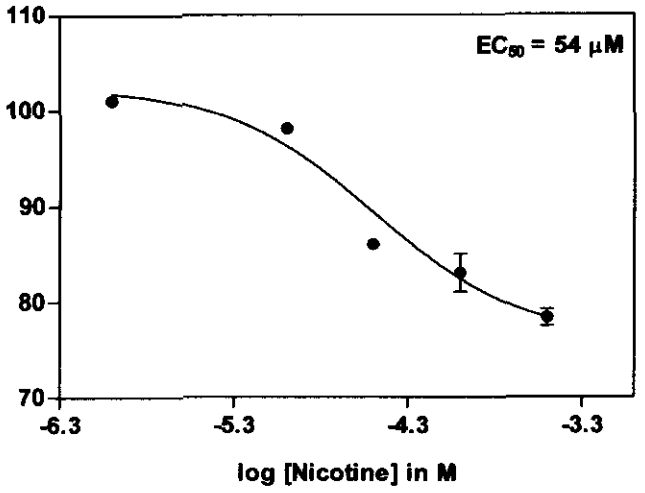

Figure 6.1: Dose-dependent release of striatal DA from synaptosomes by (*S*)-nicotine. The maximum DA release occurred at 300 µM (-3.5 log M) as opposed to the minimum release of DA at 1 µM (-6.0 log M). Data are represented as mean ± SEM of three experiments, each done with triplicate determinations (where SEM is not visible, the SEM is contained within the symbol).

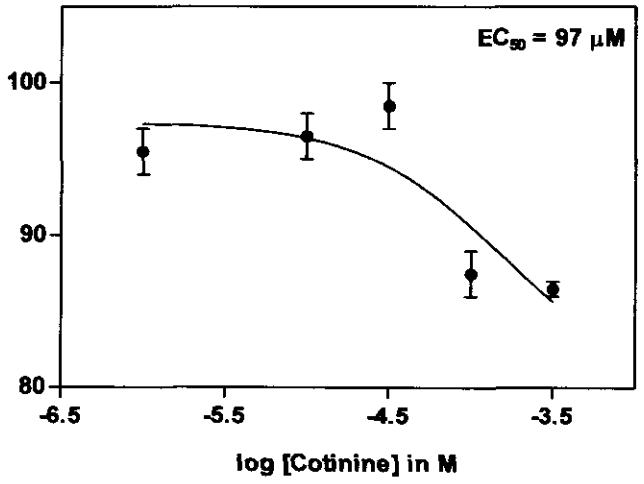

Figure 6.2: Dose-dependent release of striatal DA from synaptosomes by (*S*)-cotinine. The maximum DA release occurred at 300 µM (-3.5 log M) as opposed to the minimum release of DA at 1 µM (-6.0 log M). Data are represented as mean ± SEM of three experiments, each done with triplicate determinations.

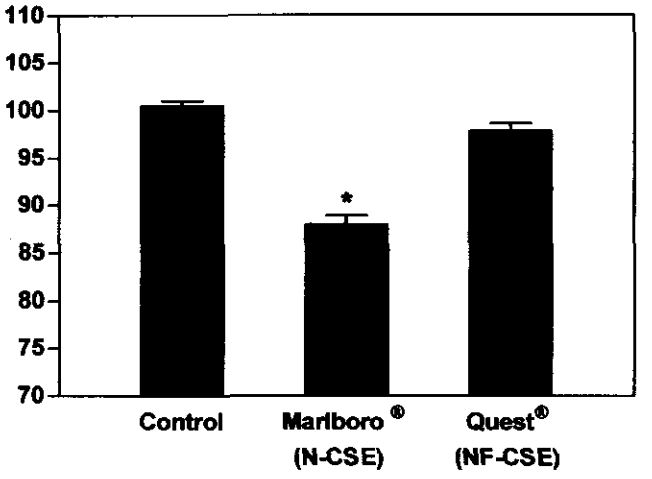

Figure 6.3: Release of striatal DA from synaptosomes mediated by CSE from N-CSE and NF-CSE. Marlboro® released 13% DA from rat striatal synaptosomes when compared to control. Each bar represents the mean + SEM of three experiments, with triplicate determinations in each. Statistical significance ($p < 0.05$) is indicated by a *.

TMN, the reversible MAO inhibitor isolated from smoke tobacco, has been shown to be neuroprotective in the MPTP-mouse model through its MAO properties.[17] Conversely to our hypothesis, it is evident from our data that TMN released striatal DA with an EC_{50} value of 87 µM (figure 6.4).

