

**The effects of a tyrosine hydroxylase activator on the
monoamine metabolism in rabbits exposed to tobacco
smoke**

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“Addiction exists wherever persons are internally compelled to give energy to things that are not their true desires. To define it directly, addiction is a state of compulsion, obsession, or preoccupation that enslaves a person’s will and desire. Addiction sidetracks and eclipses the energy of our deepest, truest desire for love and goodness” – (*Addiction and Grace*)

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Abstract

Nicotine is the most commonly used drug in the world today, with over 1.1 billion users worldwide, while smoking remains the single largest preventable cause of disease and premature death. Being the prime cause of cancer and heart disease, smoking also causes many other fatal conditions and chronic illnesses among adults. Of the 17 million smokers that try to quit smoking each year, fewer than one out of ten actually succeed. The success rate is only in the order of 2.5 % (WHO, 2002).

Cigarette smokers have been shown to have lower platelet MAO levels than non-smokers because of possible decreased MAO synthesis, the presence of MAO inhibitory compounds in smoke or the fact that low MAO individuals are more vulnerable to smoking (Oreland, 1981). Through positron emission tomography (PET) it was discovered that MAO-A levels are reduced by 30 %, and MAO-B levels by 40 % in smokers compared to non-smokers and ex-smokers (Fowler *et al.*, 1996). Nicotine only inhibits MAO at levels 200 times higher than those found in smokers. Thus, smoking addiction is not induced by nicotine alone but also by other compounds in cigarette smoke that increase neurotransmitter amine concentrations (Fowler *et al.*, 1998).

Nicotine and MAO inhibition increase dopamine (DA) levels in several areas of the brain. The continuous increases in DA causes shifts from pleasure towards a wanting process such as drug cravings. Thus, the motivation for smoking may be directed to either restoring a homeostatic imbalance, or the enjoyment of behaviour associated with DA release (Nestler, 2004b). A direct link to serotonin (5-HT) involvement in the regulation of drug intake is provided by findings showing that manipulations which decrease brain 5-HT neurotransmission elevate self administration of several different drugs. An increase in 5-HT neurotransmission could reduce drug consumption by strengthening inhibitory control (Opitz & Weischer, 1988).

Bupropion (BUP) (Zyban®) is a phenylaminoketone, atypical anti-depressant in sustained release formulation, approved by the Food and Drug Administration (FDA) as the first non-nicotine pharmacotherapy for smoking cessation. The mechanism of action is not yet proven, but is likely to involve the modest blockade of DA and norepinephrine (NA) reuptake (Butz *et al.*, 1982), and the antagonism of high-affinity nicotinic acetylcholine receptors (Slemmer *et al.*, 2000). Bupropion was found to have a modulating effect on MAO, DA, NA and 5-HT and thus helps with smoking cessation (Xi-Ming Li *et al.*, 2001).

A potential tyrosine hydroxylase activator (THA), nicotinamide adenine dinucleotide (NAD), has already been used successfully since 1939 for the short-term treatment of alcoholism and various types of substance dependencies (Cleary, 1986). A THA will hypothetically terminate tobacco cravings due to its activation of tyrosine hydroxylase, which is important in the synthesis of DA. Recently, research confirmed that NAD had a normalizing effect on the neurotransmitters which causes the homeostatic imbalance to return to normal. NAD also plays a role in the production of 5-HT in the brain (South, 1997).

We hypothesised that NAD will terminate the craving effects of tobacco addiction, having the same normalising effect as bupropion on DA and 5-HT levels and that it may be a MAO-B inhibitor.

The rabbit was used as animal model for nicotine withdrawal. Rabbits were divided into four groups (n=8) and implanted subcutaneously with two Alzet® osmotic pumps, three groups containing smoke extract and one vehicle (propylene glycol). After twenty-seven days the pumps were removed and the three smoke groups (group 1, 2 and 3) treated with saline, NAD and bupropion respectively for four days and the control group (group 4) with NAD for four days. Blood samples were taken three times a day during the four days of treatment. The rabbits were decapitated on day five and the nucleus accumbens removed

and frozen at -86 °C until analysed. MAO activity as well as DA and 5-HT levels, including their metabolites, were determined in plasma and brain tissue.

Groups receiving NAD and bupropion had higher DA and DOPAC blood levels compared to the control group 1, suggesting that NAD increases the DA levels to avoid a decrease of DA during withdrawal that leads to craving. Thus, NAD could have the same normalizing effects as bupropion, thereby identifying it as a potential drug for the treatment of smoke addiction.

In the nucleus accumbens (NAc) no significant difference could be detected in the DA, 5-hydroxyindoleacetic acid (5-HIAA) or 5-HT concentrations between group 1, 2, 3 and 4 after four days of treatment, indicating that mono-amine levels have normalised after 4 days of treatment. DOPAC concentrations, however, were significantly lower on day 32 in the NAD treated group (group 2) and the bupropion treated group (group 3) compared to the control group. This decrease in DOPAC concentration was probably due to downregulation in an attempt to normalize DA levels.

The weight of each rabbit was determined on day 1, 27 and 31 to determine the effect of NAD and bupropion on the weight of the rabbits. The two groups treated with NAD gained less weight than the saline- and bupropion-treated group, indicating that NAD might be valuable in the prevention of disproportionate amount of weight gain during smoking cessation.

NAD did not inhibit either bovine or baboon MAO-B. Inhibition of MAO-B can thus be eliminated as the possible mechanism by which NAD elevated DA levels.

The results of Nel (2003) measuring withdrawal symptoms (e.g. locomotor activity, acoustic startle) in smoking rats treated with NAD, clearly indicated that NAD do reduce withdrawal symptoms in rats. Above results indicate that NAD has the same effect on monoamine levels as bupropion, preventing DA levels to fall below normal levels – the cause for craving and withdrawal symptoms. NAD

treatment shows promising results and potential in the treatment of tobacco addiction and further research into its mechanism of action is paramount.

Opsomming

Nikotien is die mees algemene gebruikte dwelmmiddel met meer as 1.1 miljard verbruikers wêreldwyd. Sigaretrook is die enkele grootste oorsaak van ongesteldheid en ontydige dood. Dit is die primêre oorsaak van kanker en kardiovaskulêre siektes, en veroorsaak ook verkeie ander kroniese en noodlottige siektetoestande. Minder as tien persent van die 17 miljoen rokers wat jaarliks probeer ophou, slaag daarin. Die suksessyfer is slegs ongeveer 2.5 % (WHO, 2002).

Navorsing toon dat sigaretrokers laer MAO-konsentrasies in hulle bloedplaatjies het as nie-rokers en persone wat reeds ophou rook het a.g.v. 'n moontlike verlaging van MAO-sintese, die teenwoordigheid van stowwe wat MAO inhibeer of weens die feit dat individue met lae MAO-konsentrasies 'n groter neiging toon om te rook (Oreland, 1981). Deur positronemissie-tomografie het navorsing getoon dat sigaretrokers se MAO-A vlakke tot 30 %, en MAO-B vlakke tot 40 % laer is vergeleke met die van nie-rokers en persone wat opgehou rook het. Nikotien inhibeer MAO slegs by konsentrasies wat 200 keer hoër is as dit wat by sigaretrokers voorkom. Sigaretverslawing word dus nie slegs deur nikotien geïnduseer nie, maar moontlik ook deur verskeie ander komponente wat in sigaretrook voorkom en kan lei tot verhoging van die monoamienkonsentrasies (Fowler *et al.*, 1998).

Nikotien en MAO-inhibisie verhoog dopamien (DA) vlakke in sekere areas van die brein. 'n Herhalende verhoging veroorsaak 'n verskuiwing vanaf genot tot 'n toestand van afhanklikheid van die dwelmmiddel. Die drang om te rook kan dus wees om die homeostatiese wanbalans te herstel, of die genotservaring van verhoogde DA vrystelling (Nestler, 2004b). Die betrokkenheid van serotonien (5-HT) by die regulering van dwelmmiddel-inname is beklemtoon deur waarnemings dat verlaagde 5-HT-neurotransmissie selfadministrasie van verskeie dwelmmiddels verhoog. 'n Verhoging van 5-HT-neurotransmissie kan dus

moontlik dwelmmiddelverbruik verlaag omdat dit inhibisiebeheer versterk (Optiz & Weischer, 1988).

Bupropioon (BUP) (Zyban[®]), is 'n fenielaminoketoon, atipiese antidepressant, bemark as 'n verlengde vrystellingsproduk en is goedgekeur deur die *Food and Drug Administration* (FDA) as die eerste nie-nikotiniese farmakoterapeutiese middel vir die behandeling van sigaretrookverslawing. Die werkingsmeganisme is nog onbekend, maar behels moontlik die gedeeltelike blokkering van DA en noradrenalin (NA) heropname (Butz *et al.*, 1982), asook die antagonisme van hoë-affiniteit nikotiniese asetiëlcholienreseptore (Slemmer *et al.*, 2000). Daar is gevind dat BUP 'n regulerende effek het op MAO, DA, NA en 5-HT en daardeur rokers help om op te hou (Xi-Ming Li *et al.*, 2001).

'n Potensiële tirosienhidroksilase aktiveerder (THA), nikotienamiedadenindinukleotied (NAD), is sedert 1939 suksesvol gebruik in die korttermyn behandeling van alkoholisme en verskeie ander dwelmmiddelverslawings (Cleary, 1986). 'n THA sal hipoteties die lus om te rook verlaag deur die aktivering van die ensiem, tirosienhidroksilase, wat betrokke is by die sintese van DA. Navorsing het onlangs getoon dat NAD 'n normaliserende uitwerking op die neurotransmitters het en die homeostatiese wanbalans na normaal herstel. NAD speel ook 'n rol in die produksie van 5-HT in die brein (South, 1997).

Ons hipotese was dat NAD die lus om te rook sal verlaag as gevolg van die normalisering van DA- en 5-HT-vlakke deur die regulering van tirosienhidroksilase- en MAO-aktiwiteit.

Die konyn is gebruik as proefdiermodel vir nikotienonttrekking. Die konyne is in vier groepe (n=8) verdeel. Twee Alzet[®] osmotiese pompies is subkutaneus in elke konyn ingeplant. Drie groepe het pompies, gevul met rookekstrak ontvang terwyl die kontrolegroep se pompies slegs propileenglikool bevat het. Na 27 dae is die pompies verwyder en die drie rookgroepe (groep 1, 2 en 3) onderskeidelik vir vier dae met saline, NAD en BUP behandel terwyl die kontrolegroep

(groep 4) vir 4 dae met NAD behandel is. Bloedmonsters is driemaal per dag (om 8h00, 12h00 en 16h30) gedurende die vier dae van behandeling geneem. Die konyne is op dag vyf onthoof, waarna die nukleus accumbens (NAc) verwyder en by -86 °C gevries is. MAO-aktiwiteit, die vlakke van DA, 5-HT, en hulle metaboliete is in plasma en breinweefsel bepaal.

Groepe wat NAD en BUP ontvang het, het hoër DA- en DOPAC-bloedvlakke gehad as die kontrolegroep 1. Spekulatief dui dit op 'n moontlike meganisme van NAD om DA-vlakke te verhoog of binne normale vlakke te hou om sodoende te verhoed dat DA-vlakke tydens ontrekking verlaag en die drang na rook verhoog. NAD mag dus oor dieselfde regulerende eienskappe as bupropioon beskik en is dus 'n potensiële terapeutikum vir die behandeling van rookverslawing.

In die NAc was geen statistiese verskil in die DA, 5-hidroksie-indoolasynsuur (5-HIAA) en 5-HT konsentrasies van groep 1, 2, 3 en 4 na 4 dae van behandeling nie, wat aandui dat die monoamienvlakke genormaliseer het na 4 dae van behandeling. Daar was wel 'n statistiese verlaging in die DOPAC-konsentrasies van die NAD-behandelde groep (groep 2) en die bupropioon-behandelde groep (groep 3) vergeleke met die kontrolegroep. Die verlaging in DOPAC kan verklaar word as 'n afregulering in 'n poging om normale DA vlakke te bereik.

Die gewig van elke konyn is op dag 1, 27 en 31 bepaal om die effek van die onderskeie behandelings op die gewig van die konyne vas te stel. Die twee NAD-behandelde groepe het 'n laer gewigstoename as die kontrolegroep 1 en die bupropioon-behandelde groep (groep 3) getoon. Die data toon dat NAD waardevol kan wees vir die voorkoming van gewigstoename en die instandhouding van gewig tydens die rookstaking.

NAD het nie bees- of bobbejaan MAO-B geïnhibeer nie. MAO-B-inhibisie word dus uitgeskakel as 'n moontlike meganisme waardeur NAD DA verhoog.

Nel (2003) het aangetoon dat NAD sekere onttrekkingssimptome (lokomotoriese aktiwiteit, akoestiese refleks) in rotte wat rookekstrak ontvang het, verminder. Bogenoemde resultate toon dat NAD dieselfde effek op die monoamienvlakke uitoefen as bupropioon, naamlik 'n voorkoming van 'n afname in DA-vlakke tot onder die normale – die oorsaak van onttrekkingssimptome.

Ons gevolgtrekking is dat NAD belowende resultate toon vir die behandeling van rookverslawing en verdere navorsing regverdig.

Abbreviations

A

ACh	acetylcholine
ACTH	adrenocorticotropin hormone
ADP	adenine dinucleotide diphosphate
AIDS	acquired immunodeficiency disease
ALDH	aldehyde dehydrogenase
ANCOVA	analysis of covariate
ANOVA	analysis of variance
ARC	Animal Research Centre
ATP	adenine dinucleotide triphosphate
AUC	area under curve

B

BUP	bupropion
-----	-----------

C

Ca ²⁺	calcium atom
cAMP	cyclic adenine mono-phosphate
CNS	central nervous system

COMT	catechol-O-methyltransferase
CRF	corticotropin- releasing factor
Cu	copper atom
D	
DA	dopamine
DAT	dopamine transporter
ddH ₂ O	double distilled water
DLPC	dorsal lateral prefrontal cortex
DNA	deoxyribonucleic acid
DOPA	3,4-dihydroxy-phenylalanine
DOPAC	3,4-dihydroxy-phenylacetic acid
DPN	diphosphopyridine nucleotide
DSM-IV	Diagnostic and Statistical Manual of Mental Health Disorders
E	
EB	erythrohydrobupropion
ECD	electrochemical detection
EDTA	ethylenediaminetetraacetic acid
EOPs	endogenous opioid peptides

F

FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration

G

GABA	gamma-aminobutyric acid
------	-------------------------

H

HB	6-hydroxybupropion
HCl	hydrochloric acid
HClO ₄	perchloric acid
5-HIAA	5-hydroxyindoleacetic acid
H ₂ O ₂	hydrogen peroxide
HPA	hypothalamic-pituitaryadrenal
HPLC	high pressure liquid chromatography
HSD	honest significant difference
5-HT	serotonin
HVA	homovanillic acid

I

ICD-10	International Classification of Disease
IP	intra-peritoneally

IS	internal standard
M	
MAO	monoamine oxidase
mAChR	muscarinic acetylcholine receptor
mRNA	messenger ribonucleic acid
MSNs	medium spiny neurons
N	
NA	noradrenaline
Na ₂	sodium atom
NAc	nucleus accumbens
nAChRs	nicotinic acetylcholine receptors
NAD	nicotinamide adenine dinucleotide
NADH	the reduced form of NAD
NaOH	sodium hydroxide
NIDA	National Institute on Drug Abuse
NRT	nicotine replacement therapies
O	
O ₂	oxygen
8-OH-DPAT	8-hydroxy-2-dipropylaminotetralin

oQ	o-quinone
P	
PD	Parkinson's disease
PE	phenylethylamine
PET	positron emission tomography
PKA	protein kinase A
PLC	prefrontal cortex
Q	
Q	ubiquinone
QH ₂	ubiquinol (or the reduced form of coenzyme Q)
R	
RSD	relative standard deviation
S	
SN	substantia nigra
SSRIs	selective serotonin reuptake inhibitors
Std.	standard
STDEV	standard error deviation
T	
TB	<i>threo</i> -hydrobupropion

TCAs	tricyclic antidepressants
TH	tyrosine hydroxylase
THA	tyrosine hydroxylase activator
TIQ	1,2,3,4-tetrahydroisoquinoline
TNP	transdermal nicotine patch
TQD	target quit date
U	
UNDCP	United Nations International Drug Control Programme
UK	United Kingdom
USA	United States of America
V	
VTA	ventral tegmental area
VTA-Nac	ventral tegmental area – nucleus accumbens
W	
WHO	World Health Organisation

Chapter 1

Introduction

Tobacco is one of the most commonly used drugs in the world, with an estimated 13 million adult smokers in the United Kingdom (UK) alone. A steady decline in the prevalence of smoking was observed over the past twenty years, but recently there has been a slight increase in the incidence of smoking, particularly among young people and women (Smoking Cessation, 2004b).

Smoking is the single largest preventable cause of disease and premature death, and is one of the main contributors to deadly conditions like strokes, heart disease and chronic lung disease. Smoking causes cancer of the lungs, larynx, oesophagus, mouth, and bladder, and contributes to cancer of the cervix, pancreas, and kidneys (WHO, 2002), resulting in 84 % of deaths from lung cancer and 83 % of deaths from chronic obstructive lung disease, including bronchitis (Smoking Cessation, 2004b). The number of people dying from cancer because of smoking is 46,500 a year in the UK alone (Smoking Cessation, 2004b). No South African statistics were available.

According to the World Health organization (WHO, 2002), the life of a smoker is reduced by an average of five minutes with every cigarette that is smoked, and half the number of long-term smokers will die from the use of tobacco. It is estimated that about every eight seconds someone dies from tobacco use, and that about 12 times more Britains have died from smoking than from World War II up to present. In America, more than one in five deaths are caused by the use of tobacco (WHO, 2002).

Globally it is estimated that smoking related-diseases kill one in ten adults, or four million deaths per year. By 2030, if current trends continue, smoking will kill one in six people (WHO, 2002). Statistical estimations predict that for every 1000 young adult smokers, one will be murdered, about six will die in road

accidents, but half will die due to a smoking related illness (about one-quarter in middle age plus one-quarter in old age) (Smoking Cessation, 2004b).

The primary reason inducing people to smoke can be attributed to the highly addictive nicotine present in tobacco smoke. Following inhalation of cigarette smoke, nicotine is rapidly absorbed through the lining of the lungs reaching high concentrations in the brain within ten to nineteen seconds. It is significant that inhaled nicotine reaches the brain faster than by intravenous injection and this rapid effect is thought to play an important role in nicotine dependence (Smoking Cessation, 2004b). As the levels of nicotine in the body start to fall, smokers experience withdrawal symptoms, which can include irritability, restlessness, craving and lack of concentration. To avoid the distress associated with these symptoms the smoker lights up another cigarette (Smoking Cessation, 2004b).

What makes smoking so harmful?

Tobacco smoke is a complex mixture of over 4000 different substances, of which some are toxic and carcinogenic, and includes nicotine, tar, irritants and carbon monoxide (Smoking Cessation, 2004b).

Nicotine is the major known addictive component of cigarette smoke. It affects the central nervous system (CNS) inducing various mood changes including decreased tension, arousal and relaxation. These effects may also be attributed to increases in catecholamine levels in the body as a result of monoamine oxidase (MAO) inhibition from substances present in cigarette smoke (Smoking Cessation, 2004b).

At least 60 of the chemicals found in tobacco smoke are known to cause cancer, for example: arsenic, chromium, cadmium, and formaldehyde. Tar narrows the airways in the lungs, irritates the lining of the lungs causing coughing and damages the small hairs (cilia) that help protect the lungs against dirt and infection (Smoking Cessation, 2004b).

Carbon monoxide binds to haemoglobin, reducing the ability of the blood to carry oxygen to the heart, brain and circulatory system and is particularly harmful during pregnancy because smoking during pregnancy and nursing carries a risk to the fetus and to the infant during the rapid phases of development, especially if there is an increase of direct exposure to the drug (Smoking Cessation, 2004b). Evidence indicates that nicotine can abnormally alter cell proliferation and differentiation, thereby affecting synaptic and circulatory activity (Dani & De Biasi, 2001). It also puts additional strain on the heart, as it has to work harder to transport oxygen around the body.

Smoking does not only present a huge problem within healthcare - economically about 15 billion cigarettes are sold daily, which makes it 10 million cigarettes every minute. On average, the cost of smoking-related diseases in the United States alone is estimated at more than \$150 billion a year. The cost of advertising for the tobacco industries amounted to about \$15 million a day (WHO, 2002).

It is obvious, if one looks at the above statistics, that smoking is a global problem that needs to be attended to as soon and as effectively as possible. It is therefore very important that more research must be done to develop methods for the effective cessation of smoking and curing of this addiction.

Chapter 2

Literature Review

2.1. Tobacco addiction

2.1.1. Introduction

“Addiction can be defined as a loss of control over drug intake, or the compulsive seeking and taking of drugs despite their adverse and devastating consequences” (Roberts *et al.*, 1997).

AIDS, lung cancer, and cirrhosis of the liver are only some of the overwhelming problems caused by drug addiction and costs society hundreds of billions of dollars due to loss of life and productivity. Unfortunately, the treatments available for addicts today, are inadequate for most of its users (Nestler, 2004b).

There are many types of complex social and psychological factors involved within addiction. However, it is, at its core, a biological process: the effect of a biological substance (drug of abuse) on a biological substrate (a vulnerable brain). Research proved that addiction is highly heritable and about 50 % of the risk for an addiction is genetic. This holds true for many different addictions, including to cocaine, heroin, alcohol, and nicotine. However, the specific genes which comprise that risk remain unknown (Nestler, 2004b).

Over the past several decades, scientists have used animal models to study the behavioural abnormalities used to define addiction, and they have done it with increasing accuracy. The availability of such models has made it possible to investigate the neurobiological basis of the addiction process (Nestler, 2004b).

Upon initial exposure, a drug of abuse enters the brain and binds to its initial protein target. This binding troubles synaptic transmission at particular synapses

in the brain and causes the acute behavioural effects of the drug (e.g., high, euphoria, sedation, activation, etc.) (Nestler, 2004b).

Addiction however, can not be explained by these acute actions of the drug alone, it requires adaptations due to repeated drug administration. These adaptations presumably involve molecular and cellular changes in particular neurons in the brain, which alter the functioning of the neural circuits in which those neurons operate to lead ultimately to the behavioural abnormalities that characterize an addicted state. As a result, the process of addiction can be viewed as a form of drug-induced *neural plasticity* (the alteration of brain systems at a neural level that systematically affects behaviour) (Nestler, 2004b).

The fact that structurally diverse drugs all cause a similar behavioural abnormality (addiction) can be explained by the fact that each drug, despite many distinct actions in the brain, converge in producing some common actions, prominent among which is activation of the **brain's reward circuitry** (see 2.1.2) (Nestler, 2004b).

The most important part of this circuitry is the mesolimbic dopamine system, comprised of dopamine neurons with cell bodies in the ventral tegmental area (VTA) of the midbrain and their projections to the limbic forebrain, in particular the nucleus accumbens (NAc) (Chao & Nestler, 2004). Drugs of abuse affect the VTA-NAc pathway with a power and persistence not seen in response to natural rewards. One likely mechanism of addiction, then, is that repeated, extreme perturbation of these neurons changes them in a way that leads to dramatic *alterations* in reward mechanisms and motivational state that underlie addiction (Nestler, 2004b).

There are several types of functional *alterations* that have been described in the addiction field:

Dependence (see 2.1.3), is an altered physiological (functional) state that develops to compensate for persistent drug exposure. Dependence gives rise to a withdrawal syndrome upon ceasing of drug exposure, and may contribute to the *dysphoria* (negative or aversive emotional state) and high rates of relapse seen during early phases of withdrawal (Nestler, 2004b).

Tolerance (see 2.1.4), or reduced drug responsiveness with repeated exposure to a constant drug dose, may contribute to the escalation of drug intake seen during the development of an addiction (Nestler, 2004b).

Sensitization (see 2.1.5), or enhanced drug responsiveness with repeated exposure to a constant dose, may contribute to the increased risk of relapse after longer withdrawal periods (Nestler, 2004b).

Drug addiction likely involves changes in many brain structures. Changes in the VTA and NAc increase or decrease an individual's sensitivity to the rewarding effects of drug exposure and lead to withdrawal symptoms when the drug is stopped (Nestler, 2004b).

2.1.2. Brain reward pathways

The mesolimbic dopamine system is the most important reward pathway in the brain and dopamine (DA) is the neurotransmitter of importance. This circuit (VTA-NAc) is a key detector of a rewarding stimulus. Under normal conditions, the circuit controls an individual's responses to natural rewards, such as food, sex, and social interactions, and is therefore an important determinant of motivation and incentive drive. In simplistic terms, activation of the pathway tells the individual to repeat what it just did to get that reward. It also tells the memory centers in the brain to pay particular attention to all features of that rewarding experience, so it can be repeated in the future. Not surprisingly, it is a very old pathway from an evolutionary point of view. The use of DA neurons to mediate

behavioural responses to natural rewards is seen in worms and flies, which evolved 1-2 billion years ago (Nestler, 2004a).

The VTA-NAc pathway is part of a series of parallel, integrated circuits, which involve several other key brain regions (Figure 2-1). The main functions of each of these brain regions are:

The **VTA** is the site of dopaminergic neurons which tells the organism whether an environmental stimulus (natural reward, drug of abuse, stress) is rewarding or aversive.

The **NAc**, also called ventral striatum, is a principle target of VTA DA neurons. This region mediates the rewarding effects of natural rewards and drugs of abuse.

The **amygdala** is particularly important for conditioned forms of learning. It helps an organism establish associations between environmental cues and whether or not that particular experience was rewarding or aversive, for example, remembering what accompanied finding food or fleeing a predator. It also interacts with the VTA-NAc pathway to determine the rewarding or aversive value of an environmental stimulus (natural reward, drug of abuse, stress).

The **hippocampus** is critical for declarative memory, the memory of persons, places, or things. Along with the amygdala, it establishes memories of drug experiences which are important mediators of relapse.

The **hypothalamus** is important for coordinating an individual's interest in rewards with the body's physiological state. This region integrates brain function with the physiological needs of the organism.

Probably the most important, but least understood, are **frontal regions** of cerebral cortex, such as medial prefrontal cortex, anterior cingulate cortex, and

orbitofrontal cortex, which provide executive control over choices made in the environment (for example, whether to seek a reward).

The **locus coeruleus** is the primary site of noradrenergic neurons in the brain, which pervasively modulate brain function to regulate the state of activation and mood of the organism.

The **dorsal raphe** is the primary site of serotonergic neurons in the brain, which, like noradrenergic neurons, pervasively modulate brain function to regulate the state of activation and mood of the organism.

These various brain regions, and many more, do not function separately. Rather, they function in a highly inter-related manner and mediate an individual's responses to a range of environmental stimuli (Nestler, 2004a).

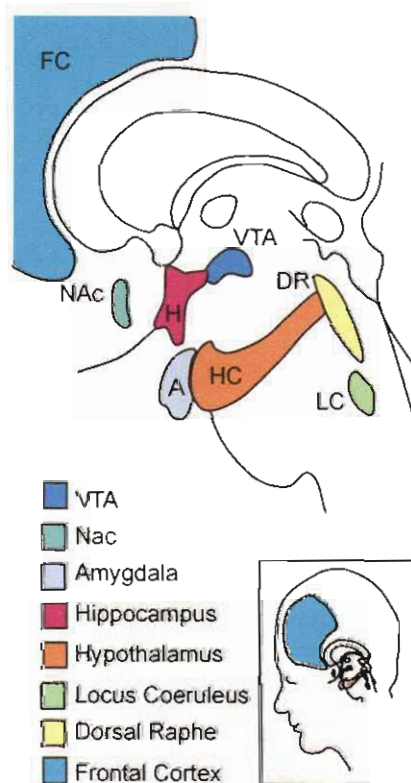


Figure 2-1 The key brain regions involved in the addiction process (Nestler, 2004a).

2.1.3. Tobacco dependence

There are two forms of dependencies currently known that exist among cigarette smokers; pharmacological and behavioural. Variations of these two components may be present between individual smokers, and ideally both forms should be targeted to overcome the dependence (Smoking Cessation, 2004a).

2.1.3.1. Pharmacological dependence

Pharmacological or physical dependence to nicotine is probably the main reason why people continue to smoke and is thus a major problem for those attempting to quit.

Physical dependence is a *state* that develops as a result of the adaptation (tolerance) (see 2.3) produced by a resetting of homeostatic mechanisms in response to repeated drug use. Drugs can affect numerous systems that previously were in equilibrium; these systems must find a new balance in the presence of inhibition or stimulation by a specific drug. A person in this adapted or physically dependent state requires continued administration of the drug to maintain normal function. If administration of the drug is stopped abruptly, there is another imbalance, and the affected systems must again go through a process of readjusting to a new equilibrium without the drug (O'Brien, 1996).

2.1.3.2. Behavioural dependence

Lighting and holding a cigarette, or blowing out smoke are rituals and behavioural patterns associated with smoking which regular smokers may repeat more than 200 times a day and this makes it very difficult to break this patterns and stop smoking (Smoking Cessation, 2004a).

Ingrained smoking behaviour, such as always smoking in positive situations (i.e. after a meal or social drinking) or in negative situations (i.e. during stress) is also very difficult to overcome (O'Brien, 1996).

2.1.4. Nicotine Tolerance

Neuroadaptation, or otherwise known as tolerance, is induced by a prolonged or repeated exposure to nicotine (Benowitz, 1998). Tolerance is defined as a state in which, after repeated doses, a given dose of a drug produces less effect than before, or in which increasing doses are required to achieve the effect observed with the first dose (Benowitz, 1988).

With the development of tolerance to nicotine, there is a decline in the prevalence of nausea, dizziness, and other characteristic symptoms despite using substantial amounts of nicotine, or a diminished effect observed with continued use of the same amount of nicotine-containing products (American Psychiatric Association, 1994).

With neuroadaptation, tolerance develops to the physiologic effects of the substance (Benowitz, 1998), which results in the need for greater and greater amounts of nicotine to achieve a physiologic response. Evidence of neuroadaptation in cigarette smokers lies in the fact that smokers progressively increase the number of cigarettes they smoke over a period of several years (Benowitz, 1999).

After neuroadaptation or tolerance has developed due to chronic nicotine exposure, the absence of nicotine results in subnormal release of DA and other neurotransmitters (Benowitz, 1999). A state of deficient DA responses to novel stimuli in general, and to a state of malaise and inability to experience pleasure may occur (Benowitz, 1999). Koob and LeMoal (1997) have termed this observation as “hedonic homeostatic dysregulation”. According to these researchers, it is not because of the self-regulation of the drug that users became drug abusers or drug dependent. They conceptualized how the regulation failure leads to addiction:

Once a person quits smoking, the body mobilizes enormous amounts of energy to maintain the homeostasis of abstinence. They argue that the first self-regulation failure (even a puff of smoking) can lead to significant emotional distress. This may explain why even a single slip might easily result in a return to compulsive drug use (Benowitz, 1999).

2.1.5. Sensitization

Sensitization is a result of repeated administration of stimulants, nicotine, opiates, or alcohol, which appears to be mediated by the mesolimbic dopamine system (Wise & Leeb, 1993).

Sensitization involves an enhanced activation of DA function in the mesolimbic system, and may represent a within-systems mechanism of neuroadaptation. For example, injections of drugs like nicotine, opiates or amphetamine directly into the ventral tegmental area that change the function of the DA neurons, produce sensitization to later injections of these drugs in the periphery (White & Wolf, 1991).

As is the case with tolerance, sensitization may develop to one particular effect of a drug and not to another. Another system that may have an important role in sensitization, representing a between-systems mechanism of neuroadaptation, involves corticotropin-releasing factor (CRF). CRF is released by the hypothalamus and the amygdala in response to stress, and causes the release of additional stress hormones into the bloodstream from the adrenal cortex (located above the kidneys) and the pituitary gland (located at the base of the brain). This stress-response system is called the hypothalamic-pituitary-adrenal (HPA) axis (Roberts *et al*, 1995).