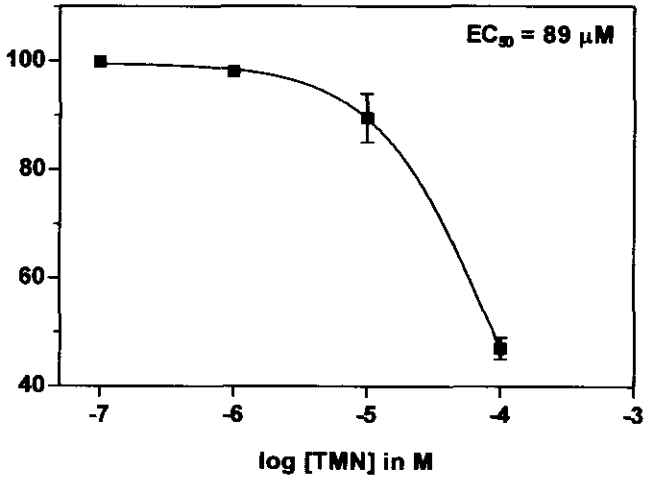

Figure 6.4: Dose-dependent release of striatal DA from synaptosomes by TMN. The maximum DA release occurred at 100 µM (-4.0 log M) as opposed to the minimum release of DA at 0.1 µM (-7.0 log M). Data are represented as mean ± SEM of three experiments, each done with triplicate determinations (where SEM is not visible, the SEM is contained within the symbol).

6.4. Discussion

The previous chapters investigated the effect of CSE on the dopaminergic system, in particular the influence of (*S*)-nicotine, (*S*)-cotinine, N-CSE and NF-CSE on TH as well as DAT regulation. Therefore, the influence of CSEs on both the synthesis as well as the re-uptake of DA, could be inferred from these data.

It is known that nicotine stimulates presynaptic nAChRs (see chapter 2, section 2.3), which causes an influx of calcium into the neuron resulting in the release of DA. In the case of addiction, the release of DA from the presynaptic neuron is responsible for the reinforcing properties of nicotine.[8,9,18] Since this release of DA is of critical importance in addiction, we investigated the effect of (*S*)-nicotine, (*S*)-cotinine, TMN, N-CSE and NF-CSE on DA release in striatal synaptosomes to elucidate whether compounds other than nicotine might be responsible for the reinforcing effects of cigarette smoke.

The effect of nicotine and cotinine on DA release has been well documented. We chose to include these compounds in our study as references to validate our *in vitro* model. The *in vitro* model for this experiment was striatal synaptosomes. Striatal synaptosomes are presynaptic neuronal vesicles that contain nicotinic receptors, Ca_{1v} calcium channels (previously designated L-type calcium channels) and DAT.[19-22]

Previous studies have shown that nicotine has a transient effect on DA release in synaptosomes,[23] necessitating us to use a 1 minute incubation time. From our studies we found that nicotine mediated striatal DA release in a dose-dependent manner (figure 6.1). Our EC_{50} value, 54 µM, for DA release by nicotine was similar to that in literature.[13-16] As expected, cotinine (figure 6.2) released DA from the synaptosomes to a lesser degree when compared to nicotine, with an EC_{50} of 97 µM.

Mainstream CSE from nicotine containing cigarettes released DA from synaptosomes to a greater extent (13%) when compared to control. Conversely, NF-CSE did not release DA in a statistically significant manner (figure 6.3). The amount of DA release was similar to what would be expected for the corresponding amount of nicotine (300 µM).[13-16]

TMN, a compound isolated from tobacco leaves, has been shown to be neuroprotective in the MPTP Parkinsonian mouse model.[17] This effect is thought to be mainly due to TMN's ability to inhibit the MAO-B enzyme.[17] The incubation time for TMN was 10 min, in contrast to that of nicotine since TMN did not exhibit the same time-dependent release of DA, as was seen with (*S*)-nicotine. Our studies show that TMN was able to mediate striatal DA release with an EC_{50} of 87

μM (figure 6.4). This indicates that TMN might be a dual symptomatic as well as neuroprotective agent, which deserves further study as possible therapy for PD patients. TMN's capability to release DA is yet to be determined, since it is known from literature that DA release is subject to nAChR stimulation.

In conclusion, we found that (*S*)-nicotine (EC_{50} = 54 M) release DA twice as strong when compared to (*S*)-cotinine (EC_{50} = 97 M) and TMN (EC_{50} = 87 M).

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6.5. References

1. Walsh, A.A. 1996. Anatomy of the neuron. [Web:] http://inside.salve.edu/walsh/neuron.jpg [Date of access: 14 November 2004].