Exposure to a variety of stressors can promote sensitization to drug effects, and the CRF mediated stress-response system has been implicated in this sensitization. For example, stress hormones released by the adrenal cortex

(i.e., corticosteroids) have been implicated in the increased locomotor response observed in mice following repeated administration of low doses of alcohol (Roberts *et al.*, 1995).

Another source of between-systems sensitization may also be characterized by excitatory neurotransmitter systems for alcohol and other drugs of abuse. Glutamate is the major excitatory neurotransmitter in the brain. The development of sensitization to psychomotor stimulants can be blocked by the administration of an antagonist of a specific glutamate receptor subtype, suggesting a role for brain glutamate systems in sensitization (Wise, 1988).

2.1.6. Dependence producing substances act as positive reinforcers

A positive reinforcer is anything that can be administered to increase the probability that a particular behavior will be repeated when the person wants to replicate the previous outcome. Substances, like nicotine, act as positive reinforcers to the degree that they activate so-called 'reward systems' in the body. In short, substance use behaviour is repeated because it is rewarded (Shadel *et al.*, 2000).

Recent research has focussed on the pharmacologic actions that substances have on DA circuitry in the central nervous system, circuitry that is thought to be involved in the regulation of reward (Spiraki, 1988; Wise & Bozarth, 1987). The reinforcing properties of nicotine may be related, in part, to nicotine's moderating effects on dopaminergic activity in the mesolimbic system (Clarke, 1990; Fuxe *et al.*, 1987; Pert & Clarke, 1987; Pich *et al.*, 1997; USDHHS, 1988).

One of the more well-articulated pathways begins in the VTA, moves through the NAc, and ends in the prefrontal cortex (Wise & Bozarth, 1987). These areas in particular are replete with DA receptors (Wise & Bozarth, 1987). When these receptors are stimulated (i.e. by dependence producing substances), increased levels of dopaminergic activity, and thus, reward follow. The potential of the

substance to produce dependence may increase with repeated use (and continued activation of dopaminergic pathways) (Bozarth, 1994; Koob, 1992).

Nicotine is a potent reinforcer (Corrigall & Coen, 1989; Goldberg *et al.*, 1981; Stolerman & Jarvis, 1995). The blood nicotine concentration reaches a plateau within six to eight hours with repeated smoking throughout the day (Benowitz *et al.*, 1982). Thus, nicotine smoked from cigarettes reaches the brain extremely quickly and therefore serves to activate reward systems within only a few seconds of use. It follows, then, that the behaviours associated with cigarette smoking (e.g. striking a match, lighting the cigarette, hand-to-mouth actions) are quickly reinforced and likely to be repeated (Shadel *et al.*, 2000).

2.1.7. Cravings

Craving is an important part of substance use, and can be defined as a general desire to use a substance (Pickens & Johanson, 1992). The mechanisms (i.e. biological, cognitive) which contribute to the rise in cravings are not clear, but cravings are generally regarded as the subjective manifestations of the felt 'need' for the substance (Niaura *et al.*, 1988; Tiffany, 1990).

Whenever a condition of deficiency arises, smokers report cravings for cigarettes that generally translate into smoking. Increasing levels of deficiency typically lead to stronger cravings (Payne *et al.*, 1996).

Cravings for cigarettes are triggered by a variety of internal (emotions, thoughts) and external (situational) cues (Abrams *et al.*, 1987; Niaura *et al.*, 1992, 1998; Shiffman *et al.*, 1996) and are commonly reported as motivators or precursors to the actual use of the substance (Marlatt & Gordon, 1985).

It is due to cravings that smokers frequently relapse to cigarettes (Killen & Fortman, 1997; Shiffman *et al.*, 1997) following a period of abstinence (Shiffman *et al.*, 1996).

2.1.8. Core conceptual features of nicotine dependence

To summarize, is a few core features of nicotine dependence:

- Dependence producing substances have dose-dependent psychoactive effects.
- Dependence producing substances act as positive reinforcers.
- Repeated exposures to a substance are necessary for dependence to develop.
- Tolerance develops to the effects of dependence producing substances.
- Withdrawal symptoms appear upon cessation of dependence producing substances.
- Cravings characterize dependence and motivate substance use.
- Compulsive use is a behavioural marker of dependence.
- Dependent users become ambivalent about their substance use.
- Dependence is a chronic condition.
- Dependence producing substances are used to manage negative affect and stress (Shadel et al., 2000).

2.2. Nicotine in tobacco smoke

Nicotine is a tertiary amine derived from the plant, *Nicotiana tabacum* L, the levo-isomer of which produces the majority of physiological effects within its users. Nicotine exists in both charged (Figure 2-2) and uncharged forms at the pH of blood. The uncharged form (Figure 2-2) can cross the blood-brain barrier very rapidly (Domino, 1998). Nicotine is buffered to physiological pH in the lungs, is

rapidly absorbed and reaches the brain in only 10 to 19 seconds (Gourlay & Benowitz, 1997).

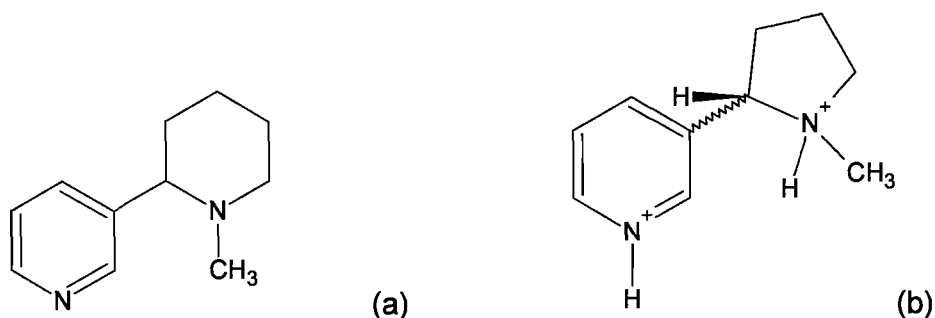


Figure 2-2 Chemical structures of nicotine (a) and diprotonated form of nicotine (b).

Nicotine alters the function of several central nervous system (CNS) neurotransmitters, including dopamine (DA), 5-hydroxytryptamine (5-HT), noradrenaline (NA), glutamate, gamma-aminobutyric acid (GABA) and endogenous opioid peptides (EOPs) (Dani & De Biasi, 2001).

Nicotine has an estimated half-life of approximately two to three hours, and it is extensively metabolized, primarily in the liver, but also in the lungs and the brain. About 70 % - 80 % of nicotine is metabolized to cotinine, which has a half-life of 14 - 20 hours. This is much longer than that of nicotine, and consequently is used as a marker of nicotine intake (USDHHS, 1988).

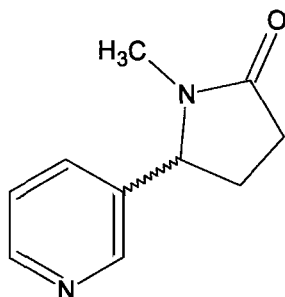


Figure 2-3 Chemical structure of cotinine.

Nicotinic acetylcholine receptors (nAChR) are receptors found in the brain. Nicotine acts via these receptors, producing its effects. These receptors are diverse members of the neurotransmitter-gated ion-channel superfamily and have crucial neuromodulatory roles in the CNS (Picciotto, 2003; Picciotto *et al.*, 2000). The endogenous neurotransmitter at nACh receptors is acetylcholine (ACh) (George & O'Malley, 2004).

2.2.1. Nicotinic acetylcholine receptors (nAChR)

Cholinergic receptors can be divided into muscarinic (mAChR) and nicotinic (nAChR), based on the agonist activities of the natural alkaloid muscarine and nicotine (Mihailescu & Drucker-Colin, 2000).

There are two subclasses of nicotinic receptors: muscle and neuronal. As mentioned before, nicotine acts through nicotinic cholinergic receptors that are present in the brain and many other organs including the autonomic ganglia (swellings on nerve trunks of peripheral autonomic neurones), and through clinical and laboratory studies it was found that these neuronal nicotinic acetylcholine receptors play an important role in complex brain functions such as memory, attention and cognition (Mihailescu & Drucker-Colin, 2000).

The hypothalamus, hippocampus, thalamus midbrain, brain stem and cerebral cortex were found to be the most predominant sites for binding of nicotine to nAChRs in the brain. Nicotine also binds to receptors in the nigrostriatal and mesolimbic dopaminergic neurons (Jain & Mukherjee, 2003).

The cholinergic receptors are relatively large structures that consist of several components known as subunits (Figure 2-4 & Figure 2-6).

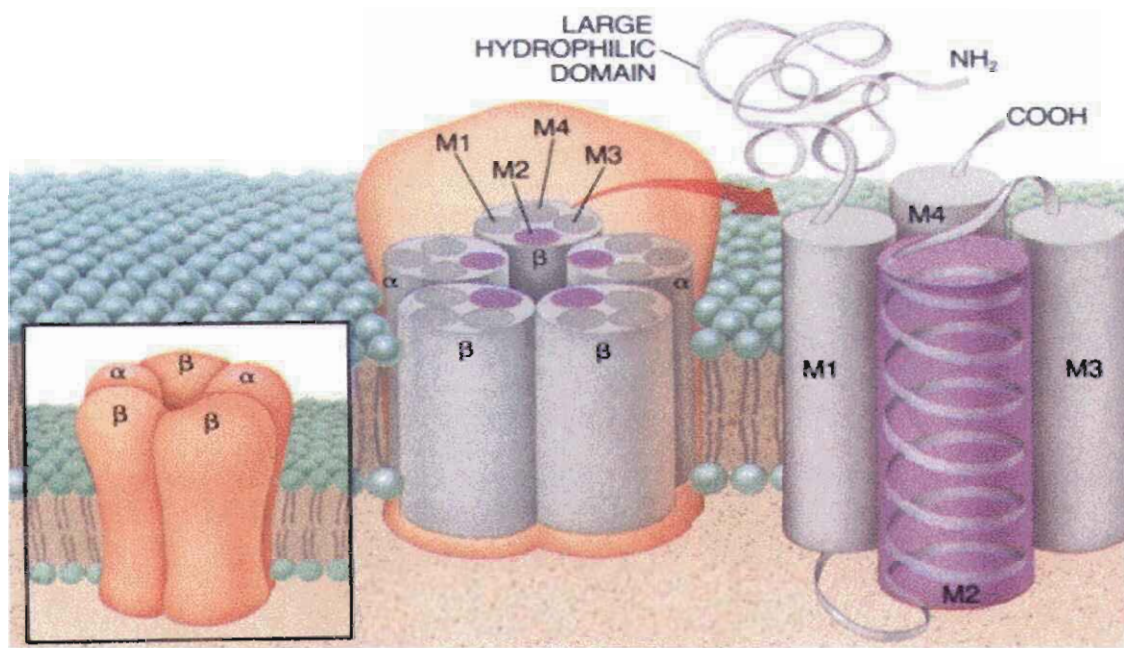


Figure 2-4 Left: Schematic representation of the pentameric arrangement of nAChR subunits in an assembled receptor. Middle: Each subunit consists of four transmembrane segments M1-M4. Right: The configuration of the four transmembrane domains. The M2 (purple) segment is thought to form the lining of the ion channel (Changeux, 1993).

These nACh receptors can be further divided into high-affinity nACh receptors, which contain $\beta 2$ -subunits, to form a heteropentameric configuration of α - and β -subunits that are sensitive to the antagonists mecamylamine and dihydro- β -erythrodine; and low-affinity nACh receptors, which are homopentameric complexes that contain $\alpha 7$ -subunits and are sensitive to the snake-venom toxin α -bungarotoxin and the selective antagonist methyllycaconitine (Figure 2-5 & Figure 2-6) (Dani & De Biasi, 2001; Picciotto *et al.*, 2000).

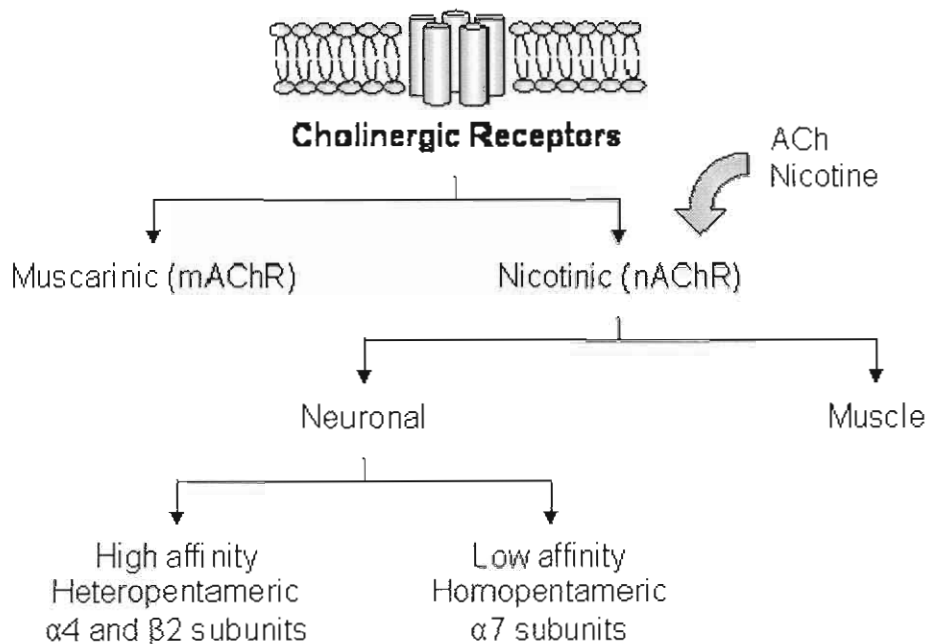


Figure 2-5 Flowdiagram of the different classes of cholinergic receptors (Dani & De Biasi, 2001).

The β subunit has recently been implicated as having a role in nicotine addiction (Jain & Mukherjee, 2003).

The nicotinic receptors release ACh, NA, DA, 5-HT, vasopressin, growth hormone and ACTH when they are stimulated. Nicotine is one of the most potent stimulants of the midbrain DA reward pathway (Jain & Mukherjee, 2003; Picciotto, 1998). A discernible increase in neurotransmitter release and metabolism were observed after stimulation of presynaptic nACh receptors on the mesocorticolimbic DA-containing neurons, by nicotine (George & O'Malley, 2004).

Heteromeric Neuronal AChRs

Subunits: $\alpha 2$ - $\alpha 6$, $\beta 2$ - $\beta 4$

$(\alpha 4)_2(\beta 2)_3$



Major Brain
Subtype With High
Affinity for Nicotine



mecamylamine and dihydro- β -erythrodine

Homomeric Neuronal AChRs

Subunits: $\alpha 7$ - $\alpha 10$

$(\alpha 7)_5$



Major Subtype With
High Affinity for α Bgt
in Both Brain and Ganglia



snake-venom toxin α -bungarotoxin
methyllycaconitine

Figure 2-6 Subunit arrangement in the homomeric $\alpha 7$ and heteromeric $\alpha 4\beta 2$ subtypes, and localisation of the ACh binding site (indicated in white). Homomeric receptors are inhibited by snake-venom toxin and methyllycaconitine and heteromeric receptors by mecamylamine and dihydro- β -erythrodine (Lindstrom, 2000).

Unlike most agonists, which downregulate receptor numbers with chronic exposure, chronic administration of nicotine leads to desensitization and inactivation of nACh receptors, and a 'paradoxical' upregulation of nACh receptor sites. After overnight abstinence, these nACh receptors are likely to resensitize and are thought to be fully responsive to nicotine as an exogenous agonist. This might explain why most smokers report that the most satisfying cigarette of the day is the first one in the morning (George & O'Malley, 2004).

Mesolimbic DA-containing neurons, which form part of the reward pathway, are particularly important because they project from the VTA in the midbrain to anterior limbic forebrain structures such as the NAc and cingulate cortex and mediate the rewarding effects of nicotine (Picciotto, 2003).

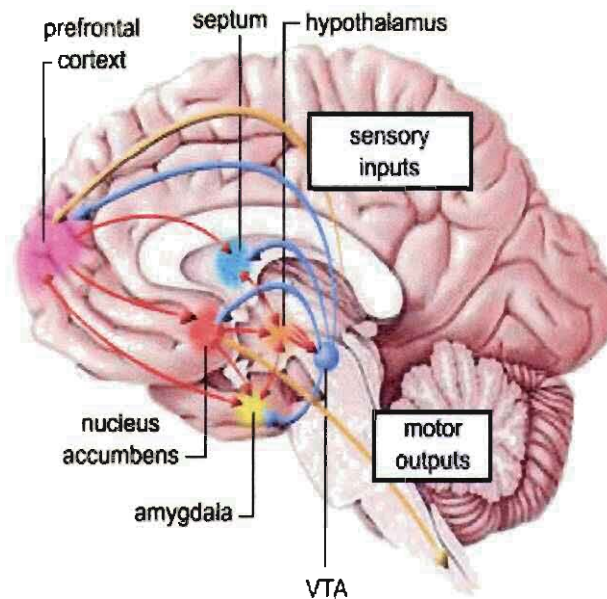


Figure 2-7 Mesolimbic DA containing neurons in the reward pathway of the brain (Dubuc, 2002).

These DA-containing neurons possess high-affinity nACh receptors on their cell bodies and terminals (Zoli *et al.*, 2002), and they receive inputs from glutamate-containing and GABA-containing neurons, which have low-affinity and high-affinity nACh receptors on their terminals, respectively (Mansvelder & McGehee, 2002).

Whenever stimulation of presynaptic nACh receptors, on DA-containing neurons that project from the VTA to the prefrontal cortex (Figure 2-7) occurs, there is an increase in DA release and DA metabolism (Marshall *et al.*, 1997). Theory also suggest that chronic nicotine administration leads to post-receptor changes (e.g. changes in gene expression, and protein synthesis and degradation) in CNS neurons (such as the mesolimbic DA system), which lead to the complex processes of nicotine dependence and withdrawal (Picciotto, 2003).

The high permeability of brain nicotinic receptors to calcium, presents nicotine with the ability to facilitate release of neurotransmitters (Rathouz & Berg, 1994).

Activation of nAChRs by endogenous ACh or pharmacologically administered nicotine is likely to result in increases in the level of intracellular calcium, and this in turn may increase neurotransmitter release at the nerve terminal. The amount of nicotine needed to affect neurotransmitter release and the electrophysiological properties of neurons were found to be extremely low, and that the low concentration levels consistent with the levels found in the blood of moderate smokers, are sufficient to produce these effects (Picciotto, 1998).

2.2.2. Nicotine Addiction

The major stronghold that nicotine has on its users can be illustrated by the fact that more than 50 % of heroin, cocaine users and alcoholics who smoke cigarettes believe that smoking is harder to quit than their other addiction. Nicotine addiction follows a classical cycle, which is very hard to break. Figure 2-8 highlights the intensity of the addiction (Smoking Cessation, 2004a).

Approximately 70 – 90 % of all smokers want to quit, but only one in three will succeed before the age of 65 years. The majority of relapses occur within the first 3 months. An estimate of 65 % of the patients trying to stop using will power alone relapse within the first week (Smoking Cessation, 2004a).

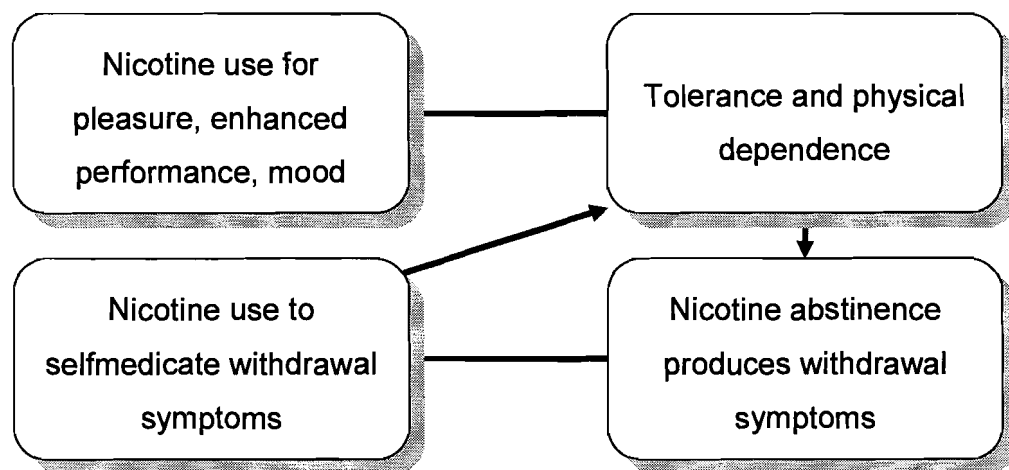


Figure 2-8 Addiction cycle of nicotine (Smoking Cessation, 2004a).

The effects of nicotine on the body

The CNS effects occur because 90 % of the nicotine inhaled into the lungs is absorbed. Nicotine present in cigarette smoke reaches the small airways and the alveoli of the lung and rapidly moves into the bloodstream. Nicotine readily crosses the blood brain barrier and reaches the target receptors in the brain within 10 – 19 seconds after inhalation. Once in the brain, smokers experience alterations in mood including pleasure, arousal and reduced tension. It is contemplated that this short time interval between inhalation and effect in the CNS, is one of the the most important factors for the conditioning response and addiction potential of nicotine (Smoking Cessation, 2004b).

Smoking also has a stimulatory effect on the cardiovascular system which contributes to several short term effects, including an increase in heart rate, stroke volume, blood pressure, cardiac output and coronary blood flow. Cutaneous vasoconstriction accompanied by a decrease in skin temperature and an increase in skeletal muscle blood flow also occurs. With continued smoking, tolerance develops to most of these effects (Smoking Cessation, 2004a).

Nicotine exerts endocrine and metabolic effects and can increase the circulating levels of catecholamines, endorphins, growth hormone, ACTH, cortisol and vasopressin (Smoking Cessation, 2004a).

Nicotine addiction and withdrawal, a disease?

Nicotine withdrawal is now a recognised disease and is included in the Diagnostic and Statistical Manual of Mental Health Disorders (DSM-IV) and the International Classification of Disease (ICD-10). It is caused by the abrupt cessation of nicotine following continuous use (Smoking Cessation, 2004a).

To identify a patient suffering from nicotine withdrawal, they should experience at least four of the withdrawal symptoms in Table 2-1, within 24 hours of cessation.

Table 2-1 Symptoms of nicotine withdrawal.

• Depression or dysphoria
• Irritability, frustration or anger
• Anxiety, tension
• Feeling light headed
• Restlessness
• Difficulty concentrating
• Insomnia
• Decreased heart rate
• Increased appetite or weight gain

Symptoms usually reach a peak of intensity about 48 hours after smoking cessation and then gradually decline over 3 - 4 weeks (Smoking Cessation, 2004a).

Summary

- Nicotine produces the majority of physiological effects within its users.
- Nicotine reaches the brain in only 10 to 19 seconds.
- Nicotine alters our central nervous system neurotransmitters.
- Nicotine acts primarily via nicotinic acetylcholine receptors (nAChR).
- The nAChR is involved in complex brain functions such as memory, attention and cognition, and plays a vital role in nicotine addiction.
- Nicotine is a very potent stimulant of the DA reward pathway and increases the release of neurotransmitters within this region.
- Chronic administration of nicotine leads to desensitization and inactivation of nACh receptors, and a 'paradoxical' upregulation of nACh receptor sites.

- Nicotine addiction is very hard to overcome, maybe more so than that of cocaine or heroin addiction.
- Nicotine withdrawal is now a recognised disease.

2.3. The effect of tobacco smoke on monoamine oxidase activity

2.3.1. Background

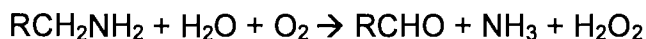
In 1928, Mary Hare isolated a new enzyme which catalyzed the oxidative deamination of tyramine (Hare, 1928), and named it tyramine oxidase. She speculated that it “may be protective and be present for the purpose of rapid detoxification of excessive amounts of tyramine absorbed from the intestine.” Blashko *et al.* (1937) discovered a few years later that this same enzyme also oxidized catecholamines.

Later Zeller proposed the general name monoamine oxidase (MAO) (Zeller, 1938), to reflect this more general reactivity. In the years that followed its discovery, MAO was further characterized along with its role in the regulation of chemical neurotransmitters. In addition MAO has become a molecular target in therapeutic drug development (Shih *et al.*, 1999).

2.3.2. Monoamine oxidase

MAO is an integral protein of outer mitochondrial membranes and occurs in neuronal and non-neuronal cells of both the brain and peripheral organs. It oxidizes the α -carbon of the amines from both endogenous and exogenous sources thereby influencing the concentrations of neurotransmitter amines as well as many xenobiotics (Richards *et al.*, 1998; Singer, 1995).

MAO catalyses the following overall oxidative deamination reaction:



MAO occurs as two subtypes, MAO-A and MAO-B. Both subtypes have different inhibitor and substrate specificities which are encoded by separate genes that are closely linked on the X chromosome. MAO-A and MAO-B both share 70 % similarity in their amino acid sequence (Bach *et al.*, 1988).

The active sites of both MAO-A and MAO-B exhibit a 93.9 % sequence identity. It is the secondary binding sites, indicated in red and blue (Figure 2-9), of the two molecules and possibly their lipid environment which confer their substrate selectivity (Fowler *et al.*, 1980). The hypothesis that MAO-A and MAO-B are derived from duplication of a common ancestral gene is strongly proposed by these results (Grimsby *et al.*, 1991).

MAO-A preferentially oxidizes norepinephrine, epinephrine, normetanephrine and serotonin (5-HT) and is selectively inhibited by clorgyline (Johnston, 1968) while MAO-B preferentially breaks down the trace amine, phenethylamine (PE) and is selectively inhibited by (R)-deprenyl (Figure 2-9) (Knoll and Magyar, 1972). Both forms oxidize DA, tyramine and octopamine (Youdim and Riederer, 1993).

The oxidative deamination of the biogenic amines (RCH_2NH_2) leads to the formation of aldehydes (RCHO) which are subsequently converted to the corresponding alcohol or acids and hydrogen peroxide ($\text{RCH}_2\text{NH}_2 + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}_2$) (Hoffman *et al.*, 1996).

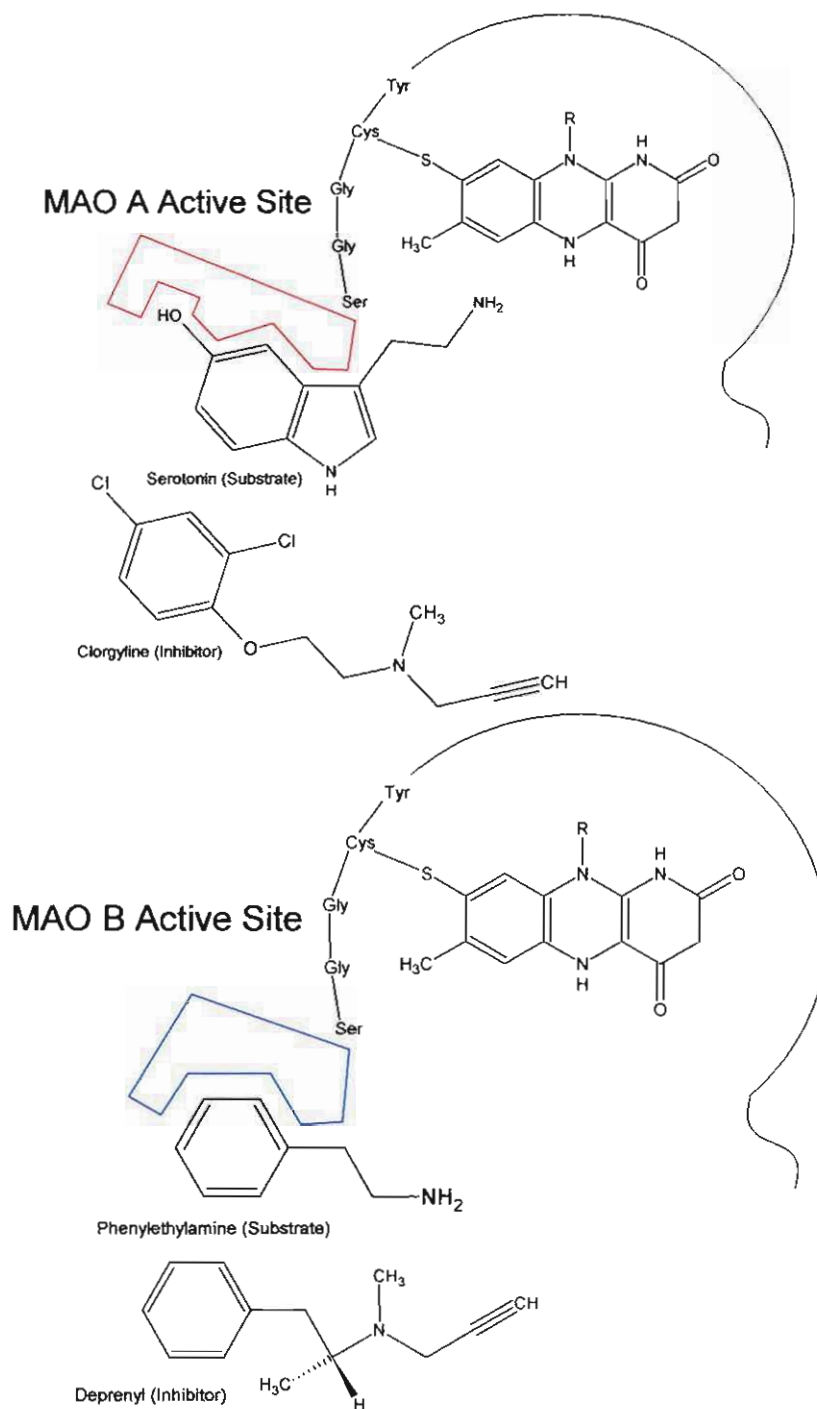


Figure 2-9 The secondary binding sites of MAO-A and B (red and blue site) differs from each other, therefore influencing their substrate and inhibitor selectivity (Fowler et al., 1980).

2.3.3. Distribution of MAO

There is a significant difference between the brain of the rodent compared to that of a primate, with respect to regional and cellular distribution of both MAO-A and B. MAO-B is abundant in both the human and guinea pig CNS, which contrasts with that of the rat and rabbit brain (where the distribution of MAO-B is less abundant) (Azzaro *et al.*, 1985; Oreland *et al.*, 1983; Ross, 1987; Saura *et al.*, 1996; Squires *et al.*, 1972). Moreover, the half-life of MAO in rats is very short when it is compared to the half-life of MAO-B in primates, which was found to be at least 30 days (Arnett *et al.*, 1987; Fowler *et al.*, 1994). These differences are of critical importance, as rodents have been employed for most laboratory studies; such results can only be extrapolated to the human CNS with caution.

In the human brain, the highest concentration of MAO-A is found in the catecholaminergic neurons of the locus coeruleus and the highest concentration of MAO-B is found in the serotonergic and histaminergic neurons of the raphe and posterior hypothalamus (Saura *et al.*, 1996; Westlund *et al.*, 1985; Westlund *et al.*, 1988; Westlund, 1994). There are especially high concentrations of both forms in the human basal ganglia (Oreland *et al.*, 1983).

Placental tissue contains predominantly MAO-A, whereas platelets and lymphocytes express only MAO-B. In humans, MAO-B is particularly plentiful in the brain and liver, while MAO-A is highest in the liver, lung, and intestine. MAO-A, but not MAO-B is expressed in the dopaminergic neurons of the substantia nigra (SN) in both rodents and primates (Kalir *et al.*, 1981).

The role of MAO may predominantly be to protect the local environment from excess levels of unfamiliar monoamines. By regulating the levels of a release-promoting substance, such as PE, MAO-B may indirectly regulate extraneuronal transmitter levels, particularly those of DA. MAO-A on the other hand, acts to maintain low intraneuronal concentrations of DA, NA and 5-HT (Youdim, 1988).

CNS MAO-B (but not MAO-A) activity increases with age in both humans and animals (Fowler *et al.*, 1997; Galva *et al.*, 1995; Kornhuber *et al.*, 1989; Saura *et al.*, 1994; Strolin-Benedetti *et al.*, 1989), perhaps as a result of the glial cell proliferation associated with neuronal loss. In humans, this increase in activity normally starts around 50-60 years of age, but is not observed in the SN (Saura *et al.*, 1997).

Norepinephrine and 5-HT are two of the monoamines linked to mood and MAO-A inhibitors have been used as antidepressants (Lecrubier *et al.*, 1990). Lack of MAO-A increases aggressive behaviour in mice (Cases *et al.*, 1995). MAO-B activity in the hypothalamus, caudate nucleus, and hippocampus and cortex gyrus cinguli has been found to be increased in patients with Alzheimer's disease, and alterations of MAO activity have been implicated with Parkinson's disease (PD) (Adolfsson *et al.*, 1980; Hotamisligil *et al.*, 1994). During oxidation, the process is accompanied stoichiometrically by the reduction of oxygen to hydrogen peroxide which has been implicated in cellular signaling as well as mitochondrial damage (Cohen *et al.*, 1997; Vindis *et al.*, 2001).

2.3.4. The role of tobacco smoke on MAO

Cigarette smokers have lower platelet MAO levels than non-smokers according to several earlier human studies. It was still unclear at the time whether this was due to decreased MAO-B synthesis in smokers, the presence of MAO inhibitory compounds in smoke or whether low MAO individuals are more vulnerable to smoking (Coursey *et al.*, 1979; Norman *et al.*, 1982; Oreland *et al.*, 1981; Von Knorring & Oreland, 1985).

A later study reporting normal MAO levels in former smokers provided evidence that low MAO-B levels may be a pharmacological effect of the smoke rather than a biological characteristic of smokers (Norman *et al.*, 1987).

A study was done in the mid-1990s where researchers compared plasma catecholamine metabolites levels in smokers and non-smokers. At that point in time all the studies in human cigarette smokers focused on MAO-B, but this study provided evidence that tobacco smoke exposure also inhibits MAO-A (Berlin *et al.*, 1995a).

In 1996, the first positron emission tomography (PET) imaging studies (Figure 2-10) were documented. These images provided information about the low brain MAO-A and B activity in smokers relative to non-smokers and former smokers (Fowler *et al.*, 1996a,b).

MAO-A and MAO-B activities are measured using carbon-11 labeled clorgyline ($[^{11}\text{C}]$ clorgyline) and carbon-11 labeled deuterium substituted L-deprenyl ($[^{11}\text{C}]$ L-deprenyl-D2), respectively, as radiotracers and PET. These radiotracers label MAO-A and MAO-B *in vivo* through the irreversible covalent attachment of the tracer to the enzyme during catalysis (Fowler *et al.*, 1987, 1995).

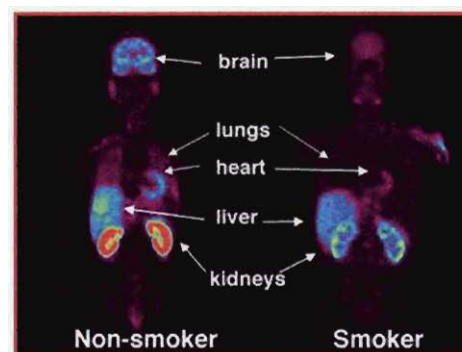


Figure 2-10 Positron emission tomography (PET) image of MAO activity in smokers compared to non-smokers (Fowler *et al.*, 1996a,b).

According to these images, human brain MAO-A and MAO-B in smokers are reduced by an average of 30 % and 40 % for MAO-A and MAO-B, respectively (Fowler *et al.*, 1996a,b).

There is also a significant variability between the degree of MAO-B inhibition in smokers relative to non-smokers, ranging between 17 % and 67 %. The degree of inhibition was not accounted for by the smoking duration or frequency (Fowler *et al.*, 1996b). Further more, no correlation between MAO-B levels and amount smoked could be found (Benowitz, 1988).

Nicotine was found to have inhibitory properties on human platelet MAO-B activity, but only at concentrations 2000 times higher than that observed in the blood of heavy smokers. Using PET imaging, (Fowler *et al.*, 1998), research determined that there was no significant decrease in MAO-B activity in the brain of a baboon treated with an acute dose of nicotine. However, a dose dependent inhibition was observed on MAO-A and MAO-B in cerebral homogenates using dimethylsulfoxide cigarette smoke extracts. Castagnoli *et al.* (2002) reported that neither (S)-nicotine nor (R,S)-N-methylanatabine (a minor tobacco alkaloid) administered via minipumps for 28 days to rats, led to a change in brain MAO-A or MAO-B activity. This shows that nicotine itself is not an MAO inhibitor in the concentrations normally achieved during smoking. The possibility arises that another component of cigarette smoke other than nicotine may be inhibiting the MAO (Jain & Mukherjee, 2003). This reinforces the need to look beyond nicotine as the only pharmacologically relevant substance in tobacco smoke (Nel, 2004).

So to summarize, MAO participates in the catabolism of DA, norepinephrine, and 5-HT (Berlin and Anthenelli, 2001), and it has been postulated that some of the clinical effects of smoking are due to MAO inhibition, leading to decreases in monoamine breakdown with a subsequent increase in monoamine availability (Berlin & Anthenelli, 2001). Thus, smoking may enhance DA availability and the rewarding properties of smoking both through DA release and MAO inhibition. Smoking may also alter mood and anxiety through MAO inhibition effects on norepinephrine and 5-HT availability and turnover.

2.3.5. MAO B inhibition

MAO inhibitors were employed clinically in the 1950s as antidepressants, but fell out of favour because of the so-called "cheese effect". These first MAO inhibitors not only had the desired effect of elevating CNS catecholamine levels, but also potentiated the sympathomimetic action of indirectly acting amines, including tyramine (a MAO-A substrate), in the periphery; hypertensive crises following the consumption of tyramine-containing foods, such as cheese and red wine, were thus a dangerous side-effect of such inhibitors (Blackwell *et al.*, 1967).

After the discovery of the existence of two MAO forms, the development of a range of inhibitors which are relatively specific for one or the other MAO form started to arise. MAO-A inhibitors have found great application in the treatment of depression, and MAO-B inhibitors in the therapy of neurodegenerative disorders, including Parkinson's disease (PD) (Gaál & Hermecz, 1993; Ramsay, 1998; Tipton & Fowler, 1984).

A distinction can be made between two classes of MAO-B inhibitors (for the kinetics of MAO inhibition): (Gaál & Hermecz, 1993; Ramsay, 1998; Tipton & Fowler, 1984):

"Reversible, competitive inhibitors are structurally related to MAO substrates, and can thus bind the active site of the enzyme, but are metabolized comparatively slowly."

"Irreversible or "suicide" inhibitors initially bind MAO in a reversible, competitive manner, but are then oxidized by the enzyme to the active inhibitor, which covalently binds the enzyme active site via the FAD cofactor, thus rendering it permanently unavailable for amine metabolism. The inhibition is more persistent than that achieved by reversible inhibitors (weeks rather than hours), as its effect can only be overcome by *de novo* synthesis of the enzyme (Abeles & Maycock, 1976)."

The first selective MAO-B inhibitor identified was *selegiline* ((R)-deprenyl) (Figure 2-11), and most investigated MAO-B inhibitors are irreversible (Knoll & Magyar, 1972). The discovery of *selegiline* served as the benchmark against which new MAO-B inhibitors are measured. After its synthesis in 1962 (Knoll *et al.*, 1965; Parnham, 1993), its lack of a “cheese effect” attracted attention even before MAO subtypes had been distinguished. The (-)-or R-isomer is 150-fold more potent than the (S)-isomer (Magyar *et al.*, 1967). As MAO-B is generally present in excess in the tissues in which it occurs, it is necessary to inhibit at least 80 % of the enzyme to achieve a pharmacological effect (Green *et al.*, 1977).

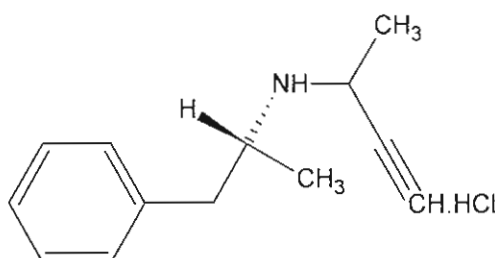


Figure 2-11 Chemical structure of *selegiline* (Baldessarini, 1996).

As mentioned before, inhibition of MAO leads to an increase of several catecholamines in the human body, due to a decrease in DA and other catecholamine metabolism. This increase in catecholamine levels, especially in the reward circuit within the brain could cause addiction and cravings for that inhibitory substance. Nicotine does not inhibit platelet MAO when it is present in the concentrations normally achieved during smoking (Oreland *et al.*, 1981).

It was demonstrated that nicotine inhibited platelet MAO activity only at concentrations 2000 times higher than that found in the blood of heavy smokers (Oreland, 1981). Thus, the use of only nicotine as a nicotine replacement therapy (NRT) for smoking cessation would, possibly, not be as valuable as using NRT in combination with a drug, a MAO inhibitor, that can maintain a suitable level of important neurotransmitters in the brain.

Summary

- MAO oxidizes amines, thereby influencing their concentrations within its users.
- MAO-A oxidizes norepinephrine, epinephrine and 5-HT.
- MAO-B oxidizes phenethylamine.
- Both forms oxidize DA.
- MAO-B regulates mostly extraneuronal transmitter levels and MAO-A maintain low intraneuronal concentrations of DA, NA and 5-HT.
- Cigarette smokers have lower platelet MAO levels than non-smokers and ex-smokers.
- Tobacco smoke exposure inhibits MAO.
- Nicotine could only inhibit human platelet MAO activity at concentrations 2000 times higher than that observed in the blood of heavy smokers.
- Nicotine itself is not a MAO inhibitor and thus inhibition is caused by other component(s) in cigarette smoke.
- A decrease in MAO activity due to inhibition by cigarette smoke leads to an increase in amine neurotransmitter levels, especially DA, and results in an increased reward within the user.

2.4. The role of nicotine in the regulation of tyrosine hydroxylase activity

2.4.1. Tyrosine hydroxylase

Tyrosine hydroxylase catalyzes the 3-hydroxy-group that is bound to tyrosine to produce the catechol-amino acid, dihydroxyphenylalanine (DOPA). Tyrosine hydroxylase catalyzes only the *L-isomer* of tyrosine, and needs tetrahydropteridine and Fe^{2+} as cofactors.

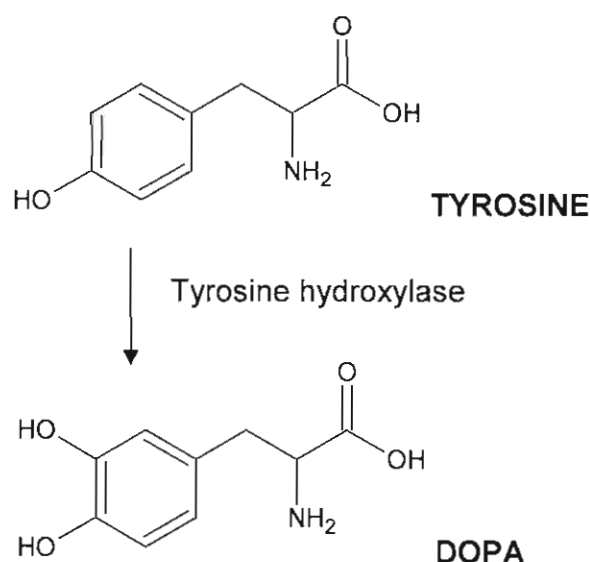


Figure 2-12 The catalyzation of tyrosine to DOPA by tyrosine hydroxylase (Hoffman *et al.*, 1996).

Tyrosine hydroxylase is synthesized in the neuron. Thus an increase in neuronal activity results in an increase in enzyme synthesis.

The hydroxylation of tyrosine to dopa is the rate limiting step in the biosynthesis of the catecholamines; therefore the formation of dopa from tyrosine is very slow (Hoffman *et al.*, 1996).

The catecholamines that are formed during their biosynthesis, inhibit the enzyme activity by competing with the enzyme for the pteridine factor. This process is referred to as product-inhibition. The activity of the enzyme is increased by a cAMP-dependent protein kinase. Neuronal activity increases the activity of adenylyl cyclase, which leads to an increase in the cAMP concentration and results in the phosphorylation of the enzyme (Hoffman *et al.*, 1996).

2.4.2. The influence of nicotine on tyrosine hydroxylase activity

Exposure to nicotine, the major pharmacologically active component of cigarette smoke, produces many physiological changes and increases the risk of coronary and peripheral vascular disease. As mentioned before, nicotine is a potent agonist of nAChRs, and creates an increase in the flux of calcium ions into the nerve cells when nicotine binds to these receptors (Sands *et al.*, 1991; Vernino *et al.*, 1992).

Nicotine triggers rapid secretion of catecholamines. The nicotine-triggered elevation in plasma catecholamines levels from the sympathetic nerve endings and the adrenal medulla are implicated in alterations in heart rate and arterial pressure (Haass & Kubler, 1997).

The nicotine induced increase in catecholamine biosynthesis, can also be attributed to the phosphorylation and rapid activation of tyrosine hydroxylase (TH), the first and major rate-limiting enzyme in catecholamines biosynthesis (reviewed by Kumer & Vrana, 1996). Similar to acute cocaine administration, acute administration of nicotine can amplify TH activity in the NAc (Arinami *et al.*, 2000). Longer-term (hours to days) responses to nicotine involve changes in gene expression (Sabban, 1997). In addition, the TH mRNA levels and activity in the locus coeruleus can increase with chronic administration of nicotine. These changes in the activity and levels of the rate-limiting biosynthetic enzyme for catecholamines are likely to be common markers for the development of

dependence for drugs of abuse (Jain & Mukherjee, 2003; Nel, 2004; Picciotto, 1998).

Calcium ions also play a vital role in the stimulation of calcium-related secondary signal pathways including kinases such as protein kinase A (PKA), Ca^{2+} /calmodulin kinase II (Braun & Schulman, 1995; MacNicol & Schulman, 1992) and Ca^{2+} - and phospholipid-dependant protein kinase (PKC) (Caldwell *et al.*, 1992; TerBush & Holz, 1986). The transcription of the TH gene might be enhanced in catecholaminergic cells as part of these kinase pathways (Goc *et al.*, 1992; Lewis *et al.*, 1987; Tank *et al.*, 1986; Vyas *et al.*, 1990).

Nicotine and nicotinic agonists also increase the protein level of TH (Craviso *et al.*, 1992; Craviso *et al.*, 1995) and the mRNA level of the TH gene in the rat brain and bovine adrenal chromaffin cells (Smith *et al.*, 1991).

Hiremagalur *et al.* (1993) recently reported that the mRNA level of TH genes in PC12 cells were increased by nicotine treatment and that the cyclic AMP response element (CRE; TGACGTCA) plays a key role in long-term nicotine-induced activation of the TH gene.

An elevation in adrenomedullary gene expression for catecholamine biosynthetic enzymes as well as several neuropeptides (neuropeptide Y and enkephalin) and other constituents coreleased with catecholamines were observed after the injection of nicotine into rats (Fossum *et al.*, 1991; Hiremagalur & Sabban, 1995; Slotkin & Seidler, 1976). The activation of nAChRs also elevates both catecholamine synthesis and release and TH gene expression in the CNS dopaminergic and noradrenergic systems (Mitchell *et al.*, 1993; Nisell *et al.*, 1996; Serova *et al.*, 1999; Smith *et al.*, 1991).

Summary

- Tyrosine hydroxylase is an enzyme that catalyzes tyrosine to DOPA.

- This hydroxylation is the rate limiting step in the biosynthesis of the catecholamines.
- Product-inhibition occurs.
- Nicotine increases catecholamine biosynthesis due to several effects on the enzyme tyrosine hydroxylase (as mentioned above).
- Nicotine increases the mRNA levels of tyrosine hydroxylase genes in PC12 cells.
- The activation of nAChRs also elevates both catecholamine synthesis and release and TH gene expression in the CNS dopaminergic and noradrenergic systems.

2.5. The effects of tobacco smoke on dopamine

2.5.1. Dopamine

Dopamine (3-hydroxytyramine; DA) is a catechol neurotransmitter widely distributed throughout the brain (Palkovits & Brownstein, 1989).

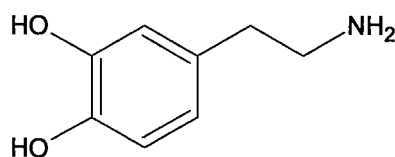


Figure 2-13 Chemical structure of dopamine (Hoffman *et al.*, 1996).

It is essential that there is a sufficient amount of this molecule within the body at any given time, and it is therefore very important that DA be kept within strict boundaries. When certain areas of the brain receive too little DA, the brain triggers the tremors and paralysis associated with Parkinson's disease. In comparison, when there is an excess DA in the brain, hallucinations and bizarre thoughts associated with schizophrenia occur (Nash & Park, 1997).

Dopamine is associated with body movement, awareness, judgement, motivation and pleasure (Swan, 1998).

2.5.2. Dopamine synthesis

The biosynthesis of DA occurs preferentially in the nerve terminals from the substrate tyrosine and through the sequential action of the enzymes, tyrosine hydroxylase and aromatic *L*-amino acid decarboxylase (L-DOPA decarboxylase) (Hermida-Ameijeiras *et al.*, 2004) (Figure 2-14).

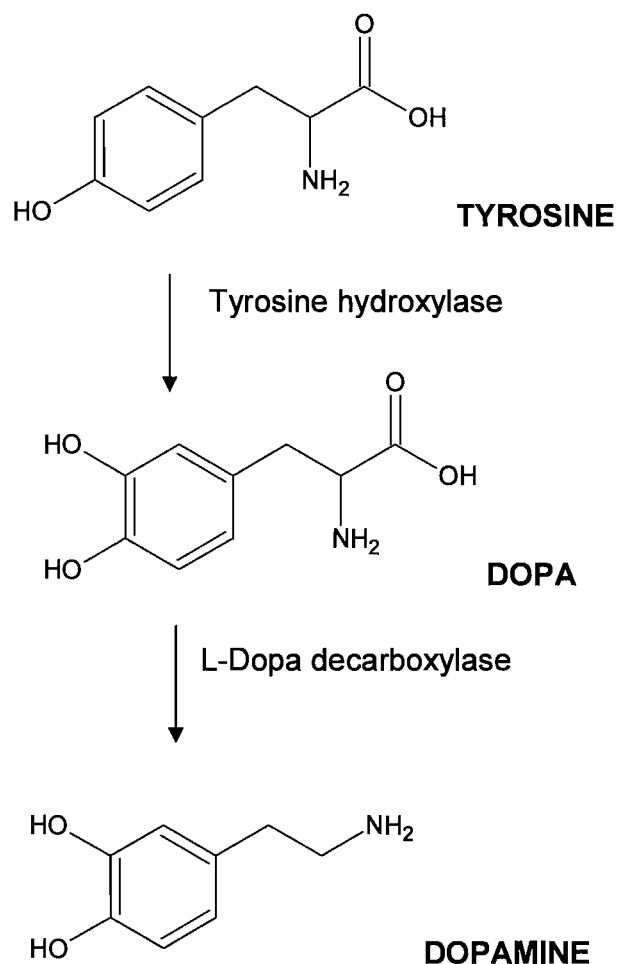


Figure 2-14 The synthesis of dopamine (Hoffman *et al.*, 1996).

Most of the DA in the brain is found in the striatum and is synthesized by the dopaminergic neurons which make up the nigrostriatal system (Palkovits & Brownstein, 1989).

The neurons of the central and peripheral nervous systems both contain DA. After the synthesis of DA, it is stored in vesicles in axon terminals and released when drugs stimulate receptors on the cell bodies of dopaminergic neurons. DA interacts with specific post-synaptic membrane receptors, especially in the NAc, which is thought to result in the perception of pleasure (Figure 2-15) (Balfour *et al.*, 1998; Walton *et al.*, 2001).

2.5.3. Dopamine metabolism

The effects of DA are terminated via two mechanisms. Firstly, by re-uptake into the presynaptic neuron by a DA transporter and stored in vesicles or diffusion away from the junction cleft and secondly, metabolic inactivation by MAO-B or catechol-o-methyltransferase (COMT) (Figure 2-15) (Reuter *et al.*, 2002).

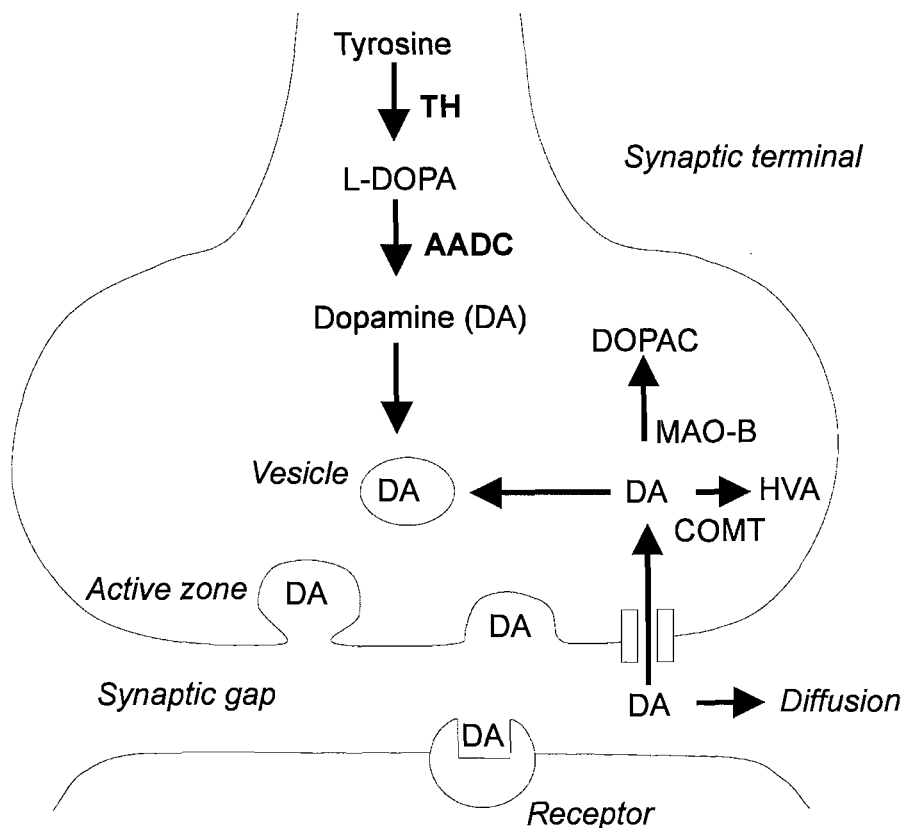


Figure 2-15 The metabolism of dopamine (Hoffman *et al.*, 1996).

Under physiological conditions, DA is non-enzymatically oxidized by molecular oxygen to produce hydrogen peroxide (H_2O_2) and the corresponding *o*-quinone (*o*Q). The *o*Q undergoes an intramolecular cyclization which is immediately followed by a cascade of oxidative reactions resulting in the final formation of a black, insoluble polymeric pigment known as neuromelanin (Graham, 1978).

In addition, DA is also enzymatically deaminated by MAO to form H_2O_2 and 3,4-dihydroxyphenylacetaldehyde. This latter compound is then oxidized by aldehyde dehydrogenase (ADH) to give 3,4-dihydroxyphenylacetic acid (DOPAC), which subsequently is methylated by COMT to form homovanilic acid (HVA) (Figure 2-16) (Fornstedt *et al.*, 1990).

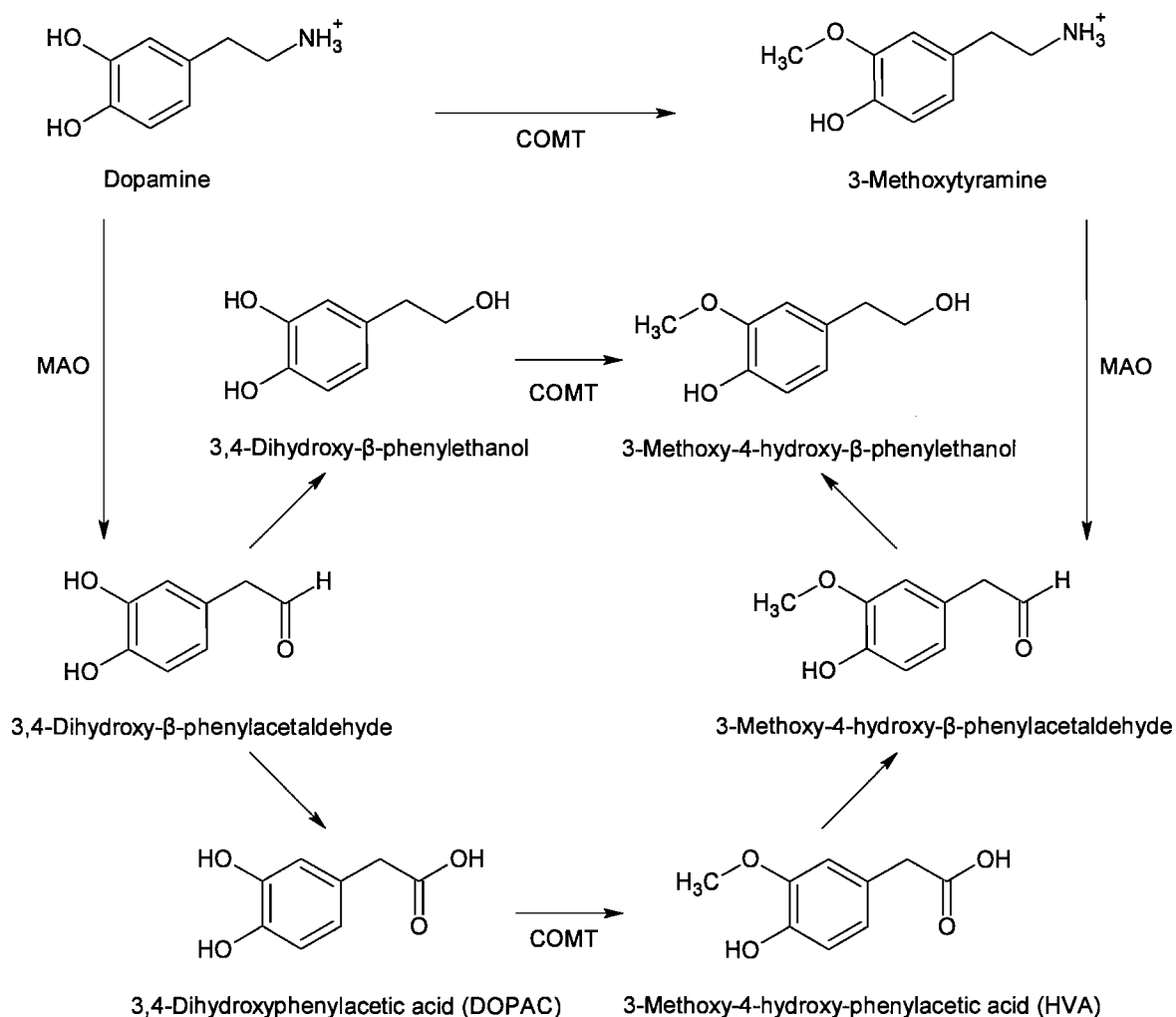


Figure 2-16 The enzymatic deamination, oxidation and methylation of dopamine (Fornstedt *et al.*, 1990).

2.5.4. Drugs affecting dopamine transmission

Many drugs affect dopamine transmission directly by either blocking or stimulating its receptors. For example, antipsychotic drugs are DA antagonists, whereas bromocriptine, used to treat hyperprolactinaemia and Parkinson's disease, is a DA agonist (Katzung *et al.*, 1998).

Several drugs of clinical importance act indirectly, such as the DA precursor, levodopa, which is converted to DA, or amphetamine, which releases DA from

terminal stores. Other drugs increase the synaptic concentration of DA by blocking its uptake or metabolism. For example, cocaine is a potent inhibitor of the DA re-uptake transporter and this may be the basis of its addictive properties. On the other hand, selegiline, a MAO-B inhibitor, elevates DA concentrations by inhibiting DA metabolism (Katzung *et al.*, 1998).

2.5.5. Dopamine and tobacco smoke

The most important pathway for the positive reinforcement associated with most, if not all, addictive drugs is the brain DA system (Koob, 1992; Leshner & Koob, 1999). Extensive evidence has illustrated that nicotine administration and smoking results in activation of the brain DA mesolimbic pathway, thereby increasing DA release and turnover in the NAc and ventral striatum. These areas are important in the reward and reinforcing properties of many addictive drugs (Di Chiara & Imperato, 1988).

Nicotine stimulates DA release via nicotinic acetylcholine receptors, in neurons originating in the substantia nigra and ventral tegmental area (Benwell *et al.*, 1994; Ryoo & Joyce, 1994; Vizi *et al.*, 1995; Wonnacott, 1997). This smoking-induced increase in the levels of DA may explain some of the clinical effects of smoking. Levels of DA in the mesostriatal and mesocorticolimbic circuits, and in turn DA receptors, are also likely to be modulated by tobacco smoking (Court *et al.*, 1998).

It may therefore be concluded that nicotine has the ability to increase the DA concentration in the synaptic cleft by binding to pre-synaptic nAChR, thereby increasing the amount of calcium into the nerve terminal, changing its polarity and causing exocytoses of DA from the nerve terminal into the synaptic cleft. This increase in DA concentration, especially in the reward and reinforcing areas of the human brain, plays a vital role in the addictive behaviour of nicotine and cigarette smoke.

Summary

- Whenever a low concentration of DA occurs, symptoms associated with Parkinson's disease develop.
- With an excess DA, schizophrenia occurs.
- Dopamine is associated with body movement, awareness, judgement, motivation and *pleasure*.
- Dopamine receptor stimulation in the nucleus accumbens is thought to result in the perception of *pleasure*.
- Dopamine systems are very important in the positive reinforcement of addictive drugs.
- Nicotine administration and smoking increases DA release and turnover, strengthening the addictive properties of the drug.
- This increase of DA in the brain, especially the reward pathway, explains some of the clinical effects associated with smoking.

2.6. The effects of tobacco smoke on serotonin

2.6.1. Serotonin

Serotonin (5-hydroxytryptamine, or 5-HT) is a monoamine neurotransmitter synthesised in serotonergic neurons in the CNS and enterochromaffin cells in the gastrointestinal tract.

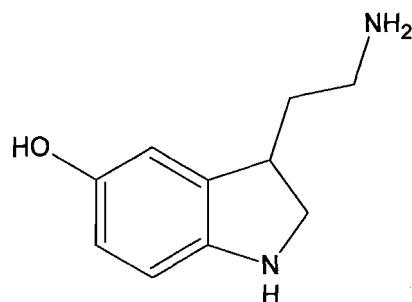


Figure 2-17 Chemical structure of serotonin (Sanders-Bush & Mayer, 1996).

Serotonin is believed to play an important part in the biochemistry of depression, bipolar disorder, drug addiction and anxiety. It is also believed to be influential on sexuality.

The name "serotonin" is something of a misnomer and reflects the circumstances of the compound's discovery. It was initially identified as a vasoconstrictor substance in blood serum - hence "serotonin", a serum agent affecting vascular tone. This agent was later chemically identified as 5-hydroxytryptamine (5-HT) and as the broad range of physiological roles were elucidated, 5-HT became the preferred name in the pharmacological field (Rang *et al.*, 2003).