2. Meyer, J.S. 2004. Synaptosome. [Web:] http://courses.umass.edu/psych732/slides/synaptosome.jpeg [Date of access: 10 December 2004].

3. Baumann, R.J., Jameson, H.D., McKean, H.E. & others. 1980. Cigarette smoking and Parkinson disease: 1. Comparison of cases with matched neighbors. *Neurology*, 30: 839 - 843.

4. Godwin-Austen, R.B., Lee, P.N., Marmot, M.G. & others. 1982. Smoking and Parkinson's disease. *J Neurol Neurosurg Psychiatry*, 45: 577 - 581.

5. Kessler, I.I. 1972. Epidemiologic studies of Parkinson's disease. 3. A community-based survey. *Am J Epidemiol*, 96: 242 - 254.

6. Kessler, I.I. & Diamond, E.L. 1971. Epidemiologic studies of Parkinson's disease. I. Smoking and Parkinson's disease: a survey and explanatory hypothesis. *Am J Epidemiol*, 94: 16 - 25.

7. Nefzger, M.D., Quadfasel, F.A. & Karl, V.C. 1968. A retrospective study of smoking in Parkinson's disease. *Am J Epidemiol*, 88: 149 - 158.

8. Leshner, A.I. & Koob, G.F. 1999. Drugs of abuse and the brain. *Proc Assoc Am Physicians*, 111: 99 - 108.

9. Koob, G.F. 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci*, 13: 177 - 184.

10. Rowell, P.P., Carr, L.A. & Garner, A.C. 1987. Stimulation of [3H]dopamine release by nicotine in rat nucleus accumbens. *J Neurochem*, 49: 1449 - 1454.

11. Pontieri, F.E., Tanda, G., Orzi, F. & others. 1996. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature*, 382: 255 - 257.

12. Corrigall, W.A., Franklin, K.B., Coen, K.M. & others. 1992. The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine. *Psychopharmacology (Berl)*, 107: 285 - 289.

13. Izenwasser, S., Jacocks, H.M., Rosenberger, J.G. & others. 1991. Nicotine indirectly inhibits [3H]dopamine uptake at concentrations that do not directly promote [3H]dopamine release in rat striatum. *J Neurochem*, 56: 603 - 610.

14. Grady, S.R., Marks, M.J. & Collins, A.C. 1994. Desensitization of nicotine-stimulated [3H]dopamine release from mouse striatal synaptosomes. *J Neurochem*, 62: 1390 - 1398.

15. Grady, S., Marks, M.J., Wonnacott, S. & others. 1992. Characterization of nicotinic receptor-mediated [3H]dopamine release from synaptosomes prepared from mouse striatum. *J Neurochem*, 59: 848 - 856.

16. Sacaan, A.I., Dunlop, J.L. & Lloyd, G.K. 1995. Pharmacological characterization of neuronal acetylcholine gated ion channel receptor-mediated hippocampal norepinephrine and striatal dopamine release from rat brain slices. *J Pharmacol Exp Ther*, 274: 224 - 230.

17. Castagnoli, K., Petzer, J.B., Steyn, S.J. & others. 2003. Inhibition of human MAO-A and MAO-B by a compound isolated from flue-cured tobacco leaves and its neuroprotective properties in the MPTP mouse model of neurodegeneration. *Inflammopharmacology*, 11: 183 - 188.

18. Kulak, J.M., McIntosh, J.M., Yoshikami, D. & others. 2001. Nicotine-evoked transmitter release from synaptosomes: functional association of specific presynaptic acetylcholine receptors and voltage-gated calcium channels. *J Neurochem*, 77: 1581 - 1589.

19. Dwoskin, L.P., Teng, L., Buxton, S.T. & others. 1999. (S)-(-)-Cotinine, the major brain metabolite of nicotine, stimulates nicotinic receptors to evoke [3H]dopamine release from rat striatal slices in a calcium-dependent manner. *J Pharmacol Exp Ther*, 288: 905 - 911.

20. Grady, S.R., Grun, E.U., Marks, M.J. & others. 1997. Pharmacological comparison of transient and persistent [3H]dopamine release from mouse striatal synaptosomes and response to chronic L-nicotine treatment. *J Pharmacol Exp Ther*, 282: 32 - 43.

21. Kirby, M.L., Barlow, R.L. & Bloomquist, J.R. 2001. Neurotoxicity of the organochlorine insecticide heptachlor to murine striatal dopaminergic pathways. *Toxicol Sci*, 61: 100 - 106.