2.6.2. The Role of Serotonin

Impulsivity, persistence and sensation seeking are all core features in an individual lacking inhibitory control. Inhibitory control is also very important for the expression of impulsive behaviour in man (Olausson *et al.*, 2001).

Clinical observations, including neuropsychological test paradigms and impulsivity questionnaires, support the view that nicotine impairs neural mechanisms involved in inhibitory control of behaviour with chronic intermittent exposure. All the cigarette smokers had higher scores in the impulsivity questionnaires compared to non-smokers (Olausson *et al.*, 2001).

The brain 5-HT systems in the neuronal circuits have recently been identified as important mediators of inhibitory control of behaviour. Depletion of 5-HT, in several experimental animals, consistently produced an impulsive behavioural pattern, anticipatory responses in various animal models and aggression (Olausson *et al.*, 2001). Whenever there is a depletion of 5-HT in the brain, followed by a reduction of 5-HT neurotransmission, the response for conditioned reward increases. Any manipulations that increase brain 5-HT neurotransmission therefore decrease the response for conditioned reinforcers.

A decrease in brain 5-HT neurotransmission elevates self-administration of several different drugs in various experiments done on the rat model. Similarly, compounds that facilitate 5-HT neurotransmission, like selective 5-HT reuptake inhibitors, decrease voluntary ethanol consumption in rats. Similar effects of 5-HT enhancing drugs have been reported on the intake of nicotine. These observations suggest that the inhibitory control can be strengthened with an increase in 5-HT neurotransmission, thereby reducing drug consumption (Opitz & Weischer, 1988).

Nicotine increases 5-HT release in the cortex, striatum, hippocampus, dorsal raphé nucleus, hypothalamus and spinal cord. Stimulation of 5-HT_{1A} receptors in the dorsal hippocampus and lateral septum mediates nicotine's anxiogenic effect, and stimulation of the 5-HT_{1A} receptors in the dorsal raphé nucleus plays a role in mediating the anxiolytic effects of nicotine. In the striatum, 5-HT₃ receptors are of significance (Seth *et al.*, 2002).

It has been reported by Helton *et al.* (1993) that nicotine withdrawal significantly increased the acoustic startle response in rats for approximately four to five days. This increased startle reactivity perhaps most closely resembles the increased irritability observed in smokers undergoing nicotine withdrawal. Systemic administration of 5-HT_{1A} receptor agonists such as 8-OH-DPAT exacerbates this response, whereas 5-HT_{1A} receptor antagonists, such as WAY-100635, alleviate

this enhanced response. Electrophysiological investigations have demonstrated that the responsiveness to 8-OH-DPAT of neurons in the dorsal raphe nucleus was significantly increased during nicotine withdrawal. Therefore, one possibility is that nicotine withdrawal increases the inhibitory influence of somatodendritic 5-HT_{1A} autoreceptors located within the raphe nuclei and thereby decreases 5-HT release into forebrain and limbic brain sites which contributes to nicotine withdrawal signs (Kenny & Markou, 2001; Nel, 2004).

Contrary to the view that nicotine withdrawal is caused by reduced serotonergic transmission, Seth *et al.* (2002) have shown that a direct administration of nicotine into the dorsal raphe nucleus, at a concentration that activates somatodendritic 5-HT_{1A} receptors, prevented an increase in anxiety observed in rats undergoing nicotine withdrawal as measured in the social interaction test. This observation suggests that there is enhanced serotonergic transmission during nicotine withdrawal that mediates the observed increases in anxiety.

2.6.3. Synthesis of Serotonin

In humans, 5-HT is synthesized by a two-step pathway from the essential amino acid L-tryptophan by various enzymes as shown in Figure 2-18.

Serotonin taken orally does not pass into the serotonergic pathways of the CNS. This is due to the blood-brain barrier preventing 5-HT in the blood stream from affecting 5-HT levels in the brain. However, the amino acid tryptophan and its metabolite 5-hydroxytryptophan, which 5-HT is synthesized from, are capable of crossing the blood-brain barrier. These agents are available as dietary supplements and may be effective serotonergic agents, though their efficacy is questionable (Birdsall, 1998).

2.6.4. The metabolism of serotonin

Serotonin is metabolized by MAO to 5-hydroxyindoleacetaldehyde, an intermediate product in the metabolism pathway, which is further oxidized by

aldehyde dehydrogenase to form 5-hydroxyindole acetic acid (5-HIAA) (see Figure 2-18).

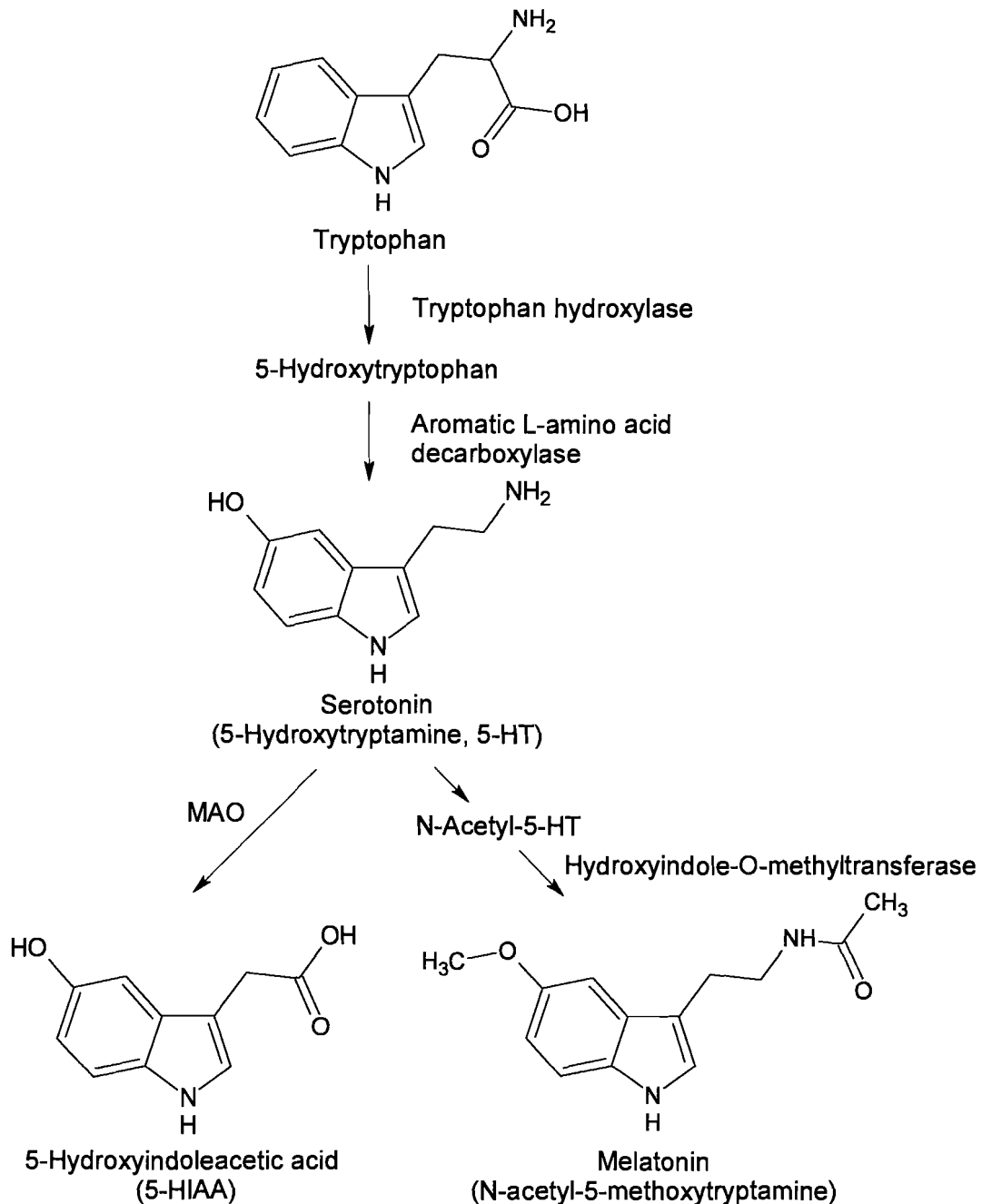


Figure 2-18 Synthesis and metabolism of serotonin (Katzung & Trevor, 1998).

When the latter enzyme (aldehyde dehydrogenase) is saturated, eg. by large amounts of acetaldehyde from ethanol metabolism, a significant fraction of the 5-hydroxyindolaecetaldehyde may be reduced in the liver to the alcohol, 5-hydroxytryptophol. The excretion of 5-HIAA, in humans consuming a normal diet, is an accurate measure of 5-HT synthesis. A significant increase in the 5-HT metabolite 5-HIAA was observed in the striatum and hypothalamus and a tendency for an increase in 5-HIAA in the cortex was reported in chronic administration of nicotine in the drinking water of mice (Gäddnäs *et al.*, 2000).

2.6.5. Neurotransmission

Serotonin is released from the terminal boutons of serotonergic neurons into the neuronal synapse. In the synapse it is free to diffuse across to and activate 5-HT receptors located on the dendrites of adjacent neurons.

Serotonergic action is terminated primarily via uptake of 5-HT from the synapse. This is through the specific monoamine transporter for 5-HT, 5-HT reuptake transporter, on the presynaptic neuron. Various agents can inhibit 5-HT reuptake including cocaine, tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) (Figure 2-19).

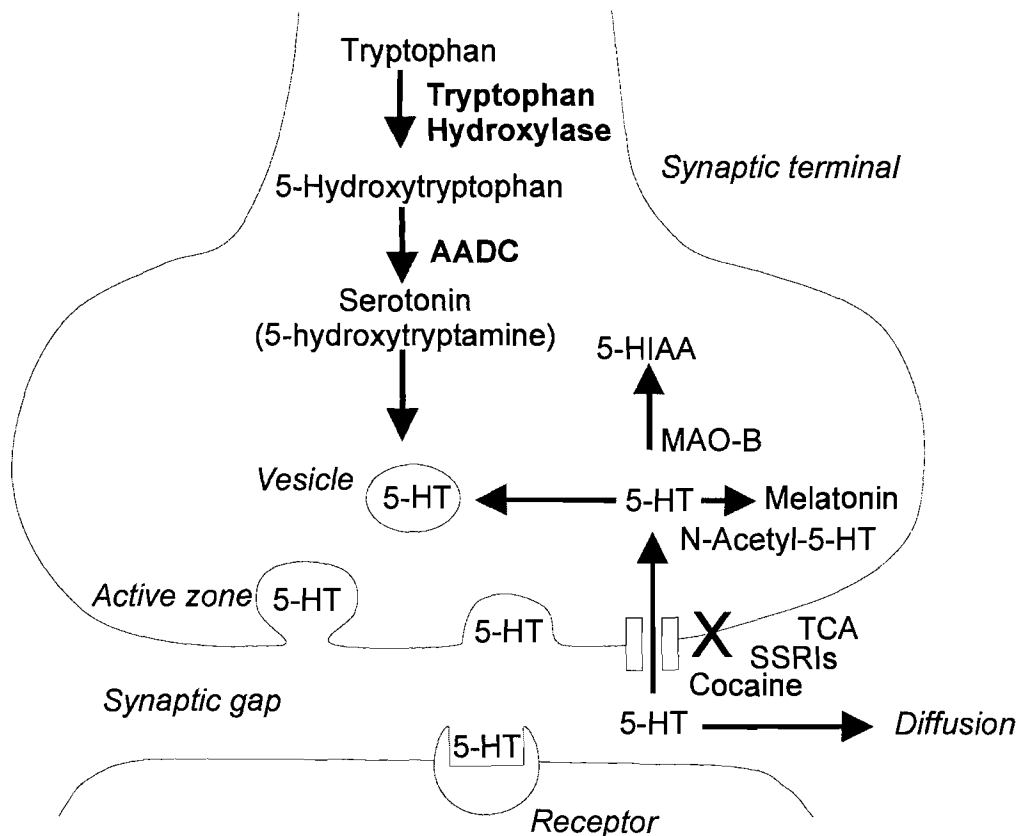


Figure 2-19 Neurotransmission of serotonin (Sanders-Bush & Mayer, 1996).

Summary

- Serotonin (5-HT) is a monoamine neurotransmitter synthesised in serotonergic neurons in the CNS and enterochromaffin cells in the gastrointestinal tract.
- Serotonin plays an important role in drug addiction.
- Nicotine impairs neural mechanisms involved in inhibitory control of behaviour.
- Brain serotonin systems are important mediators of inhibitory control of behaviour.

- Decreased brain 5-HT neurotransmission elevates self-administration of several drugs.
- Therefore, an increase in 5-HT neurotransmission decreases self-administration of drugs.

2.7. Pharmacological interventions for the treatment of tobacco addiction

2.7.1. Introduction

With over 1.1 billion tobacco users worldwide, nicotine is definitely the most commonly used drug in our society today, and the single largest preventable cause of disease and premature death. The physiological dependency caused by the use of this drug can be attributed to the large number of harmful carcinogenic substances present in cigarette smoke.

In the past, nicotine replacement therapy (NRT) was used as the primary pharmacological intervention for the alleviation of the abstinence syndrome caused by cessation of smoking (Balfour *et al.*, 2000). Of the 17 million smokers that try to quit smoking each year, fewer than one out of ten actually succeed. It has also been estimated that only 2.5 % of unaided quit attempts are successful (Cohen *et al.*, 2001; Malin, 2001). This success rate is disappointingly low.

2.7.2. Current Pharmacological treatments for tobacco addiction

The goal of NRT and intervention is to assist the smoker in the attempt to quit smoking. NRTs provide an alternative source of nicotine to the smoker. In many smokers, treatment with NRTs during smoking cessation is ineffective, but it nevertheless helps prevent severe nicotine withdrawal symptoms during cessation (Froelicher & Kozuki, 2002).

Table 2-2 Pharmacotherapies for nicotine dependence (George & O'Malley, 2004; Nel, 2004).

Medication	Clinical and pharmacological mechanisms
Nicotine patch, gum and lozenge	Nicotine replacement therapy (NRT): reduces nicotine craving and withdrawal.
Nicotine nasal spray and vapour inhaler	NRT: nACh receptor stimulation rapidly reduces nicotine craving and withdrawal symptoms.
Bupropion hydrochloride (sustained-release)	Blocks re-uptake of DA and NA; high affinity, non-competitive nACh receptor antagonist reduces nicotine reinforcement, withdrawal and craving.
Clonidine	α_2 -Adrenoceptor agonist reduces nicotine withdrawal symptoms.
Tricyclic antidepressants (nortriptyline, doxepin)	Blocks re-uptake of NA and 5-HT; probably reduces withdrawal symptoms and co-morbid depressive symptoms.
Mecamylamine	Non-competitive, high-affinity nACh receptor antagonist combined with TNP reduces nicotine reinforcement, withdrawal and craving.
Naltrexone	Endogenous μ opioid peptide receptor antagonist appears to reduce nicotine craving and withdrawal in combination with TNP.
Buspirone	Partial agonist of 5-HT _{1A} receptors reduces 5-HT release; might be effective in smokers with co-morbid anxiety symptoms.
Moclobemide	Reversible MAO-A inhibitor increases NA and 5-HT levels; might be helpful for smokers with co-morbid mood disorders.
Selegiline	Irreversible MAO-B inhibitor increases synaptic DA levels; might reduce nicotine reinforcement, withdrawal and craving.

Five nicotine formulations are currently available for replacement. Transdermally delivered nicotine (nicotine patch), nicotine polacrilex (nicotine gum), lozenge, nicotine vapour inhaler and nasal spray (George & O'Malley, 2004).

Using NRT does increase the odds of smoking cessation 1.7 times over control and decreases severity of nicotine withdrawal symptoms (Silagy *et al.*, 1994; Silagy *et al.*, 2001).

All forms of NRT are safe when it is used properly, and do not increase the risk of acute myocardial infarction, even when used during cigarette smoking, contrary to popular misconceptions (Working Group for the Study of Transdermal Nicotine in Patients with Coronary artery disease, 1994). Psychological counselling is considered to be an essential feature of the therapy.

These delivery systems are thought to be equally effective, with about 20 % of those that received therapy not smoking at one year and up to 10 % remaining non-smokers if treatment is continued (Britton & Jarvis, 2000).

Listed in Table 2-2 are some alternative medication that could also be used for the treatment of tobacco addiction.

In this study we will investigate two possible pharmacotherapies for smoking cessation. The first, bupropion, has already been employed for a few years to treat nicotine dependency, while the second drug under investigation, nicotinamide adenine dinucleotide (NAD) has not yet been evaluated as a possible agent to treat smoking addiction. These two drugs will be discussed further in the next two chapters.

Summary

- Nicotine is the most widely used drug in the world.
- Nicotine causes several diseases and even premature death.

- There are over 17 million people that are trying to quit smoking each year, but only 2.5 % of the attempts are successful.
- Nicotine replacement therapy (NRT) is not very effective with smoking cessation but helps control withdrawal symptoms.
- Five formulations of NRT are available.

2.8. Bupropion (Zyban™)

2.8.1. Bupropion

Bupropion (BUP) is a phenylaminoketone atypical antidepressant administered in a sustained-release formulation. Bupropion is better known under its brand names, Zyban® or Wellbutrin®. Bupropion was approved in the USA in 1997, by the Food and Drug Administration (FDA), as the first non-nicotine pharmacotherapy for smoking cessation and is now considered a first-line pharmacological treatment for nicotine-dependent smokers (Khurana *et al.*, 2003).

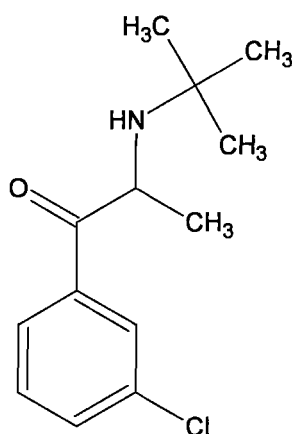


Figure 2-20 Chemical structure of bupropion.

The mechanism of action of bupropion in the treatment of nicotine dependency is not fully understood and proven yet, but is likely to involve its modest blockade of

DA and NA reuptake (Ascher *et al.*, 1995), and might also relate to its antagonism of high-affinity nACh receptors (Slemmer *et al.*, 2000).

2.8.2. Metabolism of bupropion

Bupropion (BUP) is metabolized to 6-hydroxybupropion (HB), the pharmacologically active main metabolite, to *threo*-hydrobupropion (TB) and erythrohydrobupropion (EB) (Figure 2-21) (Laizure *et al.*, 1985)

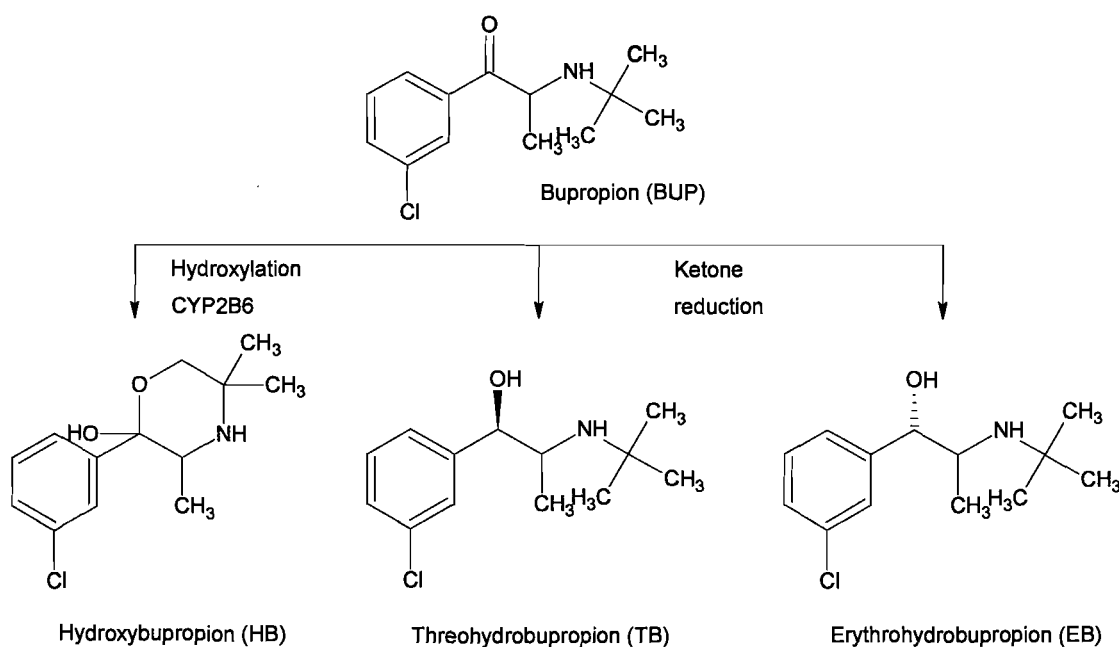


Figure 2-21 Metabolism of bupropion (Borges *et al.*, 2004).

The recommended target dose of this agent in nicotine dependence is 300 mg day⁻¹ (two tablets), and is started 7 days before the target quit date (TQD) at 150 mg day⁻¹ (one tablet), and increased to 150 mg twice daily after 3 days. The reason for doing this is that steady-state levels are achieved before the quit attempt. It is not known whether the major metabolite, 6-hydroxybupropion, which is formed by cytochrome P450 2D6 metabolism (Ascher *et al.*, 1995), contributes to the anti-smoking actions.

Unlike the NRTs, with the use of BUP, there is no absolute requirement that smokers cease smoking by the TQD. Many smokers using BUP as an aid to stop smoking, have reported a significant reduction in urges to smoke and craving, which facilitates cessation. Some smokers gradually reduce their cigarette smoking over several weeks before quitting (Ascher *et al.*, 1995).

Past history of seizures of any etiology, is the main contraindication for the use of BUP. The rates of *de novo* seizures are low with this agent (~0.1 %), and observed mainly when the dose exceeds 450 mg a day (George & O'Malley, 2004).

The primary side-effects reported with the related use of BUP are headache, jitteriness, dry mouth, initial insomnia and gastrointestinal symptoms (George & O'Malley, 2004).

Considering the fact that BUP has limited effectiveness in many smokers and, given its high cost, there is definitely a need for the development of better and more cost effective treatments for this disease called nicotine withdrawal.

Summary

- Bupropion was approved by the Food and Drug Administration as the first non-nicotinic pharmacotherapy for smoking cessation.
- Mechanism of action is likely to involve the modest blockade of DA and NA reuptake, and its antagonism of high-affinity nACh receptors.
- Target dose is 300 mg/day.

2.9. The possible use of NAD for treatment of smoking addiction

2.9.1. What is NAD?

Nicotinamide adenine dinucleotide (NAD) is a coenzyme which was discovered in 1904 by Harden & Young (Mahler & Cordes, 1966). NAD is very important in the manufacturing of cellular energy in living organisms, and has more than 100 metabolic functions in human biochemistry. An example is the Krebs cycle, which is restricted when NAD and NADP are absent. NAD can also occur as NADP and NADPH and is present either as NAD or NADH. The main source of NAD is food (Brody, 1994), but NAD can also be produced in the body, particularly in the liver, under control of adrenal gland hormones, from certain nutritional elements (White, 1982). Vitamin B3, nicotinamide and tryptophan are all important precursors of NAD (Figure 2-22) (Verwey, 2002).

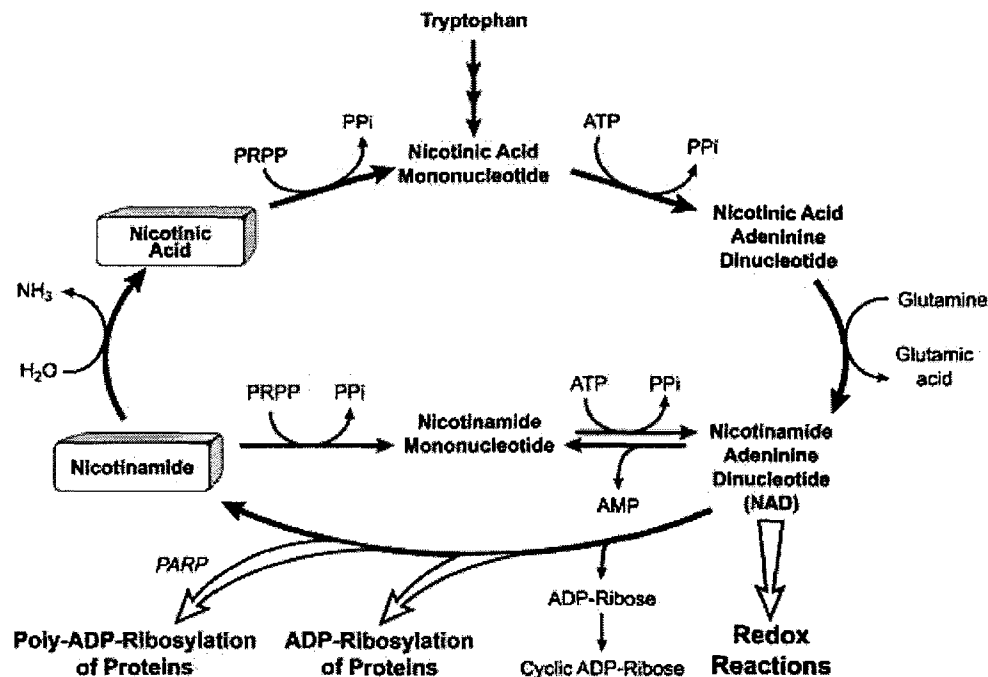


Figure 2-22 The metabolic pathway of NAD synthesis (Oregon State University, 2006).

2.9.2. The function of NAD

NAD and nicotinamide adenine dinucleotide phosphate (NADP) are two of the most important coenzymes in the cell. NADP is formed when a third phosphate group is attached to NAD.

Because of the positive charge on the nitrogen atom in the nicotinamide ring, the oxidized forms of these important redox reagents are often described as NAD^+ and NADP^+ respectively (Figure 2-23).

NAD participates in many redox reactions in cells, like in the citric acid cycle of cellular respiration and also in glycolysis.

NADP is produced by photosynthesis due to light reactions, is consumed in the Calvin cycle of photosynthesis, and is the reducing agent in many other anabolic reactions in both plants and animals (Kimball, 2006).

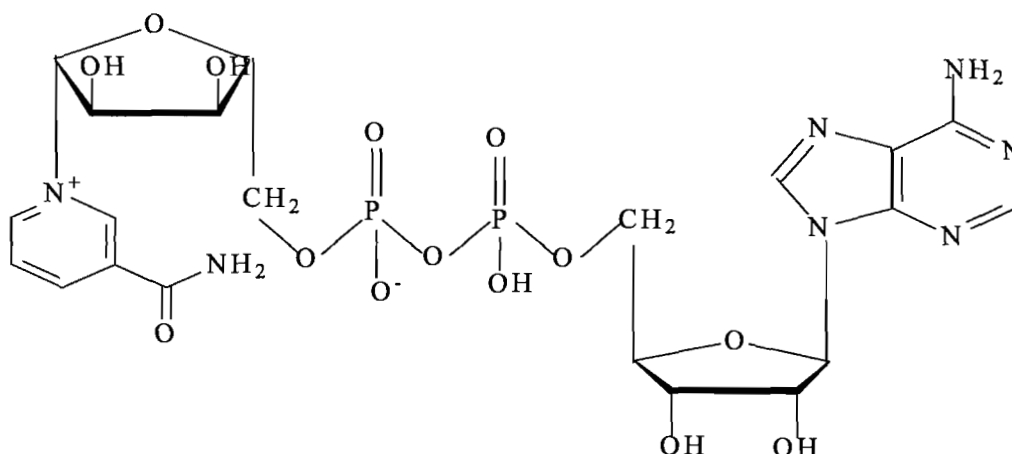


Figure 2-23 Chemical structure of NAD (Kimball, 2006).

2.9.3. NAD deficiency

As mentioned before, NAD has a fundamental part to play in many enzyme reactions involved in cellular energy production. NAD and its derivatives, NADH,

NADP⁺ and NADPH have regulatory functions in the generation of triose phosphates and pyruvate from glucose (Wahlberg *et al.*, 2000).

Primary defects of pyruvate metabolism from defects of electron transport (or oxidation), are easily distinguished using the ratio of lactate to pyruvate which is a reflection of the NAD⁺/NADH ratio (CIDEM, 2000). A higher level of lactate to pyruvate is characteristic of NAD deficiency which can lead to a lowering of energy in cells and the development of various diseases (Cleary, 1986).

2.9.4. Symptoms of NAD deficiencies

Memory deficiencies, apathy, irritability, lack of concentration, depression, reduced mental energy, increased anxiety and panic, increased craving for alcohol, sugar and *nicotine*, reduced sex drive, fatigue and increased premenstrual tension are all common symptoms associated with NAD deficiency. Clinical experience also indicates that deficiencies of NAD are chronic, and often require chronic treatment (Verwey, 2002).

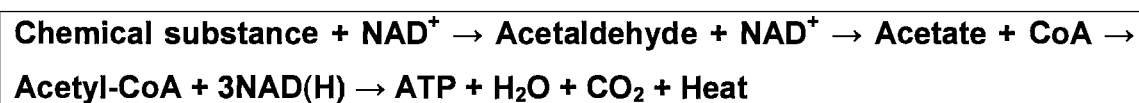
2.9.5. Metabolic Detoxification of chemical substances

NAD was first introduced in 1939 as a successful short-term treatment of alcoholism (Cleary, 1986). The treatment of various types of substance dependencies with the aid of NAD supplements was later introduced by O'Halleren. He used NAD to treat alcohol-, heroin-, cocaine-, morphine-, meperidine-, codeine-, amphetamine-, barbiturate- and sedative dependents (O'Halleren, 1961). NAD treatment for nicotine addiction has not yet been reported. NAD does not have the same significant side-effects, like serious flushing and the release of histamine, when compared to nicotinic acid at high dosages (Smith, 1978).

A variety of chemical substances, including alcohol, requires NAD or derivatives thereof for intracellular metabolism. When alcohol is taken, 90 % is absorbed

almost immediately in the body's cells, and the remaining 10 % is discharged mainly in the urine. Acetaldehyde is the first metabolite produced in various chemical substances, including alcohol (Light, 1986; O'Halleren, 1961; Peters & Preedy, 1998; Svensson *et al.*, 1999; Volpi *et al.*, 1997).

Acetaldehyde is also formed during stress. The citric-acid cycle is the last step in the metabolic detoxification process where three NADs are involved to complete the process. This cycle is also responsible for the conversion of proteins, carbohydrates and fats into ATP (Verwey, 2002). This is a purely biochemical autonomic reaction, and can be simplified as follows:



2.9.6. Improving brain functions

Derivates of niacin are found abundantly in brain tissue, mainly in the form of NAD and NADP coenzymes. A sharp decline in the the brain's supply of NAD is observed in the case of niacin deficiency, and the functioning of the brain is disturbed. Malfunctioning of the brain (dementia) is indeed one of the primary characteristics of pellagra, and permanent brain damage develops with an extended period of deficient NAD (Meij & Meyer, 1988).

Scientists have discussed the possible use of NAD for the treatment of neurodegeneration and the improvement of brain functions (Verwey, 2002), due to its role in the synthesis of the neurotransmitters, i.e. NA and DA, which are important for maintaining a positive state of mind (Burke, 1998).

Research on NAD, which was conducted in South African, also confirmed the normalizing effect of NAD on the neurotransmitters, i.e. DA, adrenaline and NA. NAD probably plays a role in the production of 5-HT and other neurotransmitters in the brain (South, 1997). The battle against tobacco addiction is far from over,

but the possibility exists that the use of NAD, could have a positive effect in the treatment of this addiction and help assist in smoking cessation.