22. Woodward, J.J., Cook, M.E. & Leslie, S.W. 1988. Characterization of dihydropyridine-sensitive calcium channels in rat brain synaptosomes. *Proc Natl Acad Sci U S A*, 85: 7389 - 7393.

23. Grady, S.R., Murphy, K.L., Cao, J. & others. 2002. Characterization of nicotinic agonist-induced [(3)H]dopamine release from synaptosomes prepared from four mouse brain regions. *J Pharmacol Exp Ther*, 301: 651 - 660.

CHAPTER 7:

CONCLUSION AND FUTURE STUDIES

7.1. Conclusion

Throughout our study, we focused on the role of specific biomarkers within two distinct dopaminergic pathways, PD and addiction. PD, a severe disabling neurodegenerative disorder, is characterized at the neuropathological level by the progressive death of dopaminergic neurons in the SNc and its axon terminals which project to the striatum.[1,2] Although the etiology of PD is unknown, the disease appears multifactorial in origin, possibly arising from complex interactions between genetics and environment.

One factor which has been linked to neurodegenerative diseases, such as PD is oxidative stress in which MAO plays a key role.[3] MAO catalyzes the metabolism of DA to H_2O_2 which produces free radicals and eventually neurodegeneration.[3] Another characteristic of MAO, in favor of neurodegeneration, is the bio-activation of a neurotoxin precursor, MPTP to MPP^+, which occurs in glial cells. DAT then selectively transports MPP^+ into the presynaptic neuron, where it exerts its effects by inhibiting mitochondrial respiration.[4]

However, smokers have lower MAO activity peripherally and centrally as oppose to non-smokers.[5,6] To elucidate which compound might be responsible for this MAO inhibition, Khalil *et al.* (2000) isolated a compound from tobacco leaves, TMN, which showed reversible inhibition against MAO-A and MAO-B.[7] Subsequently in 2001 Castagnoli *et al.* showed that TMN proved to be neuroprotective in the MPTP model.[8]

Regardless of etiology, the signs and symptoms that PD patients experience arise from dopaminergic neuronal loss, in which case DA synthesis, metabolism and function are reasonable targets for PD associated studies. The rational for our study came from epidemiological findings that PD is less prevalent among smokers.[9-13] This is indeed one of the most robust observations that linked environment with PD.

Another pathway of interest is the mesocorticolimbic dopaminergic system, which forms the core around cigarette addiction. The effect of nicotine in the CNS has been the subject of numerous experimental studies where immense progress has been made on the role that nicotine plays in

cigarette smoke addiction. DA release from presynaptic neurons has been shown to be responsible for the addictive properties of cigarette smoke.[14,15] Therefore, as in the case of PD, DA synthesis, metabolism and function are reasonable targets for addiction related studies.

In this study we aimed to determine the effect of various CSE components on the dopaminergic system, in an attempt to elucidate the low incidence of PD among smokers as well as to investigate the probable affect on addiction. Figure 7.1 provides an overview of our **hypotheses** and how it interacts with the dopaminergic system.

1. Our first hypotheses consisted of two aims. Our first aim was to determine whether cotinine does in fact cross the BBB. Although cotinine has been detected in brain after nicotine exposure,[16-18] indirect data from literature suggest that the presence of cotinine in brain may be the result of central nicotine metabolism by CYP2B1 in rats.[19] Secondly, since chronic nicotine exposure changes both BBB function and morphology, we aimed to determine whether cotinine and nicotine transfer across the BBB would vary after chronic nicotine exposure.

   From our studies we found that cotinine not only crosses the BBB but that with chronic nicotine treatment, transfer of cotinine and nicotine across the BBB is not altered. This study was of significant importance since the transfer of cotinine across the BBB has not yet been reported.

2. TH, the rate-limiting enzyme in the DA synthesis pathway, is subject to both long- and short-term regulation. The finding that the long- and short-term regulation of TH involve similar second messenger signals raised the possibility that these pathways are interdependent.[20] Our second hypothesis entailed us to determine the effect of nicotine, cotinine, N-CSE, NF-CSE and TMN treatment on the regulation of the dopaminergic biomarkers, TH and DAT *in vitro*.

   Our data support previous findings[21,22] of increased TH protein levels after nicotine treatment.[21] In addition, we found that cotinine does not affect TH regulation *in vitro*. To our knowledge, this is the first report detailing the effect of cotinine on TH regulation. Of importance is that TMN, a compound to be assumed to be present in both Quest® and Marlboro®, did not upregulate TH. Since the N-CSE but not the NF-CSE upregulated TH we conclude that TH upregulation is mainly due to nicotine, assuming that the content of Quest® and Marlboro® cigarettes contents are consistent. In conclusion, since TH regulation is nAChR mediated,[23] we propose from our results, that TH regulation is

dependent on full nAChR agonism. In addition, this might explain why cotinine, a weak nAChR agonist and TMN, a MAO inhibitor did not affect TH regulation.