Summary

- NAD (Nicotinamide Adenine Dinucleotide) is a coenzyme found in the human body, and has over 100 metabolic functions e.g manufacturing of cellular energy.
- NAD is obtained from food, but is also produced in our bodies, particularly in the liver.
- NAD deficiency can lead to various symptoms, including an increased craving for alcohol, sugar and nicotine.
- O'Halleren has already used NAD supplement in the treatment of various substance dependencies.
- No NAD treatment for nicotine addiction has yet been evaluated.
- NAD plays a vital role in the synthesis of neurotransmitters e.g dopamine, and it also has a normalizing effect on them.
- We hypothesise that NAD will therefore increase neurotransmitter levels (e.g DA), in important brain regions, like the reward pathway, in patients addicted to cigarette smoke, thereby decreasing cravings for the drug.

Chapter 3

Experimental Procedures

The development and undertaking of this protocol were done in accordance with the guidelines stipulated by the Ethics Committee for Use of Experimental Animals at the North-West University, Potchefstroom Campus (approval number 00D25).

3.1. Experimental Animals

Male and female New Zealand rabbits were used in this study. The rabbits were obtained from the St. Peter's catholic school in Pretoria, and housed in the Animal Research Centre (ARC) of the North-West University. The weight of these rabbits varied from 1343 - 2310 g, on the first day, to 1562 - 2805 g at the end of the study, with removal of the Alzet osmotic mini-pumps.

The conditions in the ARC were kept constant, with a temperature of 25 °C and humidity (55 ± 5 %) and with a 12:12 h light-darkcycle. Each rabbit was placed in its own cage with free access to food and water, after implantation of the Alzet osmotic mini-pumps.

New Zealand rabbits were chosen for this study because daily blood samples were needed to determine the catecholamine concentrations associated with nicotine withdrawal. Although Wistar rats were used in previous studies (Nel, 2004), the volume of blood received from these rats was not sufficient, therefore rabbits were used as an alternative.

3.2. Preparation of tobacco smoke extract

The smoke needed for the preparation of the extract was obtained from Peter Stuyvesant cigarettes (king size, filtered) containing 15 mg tar and 1.4 mg nicotine per cigarette.

To smoke the cigarettes and trap the compounds that are usually inhaled by smokers, a special smoking device was designed. The device consisted of a specially designed glass structure used to load the cigarettes (eight at a time) (1), attached to a flask containing acetone (2). This device was then connected to another flask that was used to trap any excess acetone that might be sucked into the pump (3), and the latter flask was connected to a relay (4) that puffed every 30 s for a duration of 3 s, to mimic the action of a human smoker (Figure 3-1).

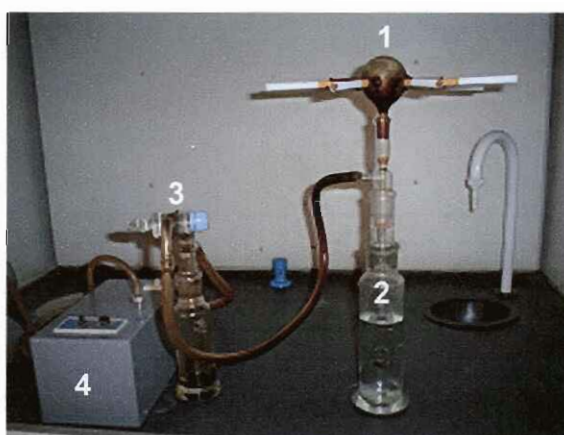


Figure 3-1 The smoking device (Nel, 2004).

The cigarette smoke compounds were thus collected in acetone (200 ml of acetone were used for sixty cigarettes). A rotary evaporator was used to evaporate the acetone and yield a solid extract. After weighing the solid extract (average: 8 mg/cigarette), the extract was dissolved in propylene glycol to achieve a concentration of 80 mg/ml. The Alzet osmotic pumps were then filled with this solution.

3.3. Alzet Osmotic Pumps

Alzet osmotic pumps are specially designed miniature, implantable pumps used for research in laboratory animals. These infusion pumps continuously deliver drugs and other test agents at controlled rates from day one to day 28. The advantage of these osmotic pumps is that the need for external connections or

frequent painful handling is reduced dramatically (Nel, 2004). Each rabbit was implanted with two Alzet pumps subcutaneously at the back of the neck during anaesthesia for systemic administration of the smoke extract solution or placebo (propylene glycol). The anaesthesia was achieved using 0.6 ml of Saffan®.

Table 3-1 **Technical description of the Alzet osmotic pump Model 2ML4 (Alzet osmotic pump Model 2ML4 leaflet, 2000).**

Pumping Rate	2.53 µl/hr (± 0.05 µl/hr)
Duration	28 days
Reservoir Volume	2097 µl (STDEV 59 µl)

The osmotic pumps were filled, using a 5 ml syringe and a 25 gauge blunt-tipped filling needle, provided with the osmotic pumps, through a 0.22 µm syringe-end acetate filter. The pumps were filled under sterile conditions in a laminar flow cabinet. The pump was held in an upright position and the filling needle was inserted through the opening at the top of the osmotic pump. The process was stopped when the solution appeared at the outlet. The needle was then removed and any excess solution was wiped off (Nel, 2004). A flow moderator was then inserted into the opening of the pump and placed in a beaker with saline. After all the pumps were filled, they were primed by placing them in a waterbath at 37 °C overnight.

Guidelines for implantation of the osmotic pumps were supplied by the Alzet company. Sterile gloves were worn during implantation. After the rabbits were given anesthesia, their skin was cleaned with 2 % chlorhexidine solution and the hair removed using a sharp razor. All techniques carried out were under sterile conditions. The weight of each rabbit was determined before implantation. A small incision was made in the skin between the scapulae. The subcutaneous connective tissues were spread apart to form a pocket for insertion of the two pumps. Each pump was inserted through the incision with the flow moderator pointing away from the opening. The skin was closed with silk stitches (Alzet osmotic pump Model 2ML4 leaflet, 2000).

The thirty-two rabbits were divided into four groups of eight each as shown in Table 3-2 below. Each group was divided into two subgroups of four rabbits each, and implantation of these subgroups (four) started on consecutive weeks to facilitate analysis. Two osmotic pumps, each containing 2 ml of the smoke extract solution, were implanted subcutaneously in groups 1, 2 and 3 (3 x 8 rabbits). Group four (1 x 8 rabbits) received 2 x 2 ml of a placebo (propylene glycol) via the Alzet osmotic pumps.

According to the Alzet osmotic pump leaflet (Alzet osmotic pump Model 2ML4 leaflet, 2000), the pumps start to release the solution upon wound closure. For a period of 27 days the rabbits were exposed to this continuous release of smoke extract solution to accomplish addiction. On day 27 the pumps were removed and group 1 (the control) was injected intra-peritoneally (IP) on four days (day 28, 29, 30 and 31) with saline. Group 2 was injected (IP) on four days (day 28, 29, 30 and 31) with NAD (1 g / 70 kg) and group 3 was injected (IP) with bupropion (300 mg / 70 kg) also on four days (day 28, 29, 30 and 31). Group 4 only received propylene glycol for the 27 days via the osmotic pumps and were injected with NAD (1 g / 70 kg) (IP) on four days (day 28, 29, 30 and 31).

All four groups were injected in the morning between 7:00 am and 8:00 am over the period of four days. Group 4 was used to evaluate the difference in monoamine levels between smoking and non-smoking rabbits in combination with the drug under investigation (NAD). Groups 2 and 3 were also compared to evaluate the difference in monoamine levels between bupropion (which is an existing and successful drug for the treatment of smoking cessation) and NAD.

Table 3-2 Treatment protocol.

DAY	Group 1	Group 2	Group 3	Group 4
1	Smoke	Smoke	Smoke	Propylene glycol
...	↓	↓	↓	↓
28	Saline	NAD	Bupropion	NAD
29	Saline	NAD	Bupropion	NAD
30	Saline	NAD	Bupropion	NAD
31	Saline	NAD	Bupropion	NAD
32	Decapitate	Decapitate	Decapitate	Decapitate

On day 27 each rabbit was weighed again upon removal of the osmotic pumps. For the next four days (day 28, 29, 30, 31) blood samples of 2 ml each were collected three times a day at 8:00 am, 13:00 pm and 16:30 pm from each rabbit. The blood was withdrawn from the rabbit's ear using a 23 G needle and a 1 ml syringe, collected in a 3 ml EDTA-containing K2E vacutainer and kept on ice.

After all the tubes were filled, the blood was centrifuged at 4 °C in a Sigma 3K-15 centrifuge for 20 minutes at 5000 g. The supernatant (plasma) was carefully removed and placed in an amber 2 ml eppendorf tube. The supernatant was immediately frozen at -86 °C until analysis.

3.4. Determination of Plasma Catecholamines

The quantification of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), in rabbit plasma was performed by a high-performance liquid chromatography (HPLC) method with electrochemical detection (De Villiers *et al.*, 1986) as modified by Brand (1996).

3.4.1. Apparatus

An Agilent 1100 series HPLC with an auto sampler was used in all the analyses.

Table 3-3 Conditions for HPLC analysis.

Stationary phase	Luna C18 reverse phase column
Detector	GBC LC 1260 EC Detector: 0.600 V & 10 nA
Pump	Isocratic
Flow rate	1.3 ml/min
Injection volume	50 µl
Temperature (column and room)	22 – 24 °C
Run Time	18 minutes
Software	Chemstation

3.4.2. Chemicals and reagents

All chemicals and reagents used for the HPLC analysis were of the highest HPLC grade available. Double distilled deionized water was used in the preparation of the mobile phase and all the buffer solutions. The reagents used for the analysis of the catecholamines were the same for both plasma and brain.

Table 3-4 Table of chemicals used, and their suppliers.

Chemical or reagent	Supplier
Dopamine hydrochloride	Aldrich
Dihydroxyphenylacetic acid (DOPAC)	Aldrich
Isopropyl-DL-noradrenaline hydrochloride (isoprenaline)	Aldrich
Methanol (MeOH)	Merck
Acetonitrile	BDH Licrosolv
Sodium hydroxide (NaOH)	Merck
Formic acid (HCOOH)	Sigma-Aldrich
Sodium heptanesulphonic acid monohydrate	Merck
Disodiumedethate (Na ₂ EDTA)	Merck
Perchloric acid (HClO ₄) 70 %	Merck
Sodium methabisulphite (Na ₂ S ₂ O ₃)	Merck

3.4.3. Mobile phase

The mobile phase comprised of 0.1 M sodium formate buffer, 0.5 mM ethylenediaminetetraaceticacid (EDTA), 5 mM sodium heptane sulphonic acid,

6 % v/v methanol and 4 % v/v acetonitrile (BDH LiCrosolv®). The EDTA forms complexes with any free metallic ions in the samples, preventing it from interfering with detection. The buffer was set at a pH of 3.0 with the addition of approximately 6.0 ml of 60 % orthophosphoric acid to 1000 ml of mobile phase. This was the only variation from the method of Brand (1996) who used formic acid to adjust the pH.

This solution was filtered through a 0.22 µm membrane filter (Millipore) and vacuum deaerated prior to utilisation.

3.4.4. Calibration solutions

The stock solutions of DA and DOPAC were made up in the same 0.1 M perchloric acid solution (**Solution A**), (containing 0.5 mM or 0.09505 g/l sodium metabisulphite and 0.3 mM or 0.1117 g/l Na₂EDTA). This solution was stored at 4 °C and aliquots of the solution were mixed to yield stock standard solutions that were used for daily calibration of the integrator.

3.4.5. Monoamine standards

3.4.5.1. Preparation of dopamine (DA) standard

3-Hydroxytyramine hydrochloride, C₈H₁₁NO₂.HCl = 189.64 MW (80.77 %) (Aldrich).

1.238 mg was weighed and dissolved in 10 ml of 0.1 M perchloric acid solution (Solution A, see 3.4.4) to yield a stock solution (**stock solution 1a**) of 100 µg/ml.

3.4.5.2. Preparation of 3,4-Dihydroxyphenylaceticacid-DOPAC

DOPAC = 168.15 MW (Aldrich).

1 mg of DOPAC was weighed and dissolved in 10 ml of solution A (see 3.4.4) to yield a stock solution (**stock solution 1b**) of 100 µg/ml.

3.4.5.3. Preparation of the internal standard – Isoprenaline

N-Isopropyl-DL-Noradrenaline-hydrochloride = 247.72 MW (Aldrich).

5 mg of Isoprenaline was weighed and dissolved in 50 ml of the calibration solution (solution A). 150 µl of this solution was then made up to 2 ml in an amber tube with solution A to give a final concentration of 7500 ng/ml. This solution was used as internal standard throughout all determinations.

Stock solution 2 was prepared by diluting 200 µl of **stock solution 1a+b** (100 µl of each) with 1800 µl of 0.1 M perchloric acid (solution A), to yield a solution with a final concentration of 10 µg/ml. **Stock solution B** was prepared by addition of 100 µl of stock solution 2 to 1900 µl of 0.1 M perchloric acid (solution A) with a final concentration of 500 ng/ml.

3.4.5.4. The composition of the standard solutions

All solutions (stock-, standard- and calibration solutions) were stored in a refrigerator at 4 °C to prevent degradation.

Table 3-5 Table of the composition of the standard solutions of DA and DOPAC.

Volume of solution				
Stock solution 2 (µl)	Stock solution B (µl)	0.1 M perchloric acid solution (µl)	Total volume (ml)	Concentration (ng/ml)
-	20	1980	2	5
-	40	1960	2	10
-	80	1920	2	20
-	120	1880	2	30
-	200	1800	2	50
20	-	1980	2	100
40	-	1960	2	200
60	-	1940	2	300
100	-	1900	2	500

3.4.6. Sample preparation

On the day of analysis, samples were thawed and then kept on ice until analysis. Amber eppendorf tubes (2 ml) were used for the preparation of samples to prevent any photolysis. To each tube an amount of ± 35 mg of acid-washed alumina (type WA-4, acid) was added. A volume of 900 µl of the plasma sample together with 500 µl tris buffer and 20 µl internal standard were mixed together. The tris buffer was constituted by adding 18.171 g of tris buffer to 100 ml of double distilled water to yield a 1.5 M solution. The pH of the tris buffer was adjusted to 8.7 by addition of approximately 3 ml of hydrochloric acid (HCl). The concentration of the internal standard was 7500 ng/ml.

The mixture was shaken vigorously for 30 min, and then centrifuged at 2000 rpm for 1 min at room temperature (Eppendorf Zentrifuge 3200). The supernatant fraction was carefully removed with a micropipette and discarded. The alumina in the tube was washed twice with 1 ml of double distilled water. The water was removed and discarded. Finally, a volume of 200 µl 0.1 M perchloric acid was added to the alumina to adjust the pH to 2. The low pH allows the catecholamines to desorb from the alumina. The mixture was kept on ice for

30 min. The mixture was vortexed (Vortex mixer K VM-300) and centrifuged for 1 min before the acidic eluate was transferred to an HPLC sample vial for analysis. The samples were all analysed on the same day.

3.4.7. Sample injection

The vials were loaded into the HPLC autosampler set to inject 50 µl of the sample solution. The flow rate was 1.3 ml/min, and the run time was set for 18 min at room temperature.

3.4.8. Standard curves

A standard curve was constructed for both DA and its metabolite DOPAC. The standard curve represents AUC-standard/AUC-internal standard, against concentration. The monoamine and metabolite were identified based on their retention time and quantified by comparing the AUC-sample/AUC-internal standard to the standards, utilizing the linear equation obtained from the standard curve.

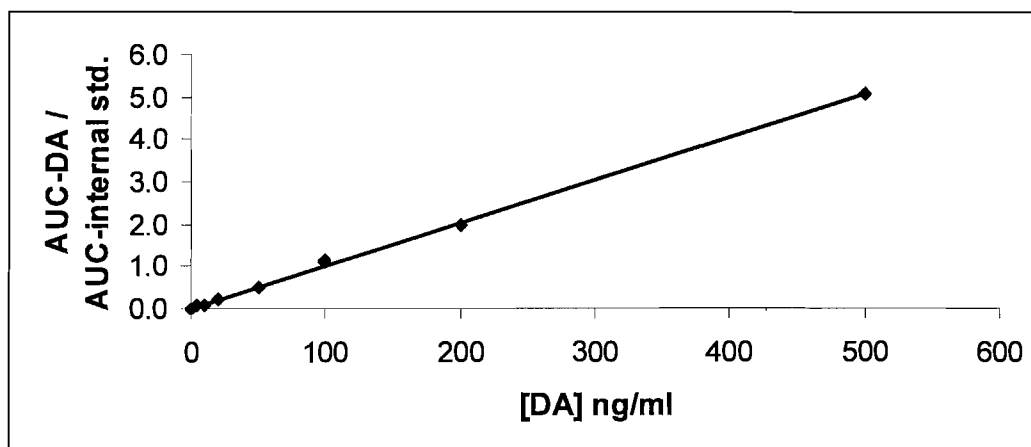


Figure 3-2 Linear curve of AUC ratio for DA against a concentration range of standard solutions for determination of plasma catecholamines.

The linear equation obtained from the DA standard curve was: $y = 0.0101x + 0.0224$. The regression coefficient was 0.9985.

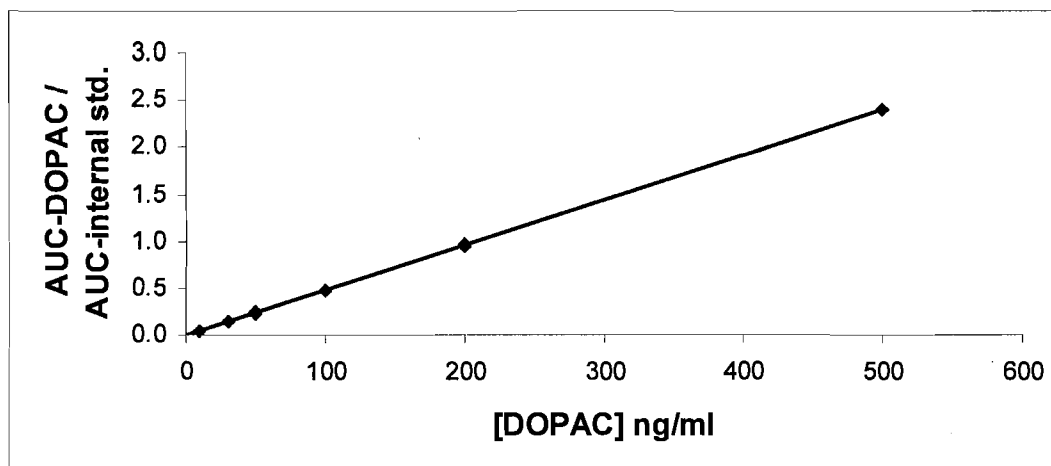


Figure 3-3 Linear curve of AUC ratio for DOPAC against a concentration range of standard solutions for determination of plasma catecholamines.

The linear equation obtained from the DOPAC standard curve was: $y = 0.00481x + 0.00538$. The regression coefficient was 0.9997.

3.5. Decapitation, Dissection and Storage

On day 32 each rabbit was gassed with CO₂ and weighed before they were decapitated. The brains were quickly removed. For each brain the NAc and striatum (also called the neostriatum or caudate putamen) were dissected out on ice. Both the NAc and striatum were placed into previously marked and weighed polypropylene containers, immediately snap frozen with liquid nitrogen and transferred to a -86 °C freezer until analysis.

3.6. Determination of Catecholamines in the brain

The quantification of the monoamines dopamine (DA) and serotonin (5-HT) as well as their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyphenylacetic acid (5-HIAA) in the NAc was performed by a high-performance liquid chromatography (HPLC) method with electrochemical detection. This method was proven to be very sensitive and selective (Brand, 1996).

3.6.1. Apparatus

An Agilent 1100 Series HPLC with an auto sampler was used in all the analyses.

Table 3-6 Experimental and technical conditions in the HPLC-analysis of monoamines and their metabolites.

Stationary phase	Luna 5 μ , C18, reverse phase
Detector	GBC LC 1260 EC Detector: 0.6 V & 10 nA
Pump	Isocratic pump
Flow rate	1.25 ml/min
Injection volume	100 μ l
Run time	40 min
Temperature (column and room)	Constant between 22 – 24 °C
Software	Chemstation

3.6.2. Chemicals and reagents

All the chemicals and reagents used for HPLC analysis were of the highest HPLC grade available. Distilled deionized water was used in the preparation of all the buffers and mobile phase (see Table 3-4).

3.6.3. Mobile phase

See 3.4.3.

3.6.4. Calibration solutions

Stock solutions of the biogenic amines and their acid metabolites were made in the same 0.1 M perchloric acid solution (solution A) (containing 0.5 mM sodium metabisulphite and 0.3 mM Na₂EDTA) that was used for tissue homogenization and determination of plasma catecholamines. This solution was kept refrigerated at 4 °C to prevent any degradation (see 3.4.4).

3.6.5. Sample preparation

Following dissection of the NAc from the rabbit brain, the tissue samples were individually placed into previously weighed and marked polypropylene tubes, snap frozen immediately in liquid nitrogen (-189 °C) and transferred to a -86 °C freezer until analysis.

On the day of analysis the samples were weighed and thawed using a Sartorius BP 211 D scale. An amount of 1 ml of a 0.1 M perchloric acid solution (containing 0.5 mM sodium metabisulphite and 0.3 mM Na₂EDTA) was added to each tube. The tissue in each tube was then ruptured by sonication (2 x 12 seconds, at amplitude 14 μ) with a MSE-Sonicator, which is the preferred method of cell disruption for small sample and volume size (Keller *et al.*, 1976).

The tubes were then allowed to stand on ice for 20 minutes to complete perchlorate precipitation of proteins and extraction of monoamines. Following this period, samples were centrifuged at 4 °C in a Sigma 3K-15 centrifuge at 15300 rpm or 21460 g for 20 minutes.

The supernatant was removed, transferred to a clean polypropylene tube and was then ready to be analysed with the HPLC. All the analyses were done on the same day as the sample preparations.

3.6.6. Sample injection

Prior to loading the samples into the auto sampler for injection into the HPLC, the pH of the samples was adjusted to pH 5 with the addition of 1 μ l of 10 M potassium acetate. The potassium acetate was prepared by dissolving 98.04 g of potassium acetate (Merck) in 100 ml of distilled water.

An aliquot of 200 μ l of the sample was placed in an HPLC sample vial. A volume of 20 μ l of the internal standard (IS) isoprenaline (7500 ng/ml) was added and the

final solution was briefly vortex. The vials were placed in the auto sampler and each was automatically injected into the HPLC at a volume of 100 μ l.

Results were expressed as ng/g wet weight of tissue.

3.6.7. Monoamine standard curves

A standard curve was constructed for each monoamine and metabolite. The standard curve represents AUC-standard/AUC-internal standard, against concentration. The monoamines and metabolites were identified based on their retention time and quantified by comparing the AUC-sample/AUC-internal standard to the standards, utilizing the linear equation obtained from the standard curve.

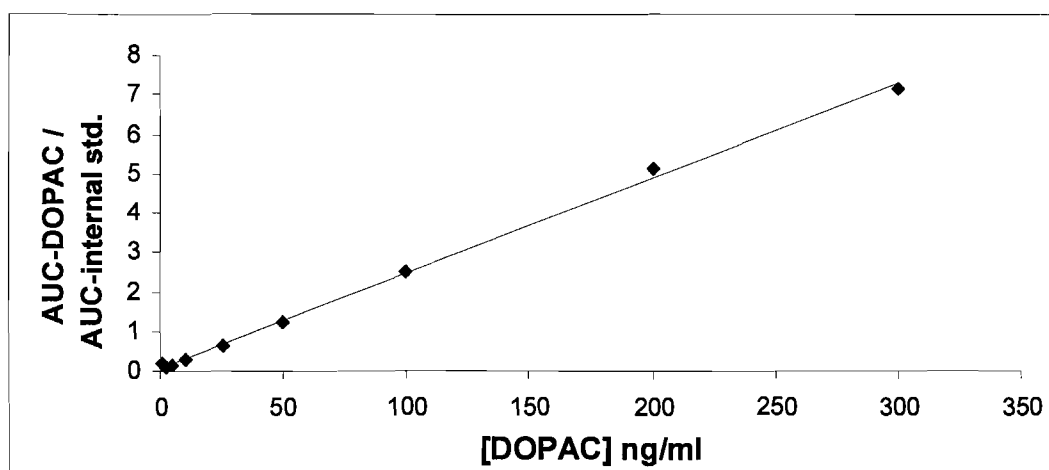


Figure 3-4 Linear curve of AUC ratio for DOPAC against a concentration range of standard solutions for determination of catecholamines in the NAc.

The linear equation obtained from the DOPAC standard curve was: $y = 0.0242x + 0.0785$. The regression coefficient was 0.9981.

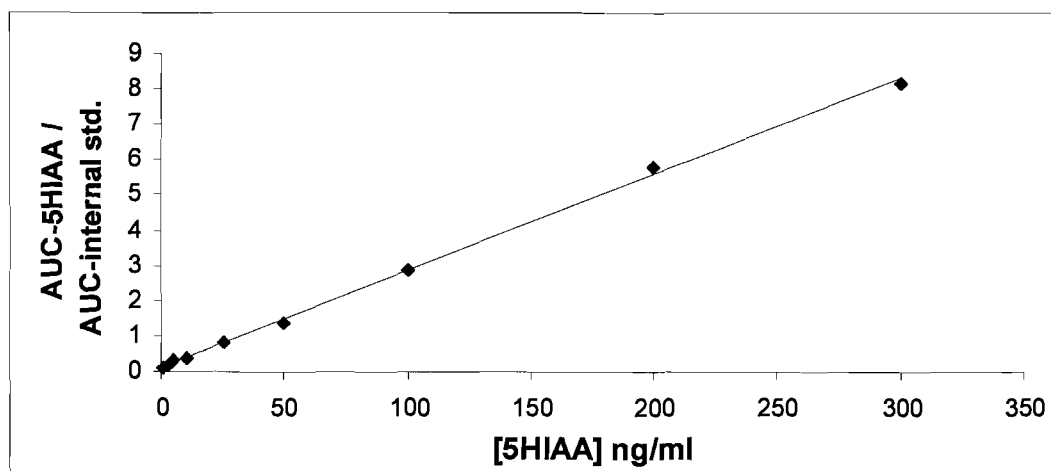


Figure 3-5 Linear curve of AUC ratio for 5HIAA against a concentration range of standard solutions for determination of catecholamines in the NAc.

The linear equation obtained from the 5HIAA standard curve was: $y = 0.0273x + 0.1159$. The regression coefficient was 0.9987.

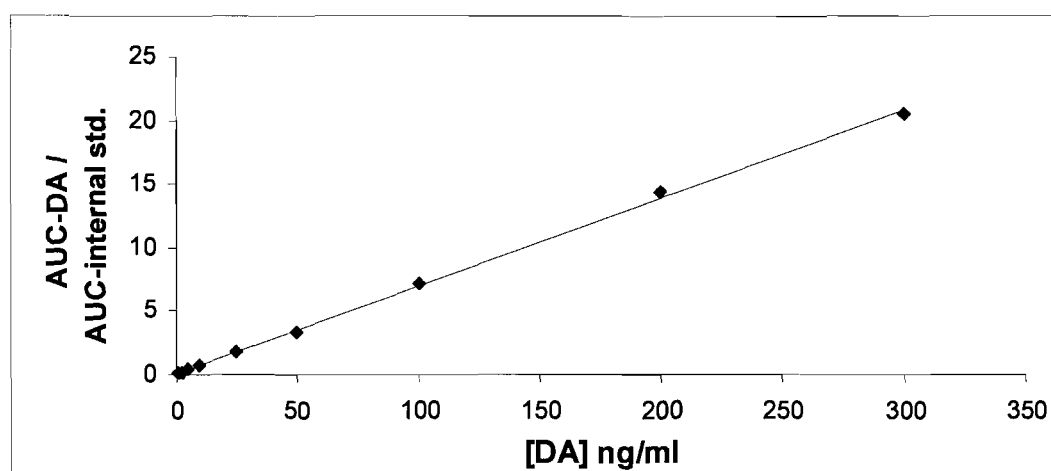


Figure 3-6 Linear curve of AUC ratio for DA against a concentration range of standard solutions for determination of catecholamines in the NAc.

The linear equation obtained from the DA standard curve was: $y = 0.0692x + 0.0815$. The regression coefficient was 0.9992.

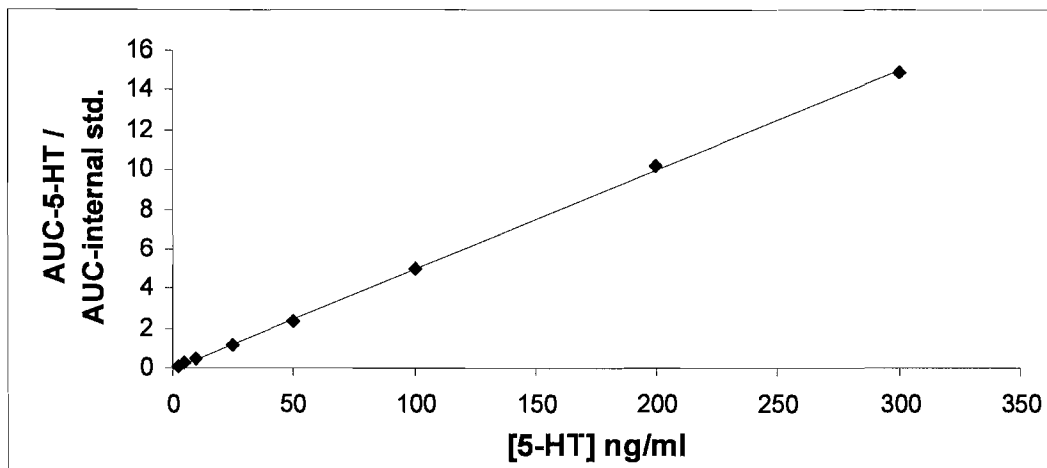


Figure 3-7 Linear curve of AUC ratio for 5-HT against a concentration range of standard solutions for determination of catecholamines in the NAc.

The linear equation obtained from the 5-HT standard curve was: $y = 0.0503x - 0.0401$. The regression coefficient was 0.9995.

3.6.8. Validation of method

In this study the method used to determine plasma DA and DOPAC concentrations was validated. The method used to determine brain monoamines and their metabolites had been used successfully in the past and had been validated (Brand, 1996).

Validation provides evidence that a process or procedure is performing consistently and is capable of delivering specified results. To validate therefore means to provide documented evidence that all causes for variation have been accounted for and, as far as possible eliminated, and that any variation present will not be excessive beyond the expected or "normal value". The above method to determine plasma monoamine concentrations was validated in terms of its selectivity, linearity, range and precision.

3.6.8.1. Specificity and selectivity

Specificity and selectivity are defined as the ability of an analytical method to accurately analyse a component in the presence of other compounds such as biological materials and other metabolic products. Figure 3-8 shows the chromatogram of the standard solution for determination of brain samples.

As observed in the chromatogram, there was no overlapping of compounds in the standard solution or in the brain samples, which indicates that his method is selective.

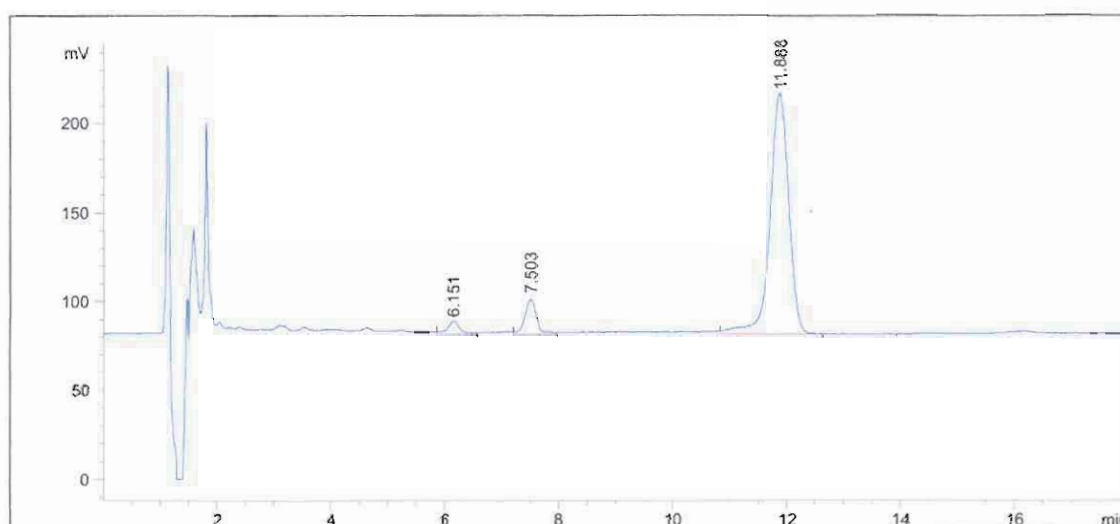


Figure 3-8 Example of a chromatograph indicating good specificity and selectivity. DOPAC, DA and isoprenaline (internal standard) are represented respectively by the peaks at 6.151, 7.503 and 11.888 min.