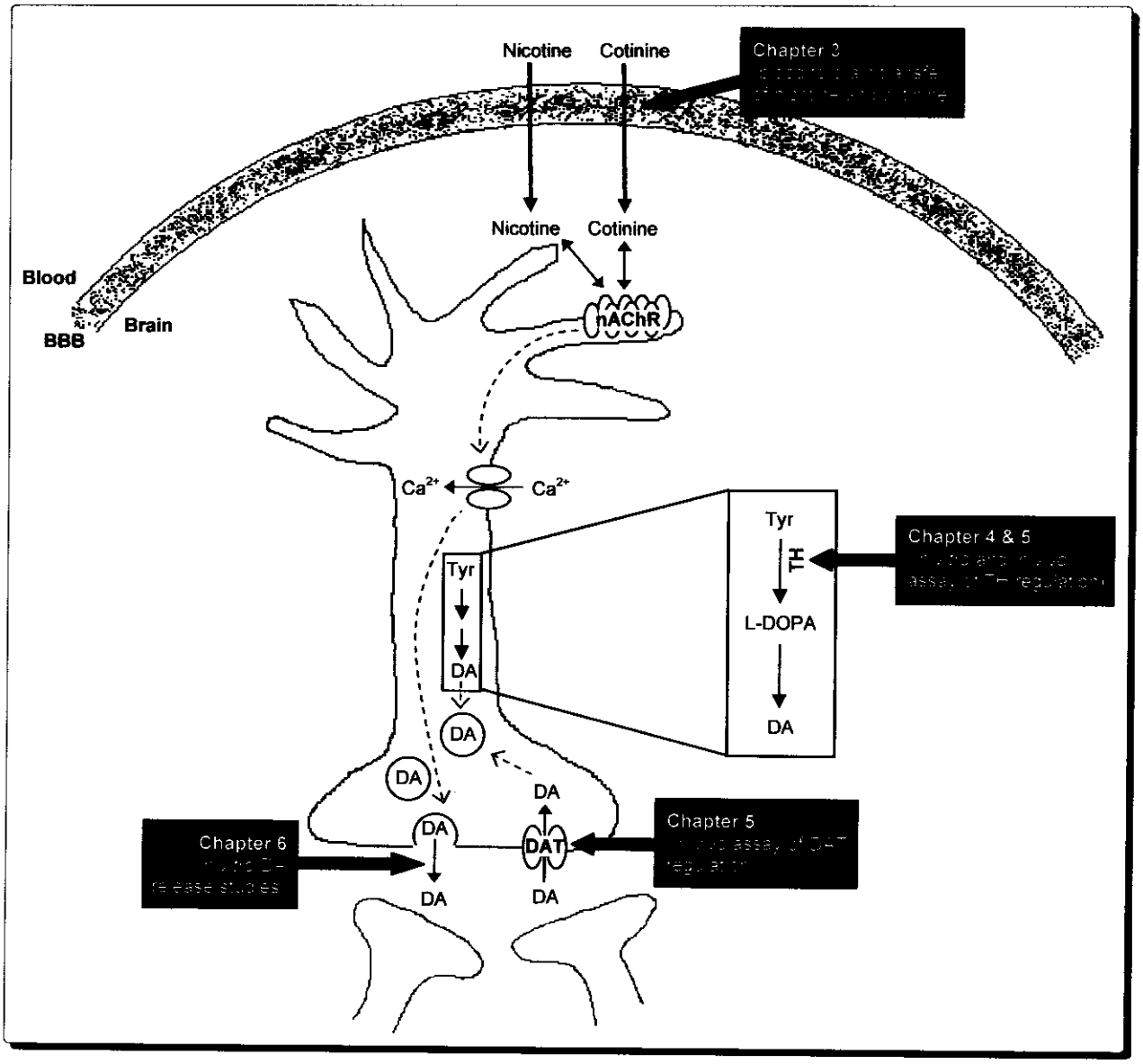

Figure 7.1: Summary of biomarkers targeted during this study.

3. After recognizing that cotinine indeed crosses the BBB (hypothesis 1, chapter 3), we determined the effect of cotinine treatment on TH and DAT regulation *in vivo* after chronic treatment. In addition to cotinine, we also assayed the effect of N-CSE, NF-CSE and nicotine on TH and DAT regulation after chronic treatment. Our results support previous

findings[23] that chronic nicotine exposure increases TH and DAT regulation in rat striatum *in vivo*.