3.6.8.2. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample. The peak surface area ratio is plotted against concentration. The best straight line through these points is drawn.

The following equation is used:

$$y = mx + c$$

Where y = peak surface area ratio; m = gradient; x = concentration in ng/ml; c = y intercept and the values are depicted in Table 3-7.

Table 3-7 The gradient, y-intercept and regression values of the standard curves.

Monoamine/Metabolite	Slope (m)	y - intercept (c)	Correlation coefficient (r²)
Dopamine (DA)	0.0105	0.0733	0.9966
DOPAC	0.0048	0.0118	0.9999

The linearity of this method was between the specified values of 0.98 – 1.00, and could therefore be accepted.

3.6.8.3. Range

The range of an analytical procedure can be defined as the concentrations between the lowest and highest detectable values. These detectable values of an analytical method must consist of a suitable level of precision, accuracy and linearity. This method proved to be sensitive enough to detect concentrations between 5 ng/ml and 500 ng/ml of the standard solutions.

3.6.8.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility (Nel, 2004).

Precision was achieved by injecting nine samples, ranging from the lowest to the highest concentrations analysed (5 ng/ml – 500 ng/ml) into the HPLC. This was repeated four consecutive times.

The surface area ratio was calculated using the area under the curve (AUC) of the standard divided by the area under the curve of the internal standard.

$$\text{Surface area ratio} = \text{AUC standard} / \text{AUC internal standard}$$

The % RSD (relative standard deviation) was calculated for each concentration after which the mean % RSD was calculated for each compound.

$$\% \text{ RSD} = \text{Standard error variation (STDEV)} / \text{Mean AUC ratio}$$

For a method to be repeatable according to USP (2002) specifications, the average % RSD must be smaller than 10 %.

Table 3-8 The average % RSD of DA and DOPAC.

Monoamine/Metabolite	Average % RSD
Dopamine (DA)	7.683636
DOPAC	6.301739

3.7. Monoamine Oxidase Activity

3.7.1. Substrate used for MAO-B catalytic activity

Benzylamine was used for the determination of MAO-B catalytic activity. Benzylamine is a selective MAO-B substrate and is, according to Krueger *et al* (1995), a good substrate to use in inhibition studies. Purified MAO-B from both bovine and baboon liver was used as an enzyme source. MAO-B oxidizes benzylamine (substrate) to benzaldehyde (product). The amount of product formed during the reaction can be measured spectrophotometrically at a wavelength of $\lambda_{\text{max}} = 250 \text{ nm}$ (Walker & Edmondson, 1994).

Background absorption, close to the preferred UV wavelength (250 nm), compromises the accuracy of the spectrophotometrical method. This problem

was solved by using a discontinuous assay where the extent of benzylamine oxidation was measured using HPLC with UV-detection.

3.7.2. Inhibition study

3.7.2.1. Materials and instrumentation

NAD was supplied by the Department of Biochemistry at the North-West University (Potchefstroom Campus), and all the analyses were performed using a Macherey-Nagel cc 125/4 Nucleosil C18 column with an Agilent 1100 HPLC system. UV-Vis spectra were recorded on a Milton-Roy Spectronic 1201 spectrophotometer.

3.7.2.2. Measurement of inhibition ability of NAD on MAO-B activity.

The MAO-B containing mitochondria were isolated from bovine and baboon liver tissue as described by Salach and Weyler (1987) and were frozen and stored at -70 °C until the day of analysis. The mitochondrial isolate was suspended in a 100 mM sodium phosphate buffer at pH 7.4, containing 50 % glycerol (w/v). The protein concentration was determined using the method of Bradford (1976).

The incubation mixture consisted of the 100 mM sodium phosphate buffer (pH 7.4), made up to a final volume of 500 µl, the substrate benzylamine (125 - 750 µM), the enzyme from mitochondrial isolate (3 mg/ml for baboon isolate and 0.15 mg/ml for bovine isolate), NAD, as possible inhibitor, (100 - 400 µM for baboon liver isolate) and (1000 - 4000 µM for bovine liver isolate). The NAD was prepared in the same 100 mM sodium phosphate buffer, pH 7.4.

The samples were incubated at 37 °C for 8 min where after the reaction was terminated with an addition of 10 µl 70 % perchloric acid (HClO₄). The samples were centrifuged for 10 min at 16,000 g. The generated benzaldehyde was measured with the HPLC-UV using only the supernatant fraction.

The HPLC was set to inject 50 μl of the sample at a flow rate of 1 ml/min. The elution time of benzaldehyde was found to be 3.97 min at a wavelength of 250 nm. The mobile phase consisted of 60 % double distilled water (containing 0.6 % (v/v) glacial acetic acid and 1% (v/v) triethylamine), 30 % methanol and 10 % acetonitrile.

3.7.2.3. Calibration curves

Standard calibration curves were obtained by preparing standard solutions of benzaldehyde. Benzaldehyde is the product of benzylamine which is used as the substrate in the reaction with MAO found in both bovine and baboon liver. The concentrations for benzaldehyde were 6.25 μM , 12.5 μM , 25 μM , 50 μM and 100 μM . Calibration curves were obtained by plotting [benzaldehyde] vs. peak height.

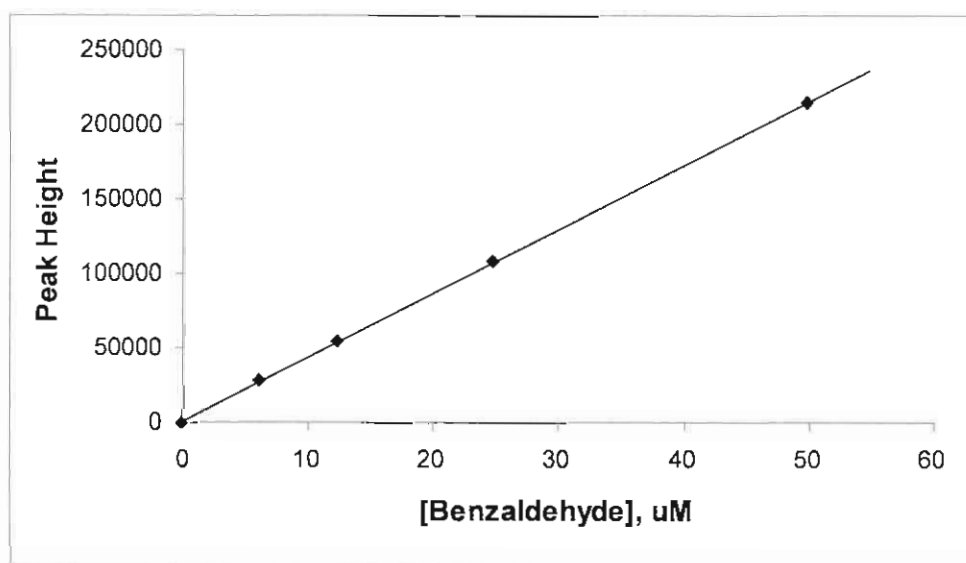


Figure 3-9 Calibration curve for the assay performed on bovine liver mitochondria.

The linear equation obtained from the bovine liver standard curve in Figure 3-9 was: $y = 4291.3x + 1266$. The regression coefficient was 0.999.

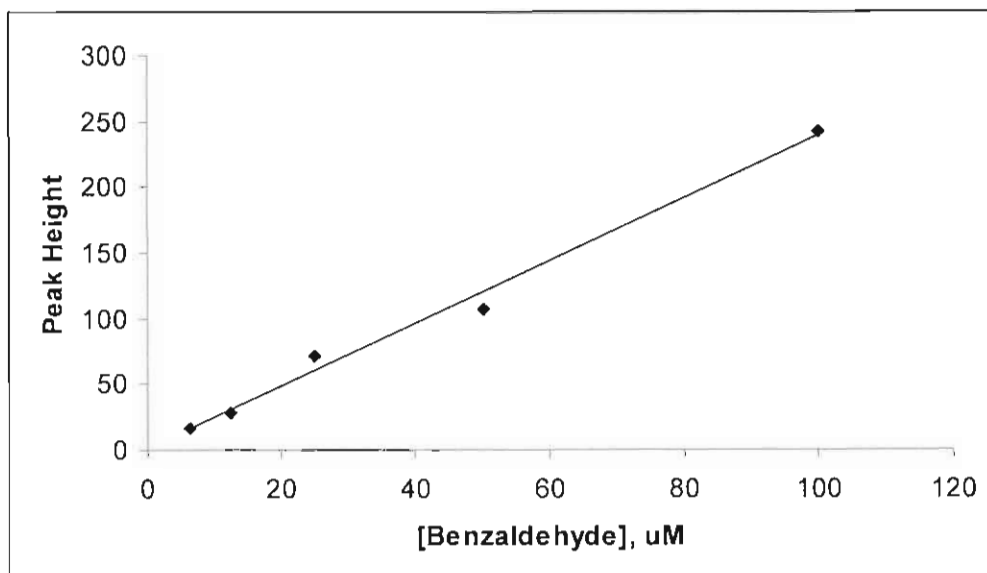


Figure 3-10 Calibration curve for the assay performed on baboon liver mitochondria.

The linear equation obtained from the baboon liver standard curve in Figure 3-10 was: $y = 2.3716x + 1.625$. The regression coefficient was 0.9912.

Chapter 4

Results and Discussion

The aim of this study was to determine if NAD inhibits MAO activity in the rabbit model, to find appropriate methods to determine monoamine concentrations in the plasma and NAc of the rabbit and to determine the effect of tobacco smoke on the weight of the rabbits in combination with the different treatment protocols for the various groups. These experiments were done to ultimately determine if NAD, which is currently being used in the treatment of alcoholism, will be effective to terminate the craving for tobacco smoke.

Groups 1, 2 and 3 received smoke extract via the alzet osmotic pumps for 27 days and group 4 (control) received propylene glycol for 27 days. The alzet osmotic pumps were removed on day 27 and group 1 was injected with saline for four days (day 28, 29, 30, 31), group 2 was injected with NAD for four days (day 28, 29, 30, 31), group 3 received bupropion injections for four days (day 28, 29, 30, 31) and group 4 was injected with saline for four days (day 28, 29, 30, 31). Group 4 was used to compare the monoamine concentrations between smoking and non-smoking rabbits. After the four days of treatment, decapitation and dissection of the NAc was performed to determine monoamine levels.

Statistical analyses were done and confirmed by the Department of Statistical Services at the North-West University (Potchefstroom Campus) using Statistica® software.

4.1. Catecholamines

4.1.1. Blood catecholamine levels

A one-way analysis of variance per day was performed. This was done on both the DA and DOPAC plasma concentrations for each of the four days during the treatment protocol. This analysis was followed by post hoc comparisons using

the unequal N HSD test (which is a generalization of the Tukey's multiple comparisons test) to compare groups within each day. A probability of $p \leq 0.05$ was employed to declare statistically significant differences.

Figure 4-1 represents the average DOPAC concentrations in the plasma of the rabbits from day 28 to day 31 (expressed as ng/ml). The average concentration for each group was determined three times per day (08:00, 12:00 and 16:30).

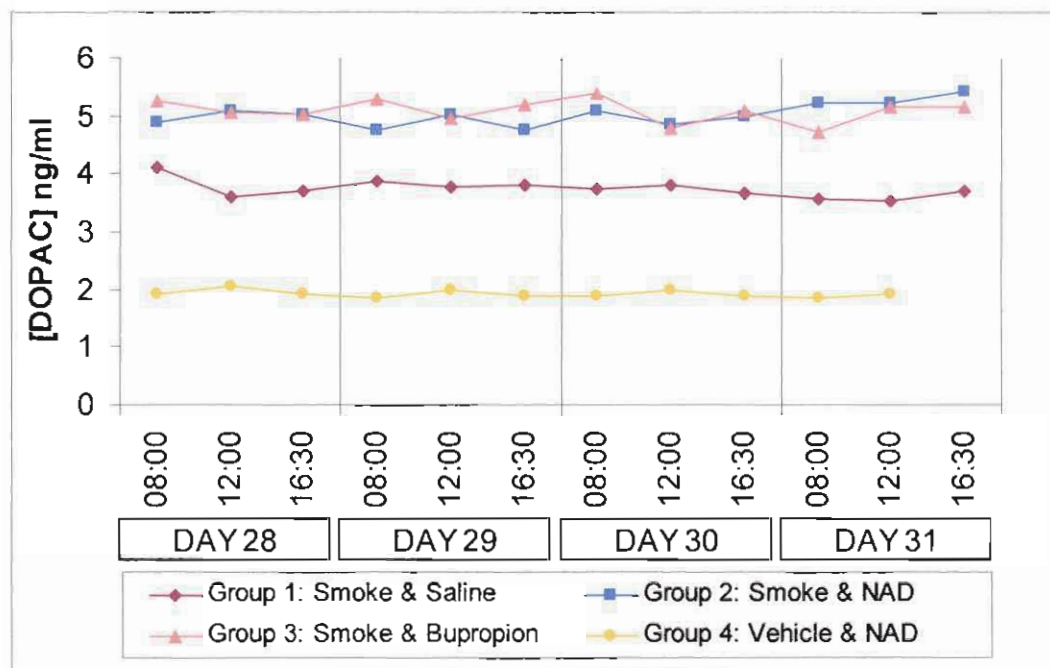


Figure 4-1 The plasma DOPAC concentrations of the rabbits from day 28 to day 31 of the treatment protocol.

Figure 4-2 represents the average DA concentrations in the plasma of the rabbits from day 28 to day 31 (expressed as ng/ml). The average concentration for each group was determined three times per day (08:00, 12:00 and 16:30).

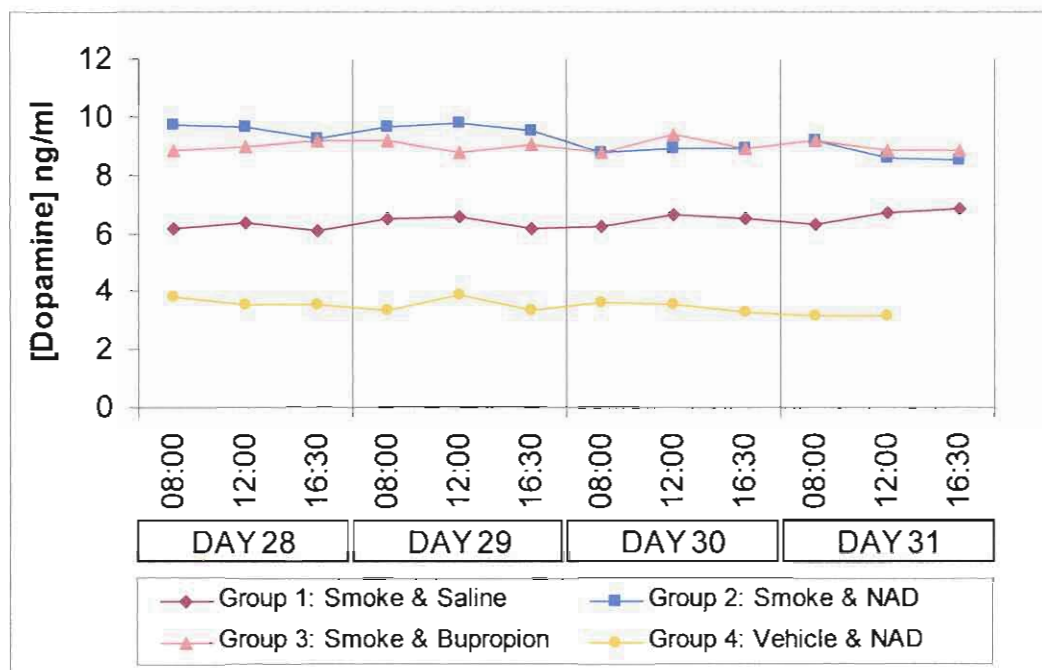


Figure 4-2 The plasma DA concentrations of the rabbits from day 28 to day 31 of the treatment protocol.

Figure 4-3 represents the average DOPAC concentrations for groups 1, 2, 3 and 4. Each bar represents the average plasma concentration for each group, per day (08:00, 12:00 and 16:30 determined together). The unequal N HSD test revealed a statistically significant difference in DOPAC concentrations on day 28 between groups 1 and 2 ($p < 0.05$, $p = 0.02867$), groups 1 and 3 ($p < 0.05$, $p = 0.00872$), groups 1 and 4 ($p < 0.05$, $p = 0.03226$), groups 2 and 4 ($p < 0.05$, $p = 0.00028$) and between groups 3 and 4 ($p < 0.05$, $p = 0.000357$).

The unequal N HSD test revealed a statistically significant difference in DOPAC concentrations on day 29 between groups 1 and 3 ($p < 0.05$, $p = 0.00530$), groups 1 and 4 ($p < 0.05$, $p = 0.00389$), groups 2 and 4 ($p < 0.05$, $p = 0.000203$) and groups 3 and 4 ($p < 0.05$, $p = 0.000198$).

The unequal N HSD test revealed a statistically significant difference in DOPAC concentrations on day 30 between groups 1 and 2 ($p < 0.05$, $p = 0.02317$), groups 1 and 3 ($p < 0.05$, $p = 0.01311$), groups 1 and 4 ($p < 0.05$, $p = 0.01765$),

groups 2 and 4 ($p < 0.05$, $p = 0.00022$) and groups 3 and 4 ($p < 0.05$, $p = 0.000306$).

The unequal N HSD test revealed a statistically significant difference in DOPAC concentrations on day 31 between groups 1 and 2 ($p < 0.05$, $p = 0.00434$), groups 1 and 3 ($p < 0.05$, $p = 0.00118$), groups 1 and 4 ($p < 0.05$, $p = 0.01249$), groups 2 and 4 ($p < 0.05$, $p = 0.00018$) and groups 3 and 4 ($p < 0.05$, $p = 0.00020$).

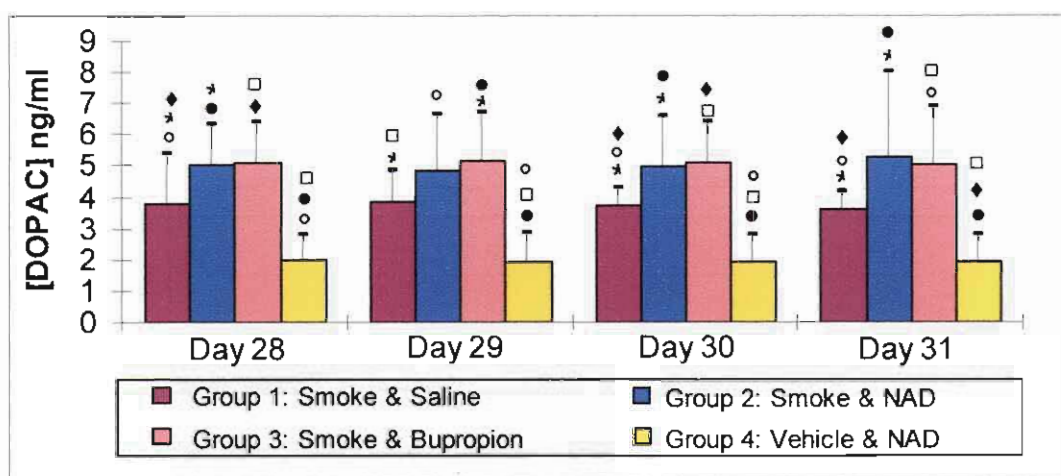


Figure 4-3 The average plasma DOPAC concentrations of the rabbits from day 28 to day 31 of the treatment protocol.

Figure 4-4 represents the average DA concentrations for groups 1, 2, 3 and 4. Each bar represents the average plasma concentration for each group, per day (08:00, 12:00 and 16:30 determined together). The unequal N HSD test revealed a statistically significant difference in DA concentrations on day 28 between groups 1 and 2 ($p < 0.05$, $p = 0.00018$), groups 1 and 3 ($p < 0.05$, $p = 0.00018$), groups 1 and 4 ($p < 0.05$, $p = 0.00196$), groups 2 and 4 ($p < 0.05$, $p = 0.000179$) and groups 3 and 4 ($p < 0.05$, $p = 0.000179$).

The unequal N HSD test revealed a statistically significant difference in DA concentrations on day 29 between groups 1 and 2 ($p < 0.05$, $p = 0.00023$), groups 1 and 3 ($p < 0.05$, $p = 0.00021$), groups 1 and 4 ($p < 0.05$, $p = 0.00067$),

groups 2 and 4 ($p < 0.05$, $p = 0.000179$) and groups 3 and 4 ($p < 0.05$, $p = 0.000179$).

The unequal N HSD test revealed a statistically significant difference in DA concentrations on day 30 between groups 1 and 2 ($p < 0.05$, $p = 0.00028$), groups 1 and 3 ($p < 0.05$, $p = 0.000183$), groups 1 and 4 ($p < 0.05$, $p = 0.000212$), groups 2 and 4 ($p < 0.05$, $p = 0.000179$) and groups 3 and 4 ($p < 0.05$, $p = 0.000179$).

The unequal N HSD test revealed a statistically significant difference in DA concentrations on day 31 between groups 1 and 2 ($p < 0.05$, $p = 0.000965$), groups 1 and 3 ($p < 0.05$, $p = 0.000196$), groups 1 and 4 ($p < 0.05$, $p = 0.000199$), groups 2 and 4 ($p < 0.05$, $p = 0.000179$) and groups 3 and 4 ($p < 0.05$, $p = 0.000179$).

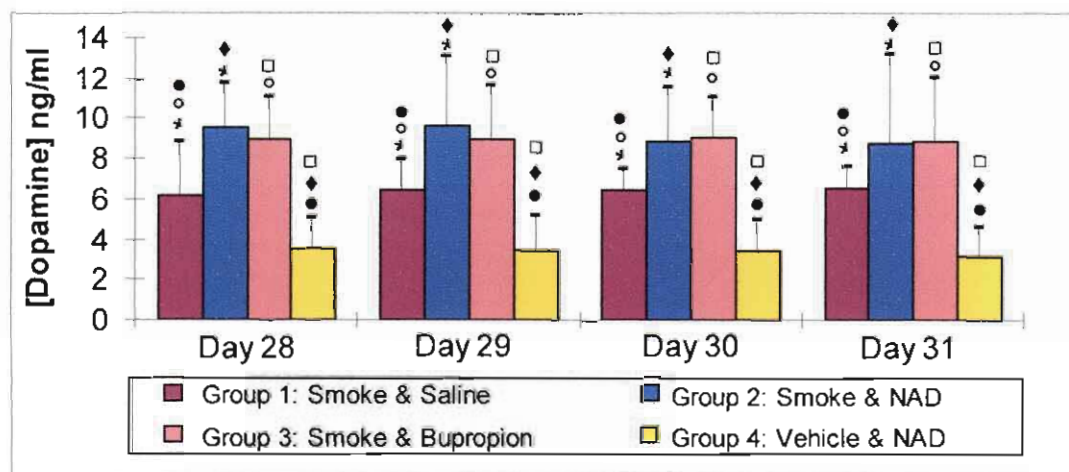


Figure 4-4 The average plasma DA concentrations of the rabbits from day 28 to day 31 of the treatment protocol.

4.1.1.1. Discussion

Groups 1, 2 and 3 in Figure 4-1 and Figure 4-2 all received identical smoke extracts until day 27 with removal of the osmotic pumps. The DOPAC and DA plasma concentrations were determined on the next day (day 28). It was

expected that all three of these groups would exhibit approximately the same concentrations on the day of removal of the osmotic pumps (day 27). One of the shortcomings of this experiment was that no concentrations were determined upon removal of the pumps on day 27 for any of the groups. This data would have made a valuable contribution to our hypothesis that NAD, like bupropion, could assist the smoker with overcoming withdrawal symptoms during smoking cessation, by increasing or maintaining DA concentrations during this period.

Laboratory studies done on animals, demonstrated that nicotine is responsible for an increase in DA concentration (Koob, 1992), and that nicotine withdrawal causes a decrease of DA.

Figure 4-1 and Figure 4-2 indicate a statistically significant increase in both DOPAC and DA plasma concentrations for group 2 (NAD) and group 3 (bupropion) when they are compared to group 1 (saline) after all of these groups had received identical smoke extract for 28 days. We speculate that NAD and bupropion increased or maintained both DOPAC and DA concentrations (when compared to group 1) during the four days of treatment, thereby possibly decreasing the withdrawal symptoms experienced during smoking cessation.

Group 4 in both Figure 4-1 and Figure 4-2 illustrated a statistically significant decrease in both DOPAC and DA concentrations compared to group 1, 2 and 3. We speculate that the reason for this might be that group 4 did not receive any smoke extract and were therefore not subjected to any external manipulation of their DOPAC and DA concentrations. During their four days of treatment with NAD, no fundamental changes could be detected in the plasma concentration of group 4, suggesting that NAD has no effect on either DOPAC or DA concentrations if they are not increased above normal.

Figure 4-3 and Figure 4-4 represent the concentrations of the average sum of all three time periods during each of the four days of treatment. Both these figures illustrate that group 2 (NAD) and group 3 (bupropion) had statistically significant

higher DOPAC and DA plasma concentrations compared to group 1 (saline) and group 4 (vehicle). It therefore seems that NAD and bupropion had similar effects on the DA and DOPAC concentrations in the test animals. From these data it could be speculated that NAD could be as effective as bupropion (Zyban®) to reduce withdrawal symptoms in patients trying to refrain from smoking.

4.1.2. Catecholamine levels in the nucleus accumbens

4.1.2.1. Groups 1, 2, 3 and 4

The data from the catecholamines were analysed using Levene's test for homogeneity of variances and was found to be equal (homogeneous). A two-way analysis of variance (ANOVA) could therefore be performed, (with groups as factor and the blocks are nested within the groups). This analysis was followed by post hoc comparisons using the unequal N HSD (honest significant difference) test, which is a generalization of Tukey's studentised range (HSD) test used in the case of unequal sample sizes. A probability of $p \leq 0.05$ was employed to declare statistically significant differences. The probability plot indicated a normal distribution of variables.

Figure 4-5 represents the average DOPAC concentrations in the NAc of the rabbits in group 1, 2, 3 and 4 (see Appendix for raw data). $n = 6$ (group1), $n = 8$ (group 2), $n = 7$ (group 3 and 4). The unequal N HSD test revealed that there was a significant difference in DOPAC concentrations between groups 1 and 2 ($p < 0.05$, $p = 0.02937$), and groups 1 and 3 ($p < 0.05$, $p = 0.01929$).

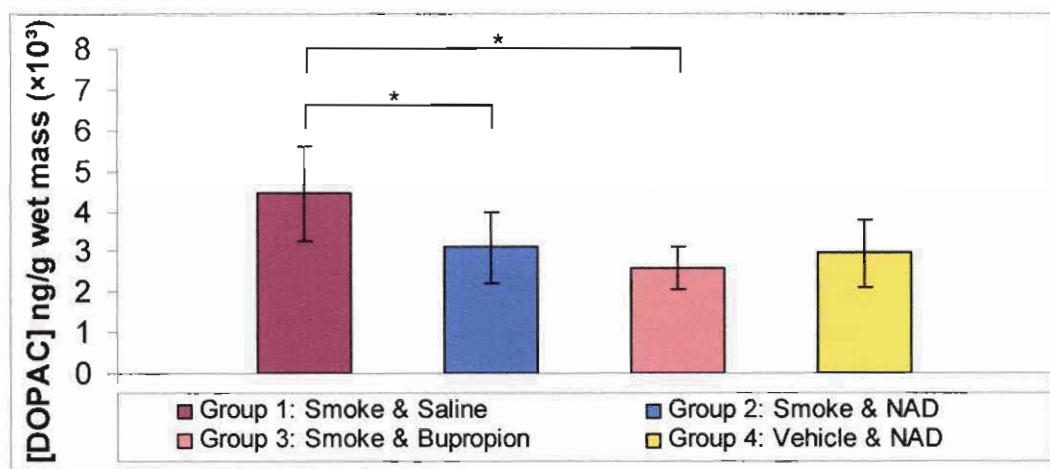


Figure 4-5 Average DOPAC concentrations in the nucleus accumbens of the rabbits on day 32 (* $p < 0.05$).

Figure 4-6 represents the average 5-HIAA concentrations in the NAc of the rabbits in group 1, 2, 3 and 4 (see Appendix for raw data). $n = 6$ (group1), $n = 8$ (group 2), $n = 7$ (group 3 and 4). There was no significant difference in 5-HIAA concentrations between groups 1, 2, 3 and 4 ($p < 0.05$, $p = 0.769$).

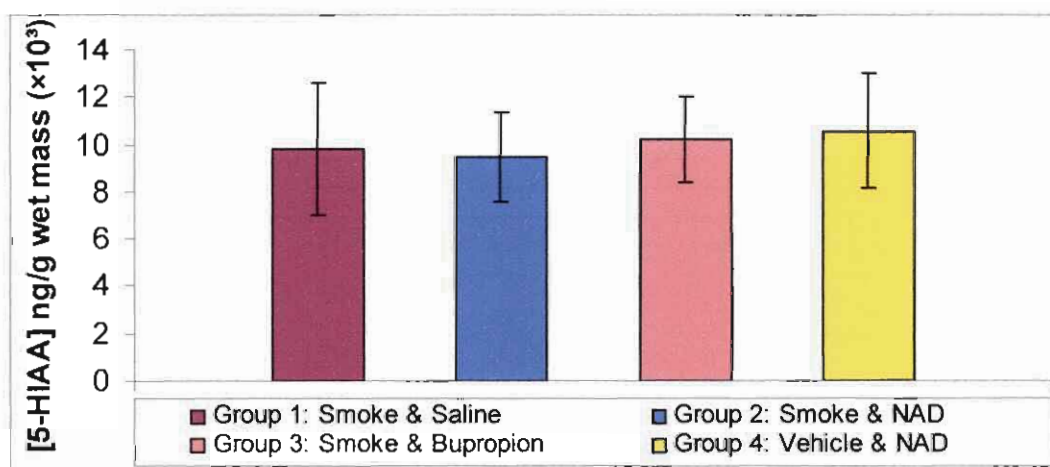


Figure 4-6 Average 5-HIAA concentrations in the nucleus accumbens of the rabbits on day 32.

Figure 4-7 represents the average DA concentrations in the NAc of the rabbits in group 1, 2, 3 and 4 (see Appendix for raw data). $n = 6$ (group1), $n = 8$ (group 2),

$n = 7$ (group 3 and 4). There was no significant difference in DA concentrations between groups 1, 2, 3 and 4 ($p < 0.05$, $p = 0.563$).