To our knowledge, this is the first report on the effect of cotinine, N-CSE and NF-CSE on TH and DAT regulation in rats. We found that neither cotinine nor NF-CSE affected TH or DAT regulation as oppose to N-CSE which upregulated TH and DAT protein. It is evident from our experiments that nicotine is mainly responsible for increased TH and DAT regulation *in vivo* assuming that both Quest® and Marlboro® cigarettes contents are consistent.

Our *in vivo* data supports our *in vitro* data seeing as TH and DAT regulation is nAChR mediated, we propose that the regulation of both dopaminergic biomarkers, TH and DAT are dependent on full nAChR agonism.

4. Not only is DA release from presynaptic neurons important for the reinforcing properties of nicotine,[14,15] but it also may play a role in PD. We therefore aimed to determine whether other compounds of CSE (including TMN) as well as nicotine's major metabolite, cotinine, might possess DA releasing capabilities.

   Our results support previous findings that nicotine releases DA from striatal synaptosomes.[24,25] However, we found that nicotine releases DA twice as strong as TMN and cotinine from striatal synaptosomes. Since nAChR binding is necessary for DA release, we ascribe the DA release of nicotine and cotinine to their nAChR binding capabilities. The question then is if TMN binds to presynaptic nAChRs or if other mechanisms can be responsible for DA release such as feedback regulation due to MAO inhibition?

   In addition we determined whether Marlboro® and Quest® are capable of releasing DA from rat striatal synaptosomes. We found that although Quest® only released a very small amount of DA, Marlboro® released 13% more DA from rat striatal synaptosomes when compared to control. The DA release mediated by Quest® was not statistically significant, but can be attributed to the dose that was chosen for the assay. From hereon, an assay of a higher dose of CSEs might prove to be meaningful.

   Nevertheless, if the content of both Marlboro® and Quest® cigarettes are consistent, we can assume that Quest® contains TMN just as Marlboro®. Therefore, the small release in DA with Quest® CSE, might be attributed to the presence of TMN in Quest® cigarettes. If this is the case and taking into account that DA plays a critical role in addiction, the usefulness of

nicotine-free cigarettes (Quest® range) as cessation therapy is a valid approach for quitting the deadly habit.

However, the probability of other substances in cigarette smoke that might antagonize or attribute to DA release is not to be disregarded.

Nevertheless, these findings are of significant importance since epidemiological studies indicated that smokers are less likely to develop PD as oppose to non-smokers.[9-13] DA release in the SN may alleviate or mask symptoms and/or protect against nigrostriatal damage in the long-term.

The possible significance of TH and DAT upregulation in addiction and PD need to be addressed.

Let us consider the significance of our results from this study from an addiction point of view. Nicotine stimulates nAChRs causing an upregulation of TH, with more DA available for release in the NAc[26-29] to induce pleasurable effects. Increased DAT upregulation ensures that the available DA in the synaptic cleft is transported back into the presynaptic neuron. And compounds such as TMN, inhibit MAO to prevent DA metabolism. The resulting effect is a massive amount of DA released with every depolarization of the membrane after nAChRs stimulation, which will intensify the reinforcing effects of cigarette smoke. In addition, since cotinine is transported into brain, it too will stimulate nAChRs causing DA release. Nicotine, with its unequivocal TH and DAT upregulation properties, is the main compound responsible for cigarette addiction.

Nearly the same argument holds true for PD as for addiction, but with emphasis on other aspects, which may render more valuable. As in the case of addiction, nicotine stimulates nAChRs, causing an upregulation in TH and increased DA synthesis in the SN.[26-29] However, the importance of TMN is more pronounced in people who might be more susceptible to oxidative stress. TMN, as a MAO inhibitor,[7,8] prevents the metabolism of DA, avoiding reactive oxygen species production. In addition, xenobiotic (MPTP) is not metabolized to neurotoxic compounds (MPP^+) through MAO catalyzation. However, auto-oxidation takes place when MAO is saturated by DA, in the synaptic cleft. On the other hand, DAT upregulation will transport DA into the presynaptic neuron and prevent it from undergoing auto-oxidation. In addition, as mentioned above, DA release may attenuate PD progression and nigrostriatal damage even under xenobiotic "attack". This can occur when the released DA competes with the neurotoxin for transport into the presynaptic neuron by DAT. Less of the neurotoxin is transported into the presynaptic neuron, where less neuronal cell damage is expected.