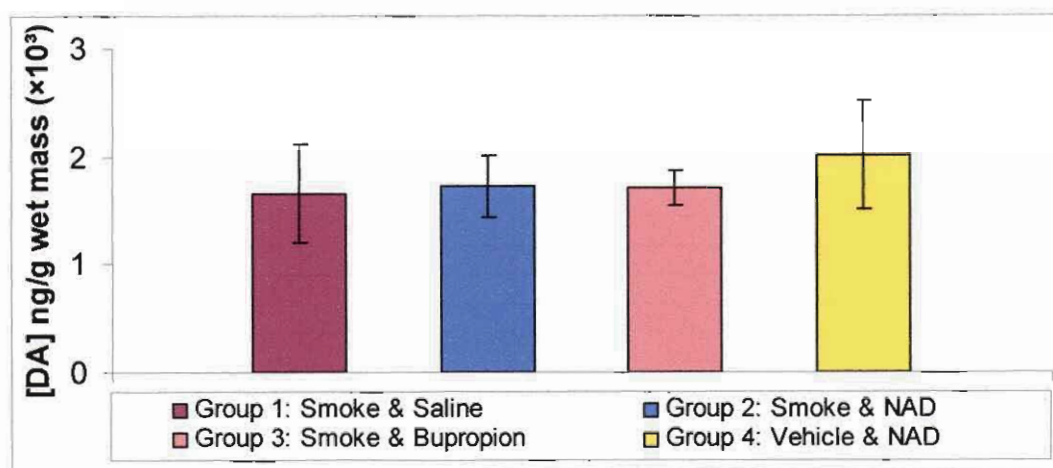


Figure 4-7 Average DA concentrations in the nucleus accumbens of the rabbits on day 32.

Figure 4-8 represents the average 5-HT concentrations in the NAc of the rabbits in group 1, 2 and 3 (see Appendix for raw data). $n = 6$ (group1), $n = 8$ (group 2), $n = 7$ (group 3 and 4). There was no significant difference in 5-HT concentrations between groups 1, 2 and 3 ($p < 0.05$, $p = 0.367$).

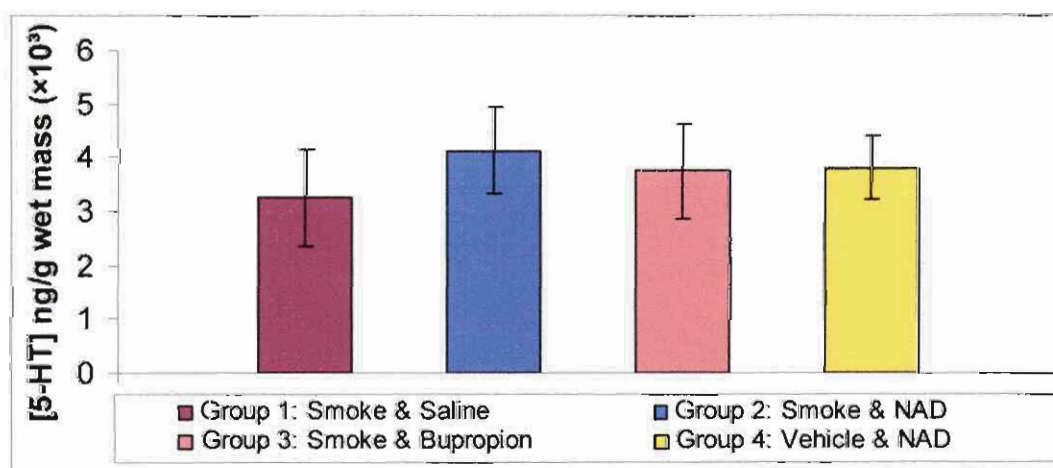


Figure 4-8 Average 5-HT concentrations in the nucleus accumbens of the rabbits on day 32.

4.1.2.2. Discussion

There was no significant difference in the NAc DA, 5-HIAA and 5-HT concentrations between group 1, 2, 3 and 4 after four days of treatment and upon sacrifice on day five. There was, however, a significant difference in DOPAC concentration on day 32 between the group treated with saline (group 1) and the group treated with NAD (group 2) and also between group 1 (saline) and group 3 (bupropion) (Figure 4-5). This decrease in DOPAC concentration indicates that NAD and bupropion might influence the DOPAC concentration in the NAc.

The DA concentration (Figure 4-7) in the NAc in groups 1, 2 and 3 (saline, NAD and bupropion) appeared to be lower compared to group 4 (the vehicle group), although not statistically significant.

The 5-HT concentration (Figure 4-8) of group 2 (NAD) and group 3 (bupropion) in the NAc appeared to be slightly higher than group 1 (saline) indicating that NAD and bupropion might influence 5-HT levels, although it was not statistically significant.

In an experiment by Gäddnäs *et al.* (2000), the effects of chronic nicotine and its withdrawal on brain monoamines were studied by administering nicotine in the drinking water to male NMRI mice. Monoamines were measured post mortem on the 50th day of nicotine administration. They found that during chronic nicotine administration the striatal DOPAC and 5-HIAA, hypothalamic 5-HIAA and NA as well as cortical NA concentrations were elevated compared to the control mice that drank tap water throughout the experiment. These effects disappeared during withdrawal.

The difference in our results when compared to those found by Gäddnäs might be due to the fact that they exposed the mice to nicotine for 50 days; whereas we only exposed them to nicotine for 27 days. Also, the brain region used by Gäddnäs, (striatum and cortex) differed from the one we used (NAc).

It is possible that the time of exposure to nicotine (27 days) might have been too short to establish addiction to nicotine. It could also have been beneficial to include a control group that was treated with saline only and not with smoke extract. This group could have been used to accurately compare the monoamine levels between smoking and non-smoking groups and the effects of NAD and bupropion on the monoamine levels.

4.2. Variations in weight

The weight of each rabbit was determined on the day of arrival at the ARC, after implantation of the alzet osmotic mini pumps on day 1. On day 27 the mini pumps were removed and the weight of each rabbit was determined again. The percentage of weight gained during this time is indicated in Figure 4-9. Each rabbit was then treated accordingly and on day 31 the rabbits received their last treatment and their weight was determined again. The amount of weight gained during these four days of treatment is indicated in Figure 4-10 (values expressed as percentage weight gained).

Groups 1, 2 and 3 received the same smoke extract for 27 days via the alzet osmotic mini pumps and was grouped together to compare to group 4 (vehicle) (Figure 4-9). The t-test for independent samples revealed that there was no significant difference between the two groups illustrated in Figure 4-9, $t(28)=-0.360(p=0.721)$. (The p-level reported with a t-test represents the probability of error involved in accepting our research hypothesis about the existence of a difference). A probability of $p \leq 0.05$ was employed to declare statistically significant differences.

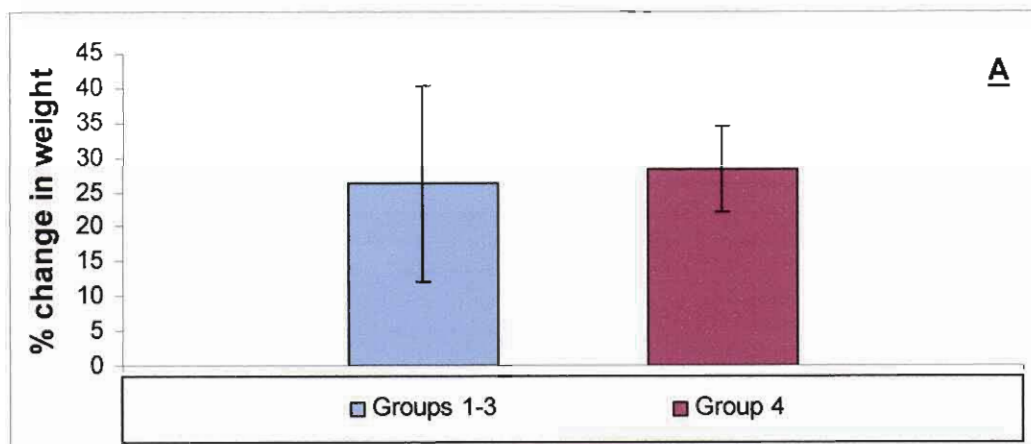


Figure 4-9 The percentage change in weight from day 1 to day 27 between groups 1,2 and 3 (receiving smoke extract) and group 4 (vehicle).

An important assumption with the analysis of variance (ANOVA) is that the variances for each group are equal (homogeneous). The Levene test of homogeneity is a commonly used test for this assumption. The Levene test was performed on the data shown in Figure 4-10 and indicated a statistical significance ($p= 0.0224$). The hypothesis of homogeneous variance was therefore rejected.

Secondly, an analysis of variance (ANOVA) was performed to determine any statistically significant differences between the groups which showed a significant difference between the groups ($p= 0.00045$).

Whenever the assumption of homogeneity of variances is violated it is advisable to use a nonparametric method to determine significant differences between groups. A nonparametric method is appropriate when the sample size is small. The nonparametric test used (the Kruskal-Wallis test) indicated significant differences between group 1 and 4 ($p= 0.0033$) and group 3 and 4 ($p= 0.0180$).

The nonparametric test was compared to a parametric test (Tukey HSD test) and the results were equivalent.

The standard probability plot indicated a normal distribution of variables.

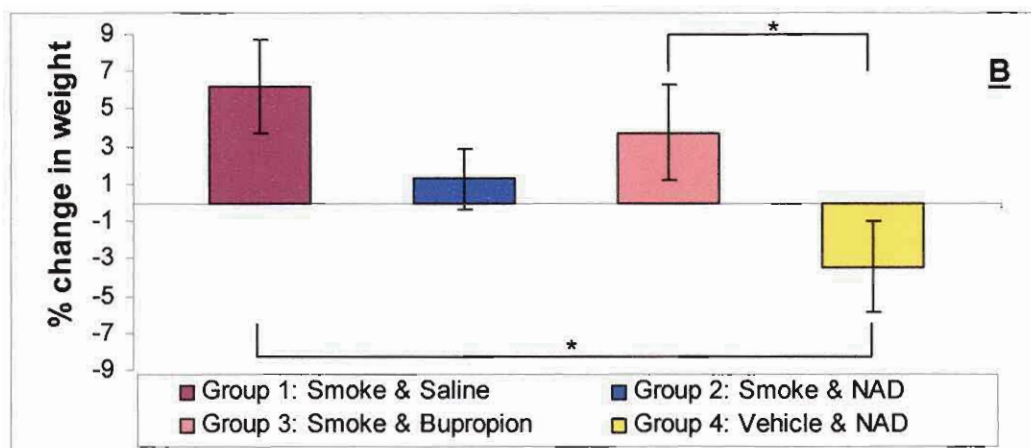


Figure 4-10 The average percentage of weight gained within each group during the four days of treatment.

4.2.1.1. Discussion

The percentage of weight gained during exposure to nicotine (A), and the four days of treatment (B) are illustrated in Figure 4-9 and Figure 4-10. There was no statistically significant difference in the percentage weight gained between the three groups that all received smoke extract compared to group 4 which only received propylene glycol (vehicle) (Figure 4-9). These results indicate that nicotine did not drastically influence the weight of the rabbits during their time of exposure to smoke extract.

After removal of the alzet osmotic pumps (Figure 4-10), group 1 (saline) gained the highest percentage of weight. According to Nelson & Gehlert (2006) biogenic amines have long been studied for their effect on the maintenance of body weight, and weight gain after smoking cessation is most likely.

The percentage of weight gained in groups 2 and 3 were undeniably lower than that of group 1, although all three groups were exposed to nicotine for 27 days. Although not significantly different, it is perceptible that NAD and bupropion might influence body weight during smoking cessation and that NAD treated rabbits

gained less weight than bupropion treated rabbits. Controlled trials also suggested that bupropion SR may be associated with weight loss. In addition, the amount of weight gained after smoking cessation was less in subjects treated with bupropion than in placebo-treated subjects (Anderson *et al.*, 2002). This appears to be similar with NAD treated subjects.

Group 4 (Figure 4-10), showed a statistically significant loss in body weight compared to groups 1 and 3. (Group 4 did not receive any smoke extract, but was treated with NAD for four days).

According to Nelson & Gehlert (2006) any agent that releases monoamines from neuronal stores has antiobesity activity, and inhibitors of neuronal reuptake of 5-HT and NE have been shown to reduce both weight and feeding, preclinically and clinically. The above results indicate that NAD might be valuable in the prevention of a disproportionate amount of weight gain during smoking cessation.

4.3. MAO inhibition

Inhibition is a term used to describe the process where an inhibitor binds to a substrate-binding site on an enzyme, thereby preventing the substrate from binding to the same area.

The Lineweaver-Burke plot (Figure 4-11) is a very good indicator of competitive inhibition. When a competitive inhibitor is added to a reaction and it inhibits the enzyme, thereby preventing the substrate to bind at the active binding site, the slope of the straight line is increased with an increase in concentration of the inhibitor, while the y-axis intercept remains unchanged.

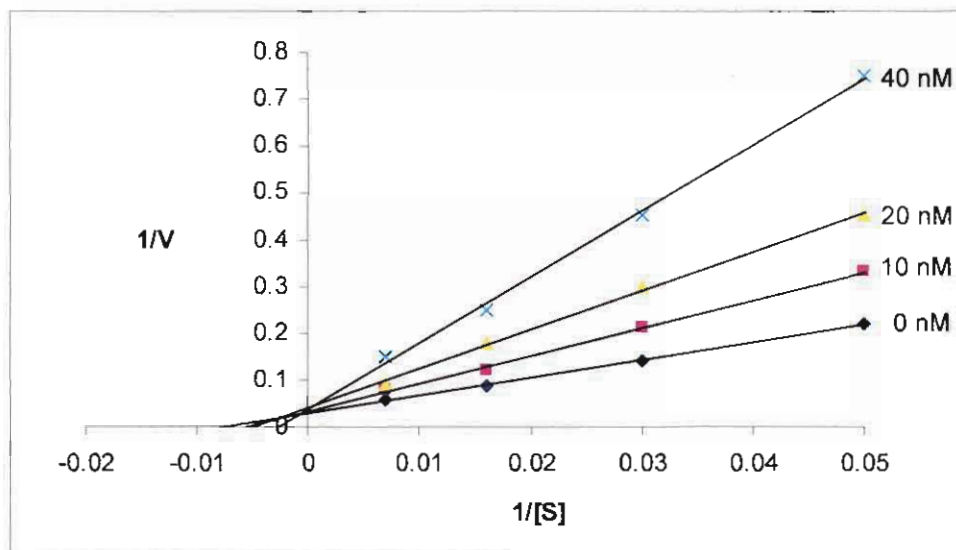


Figure 4-11 An example of competitive inhibition illustrated by the Lineweaver-Burke plot.

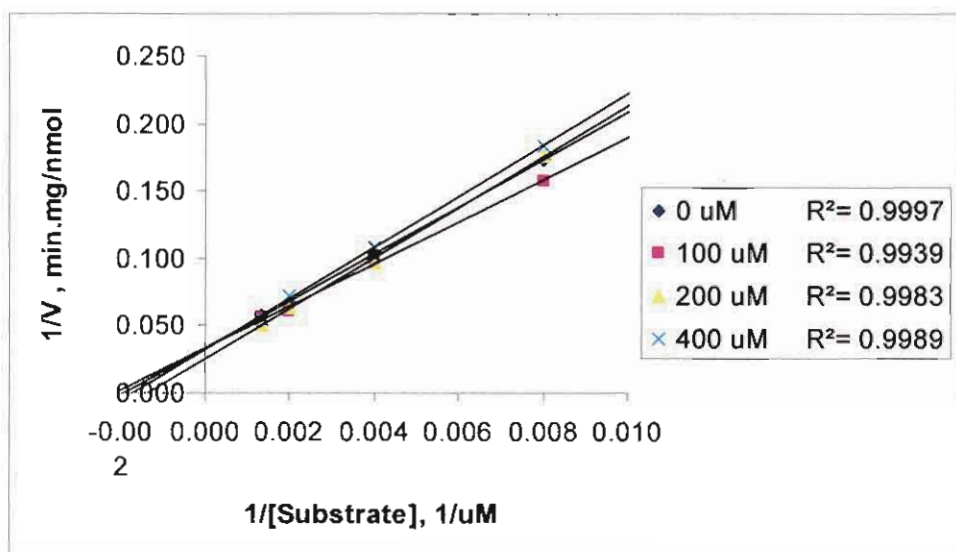


Figure 4-12 The Lineweaver-Burke plot illustrates the oxidation of benzylamine in the absence (0 μM) and presence of various concentrations of the test inhibitor, NAD. The concentration of the baboon liver mitochondria preparation was 3.0 mg/ml and the rates are expressed as nmoles.mg protein⁻¹.min⁻¹ of benzaldehyde formed.

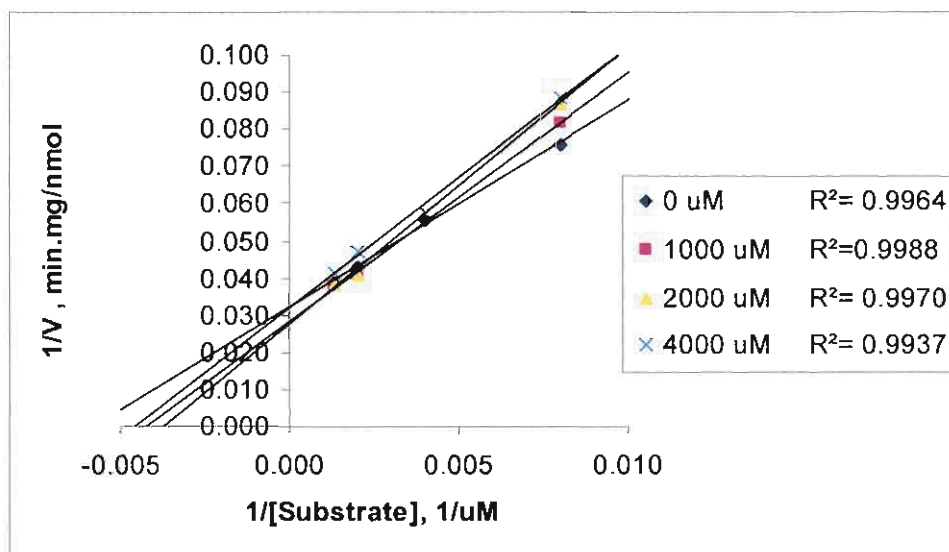


Figure 4-13 The Lineweaver-Burke plot illustrates the oxidation of benzylamine in the absence (0 μM) and presence of various concentrations of the test inhibitor, NAD. The concentration of the bovine liver mitochondria preparation was 0.15 mg/ml and the rates are expressed as nmoles.mg protein⁻¹.min⁻¹ of benzaldehyde formed.

The example shown in Figure 4-11 clearly indicates competitive inhibition due to the increase in slope with an increase in concentration of the inhibitor from 0 nM to 40 nM.

The Lineweaver-Burke plot in Figure 4-12 illustrates a lack of inhibitory properties of NAD on the MAO-B in the baboon liver mitochondria.

The Lineweaver-Burke plot in Figure 4-13 also illustrates a lack of inhibitory properties of NAD on the MAO-B in the bovine liver mitochondria.

4.3.1.1. Discussion

The Lineweaver-Burke plot shown in Figure 4-11 is a perfect example of competitive inhibition. When the possible inhibitor causes an increase in the slope of the Lineweaver-Burke plot, due to an increase in inhibitor concentration, it is a good indication that the substance is a potential inhibitor of MAO-B activity.

The treatment of smoking addiction and assistance in smoking cessation consists of a multiple treatment model, and inhibition of MAO-B is suggested to be one of the potential components in treating the addiction (Berlin *et al.*, 2001).

There was no significant inhibition of MAO-B activity in either the baboon liver preparation illustrated in Figure 4-12 or the bovine liver preparation illustrated in Figure 4-13. These results conclude the fact that NAD is not a potential inhibitor of MAO-B.

It is interesting to note that bupropion, the first non-nicotinic agent approved by the FDA for smoking cessation, also has no inhibitory effect on MAO-B activity (Ascher *et al.*, 1995). Numerous studies, however, indicated that bupropion has substantial merit in the treatment of smoking addiction and cessation of smoking (Mitrouska *et al.*, 2005).

Chapter 5

Conclusion

Tobacco is the most popular and widely used drug in the world. This contributes to the fact that smoking is the most common cause of avoidable illness and death. Smoking has evolved into an enormous global health problem, and with increased availability to the youth, this disease is destined to become an even bigger crisis if a solution is not found. Despite the availability of treatments for nicotine addiction, like bupropion and nicotine replacement therapies, smoking remains ubiquitous and it includes serious health consequences. To effectively contest this problem, it is of great importance that we study new pharmacological agents against smoking addiction, thereby reducing the number of smokers and substantially reduce morbidity and mortality associated with cigarette smoking.

The aim of this study was to determine the effects of NAD on plasma DOPAC and DA concentrations, and monoamine concentrations in the nucleus accumbens, compared to that of bupropion which is an existing treatment for smoking cessation. Secondly, to determine if NAD plays any role in MAO inhibition and thirdly, to determine the effect of NAD on the body weight of the patient during withdrawal. Studies have shown that smokers have lower levels of brain MAO-A and MAO-B activity and lower MAO-B platelet activity than non-smokers (Fowler *et al.*, 2003; Mihailescu & Drucker-Colin, 2000).

The results indicate that NAD may prove to be a valuable contributor to the potential treatment of smoking addiction. According to O'Hallaren (1961), NAD has also been used to treat various other drug addictions like alcoholism and heroin. The mechanism of action of NAD is still unclear.

The results also indicate that NAD shows definite potential in controlling the unwanted increase in body weight during smoking cessation, and does not inhibit

MAO activity, and therefore does not contribute to that component of treatment during smoking cessation.

Proposals for future studies:

- To determine the mechanism of action of NAD.
- To determine the effects of NAD on tyrosine hydroxylase activity and catechol-o-methyl-transferase activity using western blots (tyrosine hydroxylase is the rate-limiting enzyme in the hydroxylation of tyrosine to DOPA in the synthesis of DA).
- To determine if NAD has an influence on dopamine transporters (DAT) during smoking cessation.
- Determine the plasma DOPAC and DA concentrations on the same day of removal of the osmotic pumps, and comparing the data in this study to a control group which only received propylene glycol with no additional treatment.
- Determine brain monoamine levels during NAD treatment using *in vivo* microdialysis.

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APPENDIX A: RAW DATA AND STATISTICAL ANALYSES

5.1. Raw Data on Blood Sample Analysis

Table A - 1 Standard curve for determination of blood monoamine levels.

Concentration (ng / ml)	DOPAC	DOPAC/IS	DA	DA/IS	I.S.
0	0.00000	0.00000	0.00000	0.00000	0.00000
0	0.00000	0.00000	0.00000	0.00000	0.00000
5	72.40469	0.02316	314.98975	0.10073	3126.91626
5	82.79441	0.02565	316.22644	0.09798	3227.41772
10	135.83653	0.04410	310.47751	0.10080	3080.16943
10	140.00571	0.04476	338.98172	0.10837	3128.14404
20	295.89612	0.10036	638.99860	0.21673	2948.40259
20	295.98648	0.10184	649.56525	0.22349	2906.41675
30	423.20993	0.13873	1655.18201	0.54259	3050.53638
30	416.89554	0.13715	1667.50342	0.54859	3039.61157
50	701.92645	0.22653	1503.20105	0.48512	3098.64600
50	727.20740	0.24091	1479.60229	0.49015	3018.64673
100	1462.47583	0.46934	3476.32471	1.11563	3116.01489
100	1453.14807	0.48130	3499.94556	1.15922	3019.21313
200	2939.99414	0.96347	6060.94141	1.98623	3051.47266
200	2926.35425	0.95215	6048.34473	1.96796	3073.40869
300	4349.65625	1.42026	11365.10000	3.71097	3062.57080
300	4308.13525	1.40635	11295.80000	3.68741	3063.33936
500	7222.11230	2.40381	15424.50000	5.13389	3004.44629
500	7216.47656	2.39577	15261.80000	5.06670	3012.17700
DOPAC			DA		
	y =	DOPAC / IS		y =	DA / IS
	m =	0.004816		m =	0.01015
	x =	[DOPAC]		x =	[DA]
	c =	0.005385		c =	0.02595
	r =	0.9997		r =	0.9989

Table A - 2 Blood Monoamine Levels for Group 1 (D) – Smoke & Saline.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 13	DAY 1	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	62.695	261.382	3168.350	0.020	2.991	0.082	5.571
		16:30	58.835	250.646	2960.831	0.020	3.008	0.085	5.784
	DAY 2	08:00	62.788	234.518	2969.076	0.021	3.273	0.079	5.225
		13:00	54.861	237.153	2703.666	0.020	3.095	0.088	6.085
		16:30	65.142	231.594	3010.378	0.022	3.375	0.077	5.023
	DAY 3	08:00	45.678	215.617	2159.715	0.021	3.273	0.100	7.279
		13:00	61.785	233.630	2891.702	0.021	3.318	0.081	5.403
		16:30	66.277	268.400	3111.825	0.021	3.304	0.086	5.941
	DAY 4	08:00	61.466	245.333	3038.937	0.020	3.082	0.081	5.397
		13:00	51.539	194.220	2319.748	0.022	3.495	0.084	5.692
		16:30	67.522	253.972	3264.793	0.021	3.176	0.078	5.107
RABBIT 14	DAY 1	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	68.428	212.291	2956.662	0.023	3.687	0.072	4.517
		16:30	50.639	162.670	1991.310	0.025	4.162	0.082	5.492
	DAY 2	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	72.975	234.371	3010.220	0.024	3.916	0.078	5.114
		16:30	71.242	240.844	3151.615	0.023	3.576	0.076	4.972
	DAY 3	08:00	70.703	320.322	3012.684	0.023	3.755	0.106	7.919
		13:00	69.328	215.267	2802.647	0.025	4.018	0.077	5.011
		16:30	70.562	256.365	3103.824	0.023	3.602	0.083	5.581
	DAY 4	08:00	50.974	206.990	2331.743	0.022	3.421	0.089	6.189
		13:00	60.019	241.482	2790.951	0.022	3.347	0.087	5.968
		16:30	66.317	313.961	3032.704	0.022	3.422	0.104	7.643
RABBIT 15	DAY 1	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	57.279	295.355	3058.596	0.019	2.770	0.097	6.957
		16:30	35.891	139.731	1883.255	0.019	2.839	0.074	4.753
	DAY 2	08:00	56.733	253.938	2790.346	0.020	3.104	0.091	6.409
		13:00	44.917	233.019	2432.469	0.018	2.716	0.096	6.881
		16:30	60.199	287.278	2982.904	0.020	3.072	0.096	6.932
	DAY 3	08:00	64.061	242.134	3101.021	0.021	3.171	0.078	5.136
		13:00	60.720	303.382	3147.641	0.019	2.887	0.096	6.939
		16:30	58.477	314.656	3360.034	0.017	2.496	0.094	6.670
	DAY 4	08:00	58.178	268.104	3081.381	0.019	2.802	0.087	6.016
		13:00	51.532	293.085	2806.111	0.018	2.695	0.104	7.734
		16:30	61.705	265.745	3164.539	0.019	2.931	0.084	5.717

Table A - 3 Blood Monoamine Levels for Group 1 (E) – Smoke & Saline.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 17	DAY 1	08:00	81.972	282.026	3029.523	0.027	4.500	0.093	6.615
		13:00	67.576	271.720	2775.992	0.024	3.936	0.098	7.087
		16:30	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	DAY 2	08:00	84.268	297.139	3157.878	0.027	4.423	0.094	6.714
		13:00	72.023	282.208	3023.378	0.024	3.828	0.093	6.640
		16:30	95.287	324.030	3553.242	0.027	4.450	0.091	6.428
	DAY 3	08:00	76.136	237.223	3120.217	0.024	3.949	0.076	4.934
		13:00	85.515	378.935	3314.203	0.026	4.240	0.114	8.708
		16:30	90.967	383.889	3490.879	0.026	4.293	0.110	8.278
	DAY 4	08:00	33.514	94.408	1210.139	0.028	4.632	0.078	5.130
		13:00	81.363	291.008	3298.476	0.025	4.004	0.088	6.135
		16:30	80.902	348.074	3121.185	0.026	4.264	0.112	8.431
RABBIT 18	DAY 1	08:00	66.914	268.130	3039.241	0.022	3.453	0.088	6.135
		13:00	70.026	316.321	2978.705	0.024	3.763	0.106	7.906
		16:30	82.975	334.720	3282.869	0.025	4.130	0.102	7.489
	DAY 2	08:00	74.358	304.678	2973.658	0.025	4.074	0.102	7.538
		13:00	75.040	310.046	2906.914	0.026	4.242	0.107	7.952
		16:30	82.344	340.236	3296.241	0.025	4.069	0.103	7.613
	DAY 3	08:00	77.672	273.351	3075.121	0.025	4.127	0.089	6.201
		13:00	86.208	351.821	3447.556	0.025	4.074	0.102	7.497
		16:30	75.274	335.561	3214.476	0.023	3.744	0.104	7.728
	DAY 4	08:00	65.661	305.563	2832.651	0.023	3.695	0.108	8.071
		13:00	63.183	302.142	2896.176	0.022	3.412	0.104	7.722
		16:30	29.055	129.996	1237.275	0.023	3.758	0.105	7.795
RABBIT 19	DAY 1	08:00	75.516	267.945	2919.476	0.026	4.253	0.092	6.486
		13:00	66.398	286.100	2956.359	0.022	3.545	0.097	6.978
		16:30	67.769	278.179	3073.719	0.022	3.460	0.091	6.360
	DAY 2	08:00	68.172	274.560	2939.114	0.023	3.698	0.093	6.647
		13:00	75.157	267.051	2911.714	0.026	4.241	0.092	6.479
		16:30	75.369	298.356	3373.952	0.022	3.520	0.088	6.156
	DAY 3	08:00	74.229	283.220	3236.691	0.023	3.644	0.088	6.064
		13:00	80.171	284.952	3141.593	0.026	4.181	0.091	6.380
		16:30	28.817	101.455	1187.467	0.024	3.921	0.085	5.861
	DAY 4	08:00	68.396	302.616	3109.305	0.022	3.449	0.097	7.032
		13:00	47.646	180.865	2120.529	0.022	3.547	0.085	5.847
		16:30	76.168	272.739	3009.706	0.025	4.137	0.091	6.371
RABBIT 20	DAY 1	08:00	77.199	239.287	2976.773	0.026	4.267	0.080	5.363
		13:00	72.924	218.339	2660.105	0.027	4.574	0.082	5.530
		16:30	81.199	274.825	2915.187	0.028	4.665	0.094	6.731
	DAY 2	08:00	76.545	256.556	2768.105	0.028	4.624	0.093	6.575
		13:00	74.557	267.902	2790.229	0.027	4.430	0.096	6.903
		16:30	93.998	302.073	3383.368	0.028	4.651	0.089	6.240
	DAY 3	08:00	81.630	270.363	3152.457	0.026	4.259	0.086	5.893
		13:00	31.750	119.769	1283.777	0.025	4.017	0.093	6.635
		16:30	84.296	252.790	3176.686	0.027	4.392	0.080	5.283
	DAY 4	08:00	71.473	263.812	2954.747	0.024	3.905	0.089	6.240
		13:00	81.366	316.724	3072.084	0.026	4.381	0.103	7.601
		16:30	89.651	327.196	3520.394	0.025	4.170	0.093	6.600