7.2. Future studies

More research in this field is necessary to fully elucidate the role of TH and DAT in PD and addiction. We are listing some studies that need to be addressed which are important to the PD or addiction realm.

Further experiments need to be performed to determine whether the results from the N-CSE TH and DAT upregulation is solely due to the effect of nicotine and not other contributing compounds found in cigarette smoke. This can be attained through *in vivo* experiments by implanting osmotic mini-pumps which contain both N-CSE and mecamylamine, a nicotine antagonist which is bioavailable in brain.

To date no research has been done on the effect of smoke on CYP2D6 in brain. Keeping in mind that CYP2D6 converts MPTP to **non-neurotoxic**, N-4-(4'-hydroxyphenyl)-N-methyl-1,2,3,6-tetrahydropyridine and 4-phenyl-1,2,3,6-tetrahydropyridine (PTP), it might be of significance to determine whether smoke or nicotine activates or stimulates CYP2D6.[30]

Our *in situ* perfusion data would not have been influenced by the conversion, if any, of nicotine to cotinine in brain. However, the degree of contribution that CYP2B1 makes towards central cotinine production needs to be addressed.

Previous studies indicate that PD patients have a **severe reduction in VMAT-2**, the common brain isoform of VMAT, in all parts of the striatum (including the caudate nucleus, putamen and ventral striatum).[31] The question now arises if VMAT-2 is upregulated in cigarette smoke addicts which might then contribute to the finding of a lower incidence of PD among smokers. In addition, this can also shed light on the possible involvement of VMAT-2 in the development of cigarettes smoke addiction.

The results of the 2001 National Household Survey on Drug Abuse showed that 18.2% of smokers over the age of 12 also use other illicit drugs. Further studies need to be conducted on the effect of illicit drugs in conjunction with cigarette smoke on the dopaminergic pathway in the mesocorticolimbic system on a neuronal level.

To further elucidate if there are any neuroprotective compounds (other than TMN) in cigarette smoke, epidemiological studies should be trained to investigate whether people smoking nicotine free cigarettes show the same "protection" as with nicotine containing cigarettes. This might also answer the question whether smoking really protects (for example through compounds such as TMN) or simply delays the symptoms of PD by means of increased DA release masking the underlying death of striatal dopaminergic neurons.

Such studies and others are important for the development of targeted drug therapies. In addition, the resolution of these and other issues should provide the key to understanding the molecular basis of neuroprotection in PD with tobacco use as well as the development of tobacco addiction.

7.3. References

1. Lang, A.E. & Lozano, A.M. 1998. Parkinson's disease. First of two parts. *N Engl J Med*, 339: 1044 - 1053.

2. Huang, Y., Cheung, L., Rowe, D. & others. 2004. Genetic contributions to Parkinson's disease. *Brain Res Brain Res Rev*, 46: 44 - 70.

3. Foley, P. & Riederer, P. 2000. Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson's disease. *J Neurol*, 247 Suppl 2: II82 - II94.

4. Gainetdinov, R.R., Fumagalli, F., Jones, S.R. & others. 1997. Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J Neurochem*, 69: 1322 - 1325.

5. Berlin, I., Said, S., Spreux-Varoquaux, O. & others. 1995. Monoamine oxidase A and B activities in heavy smokers. *Biol Psychiatry*, 38: 756 - 761.

6. Fowler, J.S., Volkow, N.D., Wang, G.J. & others. 1996. Brain monoamine oxidase A inhibition in cigarette smokers. *Proc Natl Acad Sci U S A*, 93: 14065 - 14069.

7. Khalil, A.A., Steyn, S. & Castagnoli, N., Jr. 2000. Isolation and characterization of a monoamine oxidase inhibitor from tobacco leaves. *Chem Res Toxicol*, 13: 31 - 35.

8. Castagnoli, K.P., Steyn, S.J., Petzer, J.P. & others. 2001. Neuroprotection in the MPTP Parkinsonian C57BL/6 mouse model by a compound isolated from tobacco. *Chem Res Toxicol*, 14: 523 - 527.

9. Baumann, R.J., Jameson, H.D., McKean, H.E. & others. 1980. Cigarette smoking and Parkinson disease: 1. Comparison of cases with matched neighbors. *Neurology*, 30: 839 - 843.

10. Godwin-Austen, R.B., Lee, P.N., Marmot, M.G. & others. 1982. Smoking and Parkinson's disease. *J Neurol Neurosurg Psychiatry*, 45: 577 - 581.

11. Kessler, I.I. 1972. Epidemiologic studies of Parkinson's disease. 3. A community-based survey. *Am J Epidemiol*, 96: 242 - 254.

12. Kessler, I.I. & Diamond, E.L. 1971. Epidemiologic studies of Parkinson's disease. I. Smoking and Parkinson's disease: a survey and explanatory hypothesis. *Am J Epidemiol*, 94: 16 - 25.

13. Nefzger, M.D., Quadfasel, F.A. & Karl, V.C. 1968. A retrospective study of smoking in Parkinson's disease. *Am J Epidemiol*, 88: 149 - 158.

14. Koob, G.F. 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci*, 13: 177 - 184.

15. Leshner, A.I. & Koob, G.F. 1999. Drugs of abuse and the brain. *Proc Assoc Am Physicians*, 111: 99 - 108.

16. Crooks, P.A. & Dwoskin, L.P. 1997. Contribution of CNS nicotine metabolites to the neuropharmacological effects of nicotine and tobacco smoking. *Biochem Pharmacol*, 54: 743 - 753.

17. Crooks, P.A., Li, M. & Dwoskin, L.P. 1997. Metabolites of nicotine in rat brain after peripheral nicotine administration. Cotinine, nornicotine, and norcotinine. *Drug Metab Dispos*, 25: 47 - 54.

18. Riah, O., Courriere, P., Dousset, J.C. & others. 1998. Nicotine is more efficient than cotinine at passing the blood-brain barrier in rats. *Cell Mol Neurobiol*, 18: 311 - 318.

19. Miksys, S., Hoffmann, E. & Tyndale, R.F. 2000. Regional and cellular induction of nicotine-metabolizing CYP2B1 in rat brain by chronic nicotine treatment. *Biochem Pharmacol*, 59: 1501 - 1511.

20. Goldstein, M. 2000. Long- and Short-Term Regulation of Tyrosine Hydroxylase. (*In* Bloom, F.E. & Kupfer, D.J., *eds.* Psychopharmacology - the Fourth Generation of Progress. 4th ed. New York: Lippincott Williams & Wilkins.

21. Hiremagalur, B., Nankova, B., Nitahara, J. & others. 1993. Nicotine increases expression of tyrosine hydroxylase gene. Involvement of protein kinase A-mediated pathway. *J Biol Chem*, 268: 23704 - 23711.

22. Sabban, E.L. & Gueorguiev, V.D. 2002. Effects of short- and long-term nicotine treatment on intracellular calcium and tyrosine hydroxylase gene expression. *Ann N Y Acad Sci*, 971: 39 - 44.

23. Li, S., Kim, K.Y., Kim, J.H. & others. 2004. Chronic nicotine and smoking treatment increases dopamine transporter mRNA expression in the rat midbrain. *Neurosci Lett*, 363: 29 - 32.

24. Mihailescu, S. & Drucker-Colin, R. 2000. Nicotine, brain nicotinic receptors, and neuropsychiatric disorders. *Arch Med Res*, 31: 131 - 144.

25. Di Chiara, G. 2000. Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur J Pharmacol*, 393: 295 - 314.

26. Rapier, C., Lunt, G.G. & Wonnacott, S. 1988. Stereoselective nicotine-induced release of dopamine from striatal synaptosomes: concentration dependence and repetitive stimulation. *J Neurochem*, 50: 1123 - 1130.

27. Rapier, C., Lunt, G.G. & Wonnacott, S. 1990. Nicotinic modulation of [3H]dopamine release from striatal synaptosomes: pharmacological characterisation. *J Neurochem*, 54: 937 - 945.

28. Grady, S.R., Marks, M.J. & Collins, A.C. 1994. Desensitization of nicotine-stimulated [3H]dopamine release from mouse striatal synaptosomes. *J Neurochem*, 62: 1390 - 1398.

29. Grady, S., Marks, M.J., Wonnacott, S. & others. 1992. Characterization of nicotinic receptor-mediated [3H]dopamine release from synaptosomes prepared from mouse striatum. *J Neurochem*, 59: 848 - 856.

30. Kalgutkar, A.S., Zhou, S., Fahmi, O.A. & others. 2003. Influence of lipophilicity on the interactions of N-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines and their positively charged N-alkyl-4-phenylpyridinium metabolites with cytochrome P450 2D6. *Drug Metab Dispos*, 31: 596 - 605.

31. Lehericy, S., Brandel, J.P., Hirsch, E.C. & others. 1994. Monoamine vesicular uptake sites in patients with Parkinson's disease and Alzheimer's disease, as measured by tritiated dihydrotetrabenazine autoradiography. *Brain Res*, 659: 1 - 9.