Table A - 4 Blood Monoamine Levels for Group 2 (F) – Smoke & NAD.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 21	DAY 1	08:00	102.020	472.101	3316.397	0.031	5.269	0.142	11.468
		13:00	111.605	420.790	3182.776	0.035	6.163	0.132	10.469
		16:30	121.865	410.752	3462.724	0.035	6.189	0.119	9.130
	DAY 2	08:00	113.699	447.174	3379.941	0.034	5.867	0.132	10.478
		13:00	108.078	454.781	3325.924	0.032	5.629	0.137	10.915
		16:30	105.021	471.240	3364.803	0.031	5.363	0.140	11.241
	DAY 3	08:00	116.138	372.484	3373.112	0.034	6.031	0.110	8.323
		13:00	107.432	415.525	3320.301	0.032	5.600	0.125	9.773
		16:30	99.384	421.256	3242.624	0.031	5.246	0.130	10.243
RABBIT 22	DAY 1	08:00	74.800	357.018	3216.049	0.023	3.711	0.111	8.380
		13:00	86.219	373.435	3159.892	0.027	4.547	0.118	9.087
		16:30	76.061	354.667	3155.686	0.024	3.887	0.112	8.516
	DAY 2	08:00	80.447	384.533	3310.625	0.024	3.927	0.116	8.887
		13:00	91.554	361.202	3488.086	0.026	4.332	0.104	7.646
		16:30	81.201	441.699	3349.487	0.024	3.916	0.132	10.436
	DAY 3	08:00	82.873	342.506	3291.058	0.025	4.111	0.104	7.697
		13:00	88.983	335.815	3382.464	0.026	4.344	0.099	7.225
		16:30	82.520	376.931	3272.113	0.025	4.118	0.115	8.793
RABBIT 23	DAY 1	08:00	82.611	382.602	3112.944	0.027	4.392	0.123	9.552
		13:00	92.824	347.960	3325.419	0.028	4.678	0.105	7.752
		16:30	85.976	379.241	3308.709	0.026	4.277	0.115	8.736
	DAY 2	08:00	88.644	346.811	3256.030	0.027	4.535	0.107	7.937
		13:00	79.385	409.933	3243.206	0.024	3.964	0.126	9.896
		16:30	87.142	373.614	3358.885	0.026	4.269	0.111	8.402
	DAY 3	08:00	90.923	360.783	3411.491	0.027	4.416	0.106	7.863
		13:00	83.456	357.233	3362.418	0.025	4.036	0.106	7.911
		16:30	91.441	384.041	3424.475	0.027	4.426	0.112	8.492
RABBIT 24	DAY 1	08:00	90.352	347.152	3325.436	0.027	4.523	0.104	7.728
		13:00	79.849	382.207	3308.265	0.024	3.894	0.116	8.826
		16:30	86.004	339.525	3087.506	0.028	4.666	0.110	8.278
	DAY 3	08:00	82.922	394.003	3380.699	0.025	3.975	0.117	8.926
		13:00	92.362	444.140	3381.907	0.027	4.553	0.131	10.382
		16:30	90.142	360.101	3305.452	0.027	4.544	0.109	8.176

Table A - 5 Blood Monoamine Levels for Group 2 (G) – Smoke & NAD.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 25	DAY 1	08:00	113.768	423.691	2963.067	0.038	6.854	0.143	11.531
		13:00	108.805	476.605	3459.408	0.031	5.413	0.138	11.017
		16:30	73.337	246.968	2039.231	0.036	6.349	0.121	9.375
	DAY 2	08:00	97.911	410.395	3101.080	0.032	5.438	0.132	10.482
		13:00	92.702	325.879	2497.342	0.037	6.590	0.130	10.300
		16:30	86.466	315.261	2620.054	0.033	5.734	0.120	9.298
	DAY 3	08:00	87.021	321.964	2254.586	0.039	6.896	0.143	11.513
		13:00	89.864	320.485	2619.361	0.034	6.005	0.122	9.498
		16:30	83.703	308.778	2546.510	0.033	5.707	0.121	9.390
	DAY 4	08:00	76.482	294.824	2329.907	0.033	5.698	0.127	9.910
		13:00	114.864	417.165	3589.529	0.032	5.526	0.116	8.893
		16:30	98.749	340.176	2976.399	0.033	5.771	0.114	8.704
RABBIT 26	DAY 1	08:00	84.215	434.140	3379.291	0.025	4.056	0.128	10.101
		13:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		16:30	60.613	277.181	2241.232	0.027	4.497	0.124	9.628
	DAY 2	08:00	60.636	284.080	2466.127	0.025	3.987	0.115	8.792
		13:00	83.463	398.273	3188.967	0.026	4.316	0.125	9.748
		16:30	64.966	301.109	2496.910	0.026	4.284	0.121	9.324
	DAY 3	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	62.718	279.004	2376.474	0.026	4.362	0.117	9.010
		16:30	59.421	281.681	2157.798	0.028	4.600	0.131	10.305
	DAY 4	08:00	84.560	383.473	3122.471	0.027	4.505	0.123	9.543
		13:00	84.277	307.112	2978.495	0.028	4.757	0.103	7.602
		16:30	92.509	408.812	3258.806	0.028	4.776	0.125	9.803
RABBIT 27	DAY 1	08:00	72.080	314.334	2596.732	0.028	4.646	0.121	9.369
		13:00	83.273	363.534	2906.272	0.029	4.831	0.125	9.767
		16:30	74.768	293.798	2468.972	0.030	5.170	0.119	9.167
	DAY 2	08:00	60.814	300.845	2305.426	0.026	4.359	0.130	10.300
		13:00	94.298	406.791	3534.828	0.027	4.421	0.115	8.781
		16:30	90.084	312.257	3184.440	0.028	4.756	0.098	7.104
	DAY 3	08:00	77.536	287.800	2574.823	0.030	5.135	0.112	8.456
		13:00	71.908	266.231	2583.698	0.028	4.661	0.103	7.595
		16:30	72.872	258.917	2438.262	0.030	5.088	0.106	7.905
	DAY 4	08:00	86.496	349.396	3120.389	0.028	4.638	0.112	8.475
		13:00	93.230	336.957	2954.904	0.032	5.433	0.114	8.678
		16:30	97.647	324.681	3209.370	0.030	5.199	0.101	7.411
RABBIT 28	DAY 1	08:00	98.440	376.224	3050.808	0.032	5.582	0.123	9.593
		13:00	100.794	387.529	2853.810	0.035	6.216	0.136	10.822
		16:30	79.201	356.253	2585.999	0.031	5.241	0.138	11.016
	DAY 2	08:00	77.181	347.827	2548.076	0.030	5.171	0.137	10.892
		13:00	108.886	438.873	3178.139	0.034	5.996	0.138	11.048
		16:30	89.697	425.638	3081.681	0.029	4.926	0.138	11.051
	DAY 3	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	84.104	347.854	2780.987	0.030	5.161	0.125	9.767
		16:30	109.031	338.831	3140.993	0.035	6.090	0.108	8.071
	DAY 4	08:00	110.322	366.454	3180.401	0.035	6.085	0.115	8.795
		13:00	84.643	326.225	2774.419	0.031	5.217	0.118	9.028
		16:30	75.387	238.126	2215.680	0.034	5.947	0.107	8.032

Table A - 6 Blood Monoamine Levels for Group 3 (H) – Smoke & Bupropion.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 29	DAY 1	08:00	83.200	333.448	3242.400	0.026	4.210	0.103	7.575
		13:00	74.387	351.379	2903.787	0.026	4.201	0.121	9.365
		16:30	73.722	297.984	2822.936	0.026	4.304	0.106	7.843
	DAY 2	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	78.284	396.775	3241.070	0.024	3.897	0.122	9.505
		16:30	80.328	384.241	2994.793	0.027	4.451	0.128	10.084
	DAY 3	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	59.362	268.898	2572.278	0.023	3.674	0.105	7.743
		16:30	78.522	345.892	3000.486	0.026	4.316	0.115	8.801
	DAY 4	08:00	75.276	398.939	3241.873	0.023	3.703	0.123	9.567
		13:00	51.445	230.159	1938.278	0.027	4.393	0.119	9.142
		16:30	80.842	324.149	3081.229	0.026	4.330	0.105	7.808
RABBIT 30	DAY 1	08:00	79.624	363.605	2822.498	0.028	4.740	0.129	10.135
		13:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		16:30	45.576	198.893	1693.707	0.027	4.469	0.117	9.013
	DAY 2	08:00	88.328	355.671	3153.580	0.028	4.698	0.113	8.555
		13:00	85.953	367.649	3168.690	0.027	4.514	0.116	8.874
		16:30	84.841	361.691	2916.902	0.029	4.921	0.124	9.660
	DAY 3	08:00	88.988	354.813	3012.573	0.030	5.015	0.118	9.047
		13:00	84.203	415.544	3260.394	0.026	4.244	0.127	10.000
		16:30	71.772	303.484	2703.798	0.027	4.394	0.112	8.502
	DAY 4	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	87.556	341.815	3078.659	0.028	4.787	0.111	8.382
		16:30	85.196	369.634	3179.257	0.027	4.446	0.116	8.898
RABBIT 31	DAY 1	08:00	82.465	311.700	2348.938	0.035	6.172	0.133	10.517
		13:00	122.026	467.491	3553.976	0.034	6.011	0.132	10.403
		16:30	100.552	384.353	2969.493	0.034	5.913	0.129	10.195
	DAY 2	08:00	80.283	298.911	2517.582	0.032	5.503	0.119	9.141
		13:00	109.127	380.689	3468.568	0.031	5.415	0.110	8.257
		16:30	102.886	353.661	3039.250	0.034	5.911	0.116	8.908
	DAY 3	08:00	108.607	396.452	3134.678	0.035	6.076	0.126	9.904
		13:00	110.594	459.744	3262.377	0.034	5.921	0.141	11.327
		16:30	113.905	368.646	3268.138	0.035	6.119	0.113	8.557
	DAY 4	08:00	108.350	401.385	3404.765	0.032	5.490	0.118	9.058
		13:00	100.712	370.501	3122.875	0.032	5.578	0.119	9.132
		16:30	100.181	338.137	2824.065	0.035	6.248	0.120	9.240
RABBIT 32	DAY 1	08:00	111.052	341.556	3245.001	0.034	5.988	0.105	7.813
		13:00	83.843	313.023	2622.942	0.032	5.519	0.119	9.201
		16:30	103.437	371.865	3098.337	0.033	5.814	0.120	9.268
	DAY 2	08:00	98.933	338.634	2950.758	0.034	5.844	0.115	8.750
		13:00	105.837	363.642	3454.116	0.031	5.244	0.105	7.816
		16:30	79.313	283.538	2640.509	0.030	5.119	0.107	8.023
	DAY 3	08:00	86.758	294.318	2679.890	0.032	5.604	0.110	8.264
		13:00	86.614	296.933	2888.509	0.030	5.108	0.103	7.571
		16:30	98.922	348.551	2928.322	0.034	5.896	0.119	9.170
	DAY 4	08:00	112.958	423.748	3600.254	0.031	5.397	0.118	9.039
		13:00	103.126	326.379	3139.239	0.033	5.703	0.104	7.686
		16:30	102.036	344.220	3361.088	0.030	5.185	0.102	7.533

Table A - 7 Blood Monoamine Levels for Group 3 (I) – Smoke & Bupropion.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 33	DAY 1	08:00	90.527	391.917	3117.575	0.029	4.911	0.126	9.829
		13:00	78.676	315.724	2812.442	0.028	4.690	0.112	8.503
		16:30	80.097	386.587	3186.495	0.025	4.101	0.121	9.396
	DAY 2	08:00	92.572	353.134	2944.574	0.031	5.410	0.120	9.259
		13:00	75.817	417.410	3053.337	0.025	4.038	0.137	10.912
		16:30	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	DAY 3	08:00	88.353	327.537	3075.477	0.029	4.847	0.106	7.936
		13:00	88.394	412.065	3256.147	0.027	4.519	0.127	9.911
		16:30	90.355	383.467	3273.052	0.028	4.614	0.117	8.986
	DAY 4	08:00	73.907	306.450	2950.208	0.025	4.084	0.104	7.677
		13:00	92.994	422.494	3478.458	0.027	4.433	0.121	9.410
		16:30	123.987	579.151	4389.577	0.028	4.747	0.132	10.442
RABBIT 34	DAY 1	08:00	108.229	476.453	3855.386	0.028	4.711	0.124	9.619
		13:00	83.437	331.900	2942.869	0.028	4.769	0.113	8.555
		16:30	81.298	351.469	3003.003	0.027	4.503	0.117	8.974
	DAY 2	08:00	103.587	416.001	3413.821	0.030	5.182	0.122	9.449
		13:00	97.168	357.301	3165.951	0.031	5.255	0.113	8.562
		16:30	90.034	363.613	2977.619	0.030	5.160	0.122	9.474
	DAY 3	08:00	76.474	323.138	2743.014	0.028	4.671	0.118	9.050
		13:00	66.628	334.474	2467.838	0.027	4.488	0.136	10.796
		16:30	101.908	485.431	3751.470	0.027	4.522	0.129	10.192
	DAY 4	08:00	102.880	470.756	3498.923	0.029	4.987	0.135	10.699
		13:00	84.802	399.783	3019.348	0.028	4.714	0.132	10.488
		16:30	112.288	454.805	3759.997	0.030	5.083	0.121	9.360
RABBIT 35	DAY 1	08:00	97.494	344.607	3521.577	0.028	4.630	0.098	7.084
		13:00	87.951	332.541	3272.138	0.027	4.463	0.102	7.456
		16:30	86.181	290.752	2936.277	0.029	4.976	0.099	7.199
	DAY 2	08:00	90.034	334.085	3239.239	0.028	4.653	0.103	7.605
		13:00	83.401	298.049	2892.316	0.029	4.869	0.103	7.596
		16:30	91.908	400.675	3304.892	0.028	4.656	0.121	9.388
	DAY 3	08:00	113.226	416.264	3711.087	0.031	5.217	0.112	8.494
		13:00	51.005	224.503	1998.846	0.026	4.180	0.112	8.509
		16:30	102.161	452.231	4009.824	0.025	4.172	0.113	8.555
	DAY 4	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	82.144	290.053	2854.377	0.029	4.857	0.102	7.455
		16:30	99.943	374.811	3241.552	0.031	5.284	0.116	8.835
RABBIT 36	DAY 1	08:00	134.190	382.276	3547.983	0.038	6.735	0.108	8.059
		13:00	97.250	355.414	2953.444	0.033	5.719	0.120	9.299
		16:30	98.232	396.563	2789.699	0.035	6.193	0.142	11.449
	DAY 2	08:00	105.866	452.669	3173.400	0.033	5.809	0.143	11.497
		13:00	121.956	387.444	3400.096	0.036	6.330	0.114	8.670
		16:30	110.266	340.393	3191.856	0.035	6.055	0.107	7.950
	DAY 3	08:00	121.953	388.011	3362.064	0.036	6.414	0.115	8.814
		13:00	105.506	350.701	2999.828	0.035	6.185	0.117	8.961
		16:30	129.414	392.448	3459.470	0.037	6.649	0.113	8.620
	DAY 4	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	121.870	373.653	3228.574	0.038	6.720	0.116	8.846
		16:30	151.372	510.906	4440.928	0.034	5.959	0.115	8.778

Table A - 8 Blood Monoamine Levels for Group 4 (J) – Vehicle & NAD.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 37	DAY 1	08:00	64.441	255.179	4055.097	0.016	2.182	0.063	3.643
		13:00	54.480	209.952	3188.636	0.017	2.430	0.066	3.930
		16:30	48.118	203.118	3078.036	0.016	2.128	0.066	3.945
	DAY 2	08:00	72.489	293.016	4386.098	0.017	2.314	0.067	4.025
		13:00	63.000	213.110	3595.022	0.018	2.521	0.059	3.284
		16:30	46.909	196.863	2996.761	0.016	2.132	0.066	3.915
	DAY 3	08:00	49.011	214.248	3057.064	0.016	2.211	0.070	4.348
		13:00	56.074	201.063	3251.627	0.017	2.463	0.062	3.535
		16:30	43.475	169.452	2576.572	0.017	2.385	0.066	3.923
RABBIT 38	DAY 1	08:00	42.071	162.433	2848.553	0.015	1.949	0.057	3.061
		13:00	41.874	143.386	2763.232	0.015	2.028	0.052	2.556
		16:30	‡ Rabbit died						
	DAY 2	08:00							
		13:00							
		16:30							
	DAY 3	08:00							
		13:00							
		16:30							
RABBIT 39	DAY 1	08:00	50.853	280.129	3766.595	0.014	1.685	0.074	4.771
		13:00	45.438	202.262	3115.099	0.015	1.911	0.065	3.840
		16:30	51.526	256.378	3711.888	0.014	1.764	0.069	4.248
	DAY 2	08:00	48.014	219.932	3657.603	0.013	1.608	0.060	3.367
		13:00	43.960	232.959	3304.016	0.013	1.644	0.071	4.390
		16:30	41.208	206.302	3157.839	0.013	1.591	0.065	3.880
	DAY 3	08:00	40.668	194.498	3036.028	0.013	1.663	0.064	3.755
		13:00	43.196	220.930	3265.505	0.013	1.629	0.068	4.109
		16:30	32.958	168.889	2494.469	0.013	1.625	0.068	4.114
RABBIT 40	DAY 1	08:00	49.391	219.289	3449.616	0.014	1.855	0.064	3.706
		13:00	46.181	231.231	3279.361	0.014	1.806	0.071	4.390
		16:30	39.776	145.948	2644.448	0.015	2.005	0.055	2.881
	DAY 2	08:00	44.698	166.391	2942.980	0.015	2.035	0.057	3.014
		13:00	39.994	189.406	2884.002	0.014	1.761	0.066	3.914
		16:30	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	DAY 3	08:00	47.307	211.401	3116.856	0.015	2.033	0.068	4.126
		13:00	46.713	199.673	3149.010	0.015	1.962	0.063	3.690
		16:30	44.912	168.961	2980.936	0.015	2.010	0.057	3.028

Table A - 9 Blood Monoamine Levels for Group 4 (K) – Vehicle & NAD.

			DOPAC	DA	Int. Std	DOPAC/IS	[DOPAC]	DA/IS	[DA]
RABBIT 41	DAY 1	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	58.975	221.712	3432.961	0.017	2.449	0.065	3.806
		16:30	40.412	138.546	2503.421	0.016	2.234	0.055	2.896
	DAY 2	08:00	43.005	219.012	3269.891	0.013	1.613	0.067	4.042
		13:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		16:30	46.981	161.707	2734.874	0.017	2.449	0.059	3.269
	DAY 3	08:00	48.504	163.789	2994.016	0.016	2.246	0.055	2.833
		13:00	58.637	233.409	3422.777	0.017	2.439	0.068	4.162
		16:30	48.269	186.556	3168.628	0.015	2.045	0.059	3.244
	DAY 4	08:00	46.049	174.360	2756.033	0.017	2.351	0.063	3.676
		13:00	47.514	191.664	2990.458	0.016	2.181	0.064	3.758
		16:30	0.000	0.000	0.000	0.000	0.000	0.000	0.000
RABBIT 42	DAY 1	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	50.627	184.926	3278.513	0.015	2.088	0.056	3.001
		16:30	51.522	231.979	3534.503	0.015	1.909	0.066	3.910
	DAY 2	08:00	40.178	126.633	2521.129	0.016	2.191	0.050	2.392
		13:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		16:30	43.168	166.538	3079.688	0.014	1.792	0.054	2.771
	DAY 3	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	46.357	157.809	3201.118	0.014	1.889	0.049	2.300
		16:30	67.772	232.424	4594.289	0.015	1.945	0.051	2.428
	DAY 4	08:00	40.128	153.975	2855.951	0.014	1.799	0.054	2.755
		13:00	58.431	195.785	3749.237	0.016	2.118	0.052	2.588
		16:30	0.000	0.000	0.000	0.000	0.000	0.000	0.000
RABBIT 43	DAY 1	08:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		13:00	42.589	190.216	3292.182	0.013	1.568	0.058	3.136
		16:30	42.667	211.027	3394.239	0.013	1.492	0.062	3.569
	DAY 2	08:00	42.477	221.840	3626.142	0.012	1.314	0.061	3.471
		13:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		16:30	35.100	150.765	2739.597	0.013	1.542	0.055	2.865
	DAY 3	08:00	34.839	172.009	2980.334	0.012	1.309	0.058	3.130
		13:00	46.814	219.707	3620.568	0.013	1.567	0.061	3.422
		16:30	56.751	272.665	4757.003	0.012	1.359	0.057	3.091
	DAY 4	08:00	36.747	170.877	2969.559	0.012	1.451	0.058	3.113
		13:00	42.946	197.140	3415.071	0.013	1.493	0.058	3.131
		16:30	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table A - 10 Summary of Blood Monoamine Levels for Group 1.

		DOPAC			DA		
		08:00	13:00	16:30	08:00	13:00	16:30
[AVG]	DAY 1	4.11828	3.60966	3.71075	6.14971	6.36375	6.10142
	DAY 2	3.86584	3.78125	3.81613	6.51798	6.57917	6.19469
	DAY 3	3.73957	3.81934	3.67880	6.20376	6.65333	6.47740
	DAY 4	3.56945	3.55448	3.69394	6.29633	6.67111	6.80916
ST DEV	DAY 1	0.45753	0.59974	0.72217	0.56226	1.18059	0.96727
	DAY 2	0.61456	0.64172	0.58530	0.74579	0.86443	0.95565
	DAY 3	0.41075	0.51157	0.64475	1.07754	1.24849	1.13575
	DAY 4	0.59453	0.53090	0.52847	0.99624	0.95886	1.19856
RSD	DAY 1	11.11	16.61	19.46	9.14	18.55	15.85
	DAY 2	15.90	16.97	15.34	11.44	13.14	15.43
	DAY 3	10.98	13.39	17.53	17.37	18.76	17.53
	DAY 4	16.66	14.94	14.31	15.82	14.37	17.60
n	DAY 1	4	7	6	4	7	6
	DAY 2	6	7	7	6	7	7
	DAY 3	7	7	7	7	7	7
	DAY 4	7	7	7	7	7	7

Table A - 11 Summary of Blood Monoamine Levels for Group 2.

		DOPAC			DA		
		08:00	13:00	16:30	08:00	13:00	16:30
[AVG]	DAY 1	4.87929	5.10588	5.03463	9.71546	9.67707	9.23076
	DAY 2	4.75493	5.03547	4.74962	9.68120	9.76203	9.55098
	DAY 3	5.09387	4.84029	4.97738	8.79602	8.89506	8.92184
	DAY 4	5.23127	5.23329	5.42329	9.18089	8.55033	8.48717
ST DEV	DAY 1	1.00043	0.86439	0.88147	1.33146	1.19155	0.84885
	DAY 2	0.74777	1.01873	0.64802	1.12454	1.20639	1.48954
	DAY 3	1.16683	0.68411	0.67875	1.40119	1.16906	0.95532
	DAY 4	0.78010	0.34297	0.53651	0.66077	0.64844	1.02376
RSD	DAY 1	20.50	16.93	17.51	13.70	12.31	9.20
	DAY 2	15.73	20.23	13.64	11.62	12.36	15.60
	DAY 3	22.91	14.13	13.64	15.93	13.14	10.71
	DAY 4	14.91	6.55	9.89	7.20	7.58	12.06
n	DAY 1	8	7	8	8	7	8
	DAY 2	7	7	7	7	7	7
	DAY 3	6	8	8	6	8	8
	DAY 4	4	4	4	4	4	4

Table A - 12 Summary of Blood Monoamine Levels for Group 3.

		DOPAC			DA		
		08:00	13:00	16:30	08:00	13:00	16:30
[AVG]	DAY 1	5.26205	5.05327	5.03432	8.82897	8.96898	9.16720
	DAY 2	5.29981	4.94519	5.18200	9.17933	8.77391	9.06957
	DAY 3	5.40626	4.78982	5.08528	8.78684	9.35242	8.92277
	DAY 4	4.73206	5.14814	5.16025	9.20816	8.81770	8.86184
ST DEV	DAY 1	0.90475	0.69101	0.82214	1.33203	0.91778	1.30996
	DAY 2	0.48354	0.79601	0.60226	1.19066	1.05182	0.81834
	DAY 3	0.65126	0.87877	0.97217	0.64141	1.38080	0.56432
	DAY 4	0.79992	0.79714	0.67760	1.08924	0.97642	0.90817
RSD	DAY 1	17.19	13.67	16.33	15.09	10.23	14.29
	DAY 2	9.12	16.10	11.62	12.97	11.99	9.02
	DAY 3	12.05	18.35	19.12	7.30	14.76	6.32
	DAY 4	16.90	15.48	13.13	11.83	11.07	10.25
n	DAY 1	8	7	8	8	7	8
	DAY 2	7	8	7	7	8	7
	DAY 3	7	8	8	7	8	8
	DAY 4	5	8	8	5	8	8

Table A - 13 Summary of Blood Monoamine Levels for Group 4.

		DOPAC			DA		
		08:00	13:00	16:30	08:00	13:00	16:30
[AVG]	DAY 1	1.91755	2.03995	1.92190	3.79537	3.52275	3.57466
	DAY 2	1.84574	1.97547	1.90139	3.38520	3.86245	3.34005
	DAY 3	1.89242	1.99129	1.89495	3.63826	3.53649	3.30438
	DAY 4	1.86729	1.93063		3.18134	3.15889	
ST DEV	DAY 1	0.20702	0.32036	0.26700	0.71203	0.63999	0.57361
	DAY 2	0.39180	0.47572	0.38404	0.62824	0.55492	0.54240
	DAY 3	0.39962	0.38623	0.35745	0.64463	0.67583	0.62168
	DAY 4	0.45376	0.38030		0.46448	0.58533	
RSD	DAY 1	10.80	15.70	13.89	18.76	18.17	16.05
	DAY 2	21.23	24.08	20.20	18.56	14.37	16.24
	DAY 3	21.12	19.40	18.86	17.72	19.11	18.81
	DAY 4	24.30	19.70		14.60	18.53	
n	DAY 1	4	7	6	4	7	6
	DAY 2	6	3	5	6	3	5
	DAY 3	5	6	6	5	6	6
	DAY 4	3	3	0	3	3	0

Table A - 14 Standard Curve for Validation of a Method for Determination of Blood DOPAC Levels.

[DOPAC] ng / ml	AUC 1	AUC 2	AUC 3	AUC 4	Average	STDEV	RSD
5	0.039078	0.037358	0.024145	0.030698	0.032820	0.006819	20.778480
10	0.056312	0.056355	0.046771	0.047048	0.051622	0.005442	10.542410
20	0.117204	0.103889	0.108274	0.115379	0.111187	0.006205	5.581135
30	0.174234	0.153451	0.150946	0.149229	0.156965	0.011642	7.417233
50	0.264375	0.257477	0.263750	0.255329	0.260233	0.004516	1.735191
100	0.544546	0.518404	0.473495	0.488509	0.506239	0.031633	6.248631
200	0.999171	0.991306	0.970042	0.958979	0.979875	0.018586	1.896788
300	1.440315	1.476679	1.455726	1.414610	1.446833	0.026145	1.807047
500	2.441729	2.454241	2.434870	2.413036	2.435969	0.017264	0.708726

Table A - 15 Standard Curve for Validation of a Method for Determination of Blood DA Levels.

[DA] ng / ml	AUC 1	AUC 2	AUC 3	AUC 4	Average	STDEV	RSD
5	0.000000	0.000000	0.101951	0.097900	0.099926	0.002864	0.286449
10	0.131461	0.124419	0.102110	0.108254	0.116561	0.013682	1.368205
20	0.221137	0.247537	0.220888	0.241497	0.232765	0.013793	1.379291
30	0.375169	0.322215	0.000000	0.000000	0.450787	0.119910	11.991000
50	0.912543	0.870227	0.000000	0.000000	0.693265	0.229426	22.942600
100	1.114286	1.062452	1.112902	1.166656	1.114074	0.042548	4.254847
200	2.041677	2.083064	1.980039	1.967957	2.018184	0.053973	5.397261
300	3.046551	3.145540	0.000000	0.000000	3.406085	0.069996	6.999579
500	5.317585	5.392110	5.155010	5.075424	5.235032	0.145335	14.533490

5.2. Raw Data on Brain Sample Analysis

Table A - 16 Standard Curve for Determination of Brain Monoamine Levels.

		DOPAC	5HIAA	DA	IS	5-HT
Retention time (min)		7.3	10.2	12.3	16.5	26.8
1.0	ng / ml	100.2232	42.47083	42.02099	486.0203	
2.5	ng / ml	41.24884	89.47989	98.65660	475.1859	63.05418
5.0	ng / ml	78.65134	153.3945	228.0736	501.3269	127.2847
10	ng / ml	153.5160	205.4692	378.8615	516.5635	233.1022
25	ng / ml	334.5018	412.7904	913.9016	513.3981	584.2418
50	ng / ml	616.2969	685.7211	1736.096	506.4636	1197.782
100	ng / ml	1190.225	1359.376	3373.959	471.8857	2360.927
200	ng / ml	2370.806	2671.222	6624.604	460.6105	4722.203
300	ng / ml	3328.014	3795.563	9523.158	464.0355	6920.153
		DOPAC / IS	5HIAA / IS	DA / IS		5-HT / IS
1.0	ng / ml	0.206212	0.087385	0.086459		
2.5	ng / ml	0.086806	0.188305	0.207617		0.132694
5.0	ng / ml	0.156886	0.305977	0.454940		0.253896
10	ng / ml	0.297187	0.397762	0.733427		0.451256
25	ng / ml	0.651545	0.804036	1.780103		1.137990
50	ng / ml	1.216863	1.353940	3.427879		2.364992
100	ng / ml	2.522274	2.880731	7.149950		5.003174
200	ng / ml	5.147094	5.799308	14.38222		10.25205
300	ng / ml	7.171895	8.179467	20.52248		14.91298
	M	0.024153	0.027330	0.069230		0.049863
	C	0.078533	0.115945	0.081537		0.471389
	R	0.999026	0.999336	0.999584		0.999774

Table A - 17 Brain Monoamine Levels.

Peak Areas						
Group	Rabbit	DOPAC	5HIAA	DA	IS	5-HT
Retention Time (min)		7.3	10.2	12.3	16.5	26.8
1	13	1207.86658	2405.22510	524.57025	508.59171	1075.09375
	14	920.44019	2289.91504	453.99072	488.97775	760.83575
	15	525.60181	600.28253	253.29393	479.00336	298.13202
	18	1759.25452	4250.38330	521.12769	469.37094	1039.97485
	19	1189.20667	2885.43457	368.06400	442.42035	873.89691
	20	862.67700	2522.20483	486.70248	509.94144	961.50598
2	21	850.64490	1973.23792	360.42444	463.66721	664.51398
	22	768.61365	2996.77661	646.76813	501.78201	1434.95068
	23	508.28079	1993.21143	358.61746	546.52295	867.49841
	24	978.13312	2885.99365	500.89279	487.37958	991.60175
	25	1026.02271	2011.65027	571.24359	471.24191	1046.80896
	26	1226.40747	3298.49268	950.54126	430.36914	1778.12378
	27	1051.91614	5797.73047	1327.58496	633.51520	2697.31250
	28	601.97406	3241.04663	735.42694	446.43805	1825.38721
3	29	772.59393	2466.84912	433.65268	473.19144	912.28339
	30	941.93591	2645.40381	579.16162	558.06769	951.92426
	32	1160.64185	4892.88672	951.94446	493.80832	2288.88403
	33	1143.15405	4306.59814	615.41992	504.16324	1303.26953
	34	1084.00073	4819.27393	944.60602	495.72461	2238.21265
	35	1003.31311	2610.78174	572.07037	534.08118	906.61182
	36	855.87946	4502.85645	790.04468	459.18762	1953.30298
4	37	664.15106	2527.87646	459.51096	509.66064	848.77393
	38	730.79346	2108.69360	546.83087	509.73120	949.10522
	39	959.25024	3956.95825	674.60004	495.63370	1556.52637
	40	1147.14062	3350.79663	794.45917	504.52573	825.90021
	41	965.84937	3714.55542	729.33301	389.62695	1583.33777
	42	951.36725	2104.43677	343.07953	481.65152	553.78961
	43	691.98053	2332.63086	490.94879	461.16553	1057.76318

Peak Area / Internal Standard					
Group	Rabbit	DOPAC / IS	5HIAA / IS	DA / IS	5-HT / IS
1	13	2.37492	4.72919	1.03142	2.11386
	14	1.88238	4.68307	0.92845	1.55597
	15	1.09728	1.25319	0.52879	0.62240
	18	3.74811	9.05549	1.11027	2.21568
	19	2.68796	6.52193	0.83193	1.97526
	20	1.69172	4.94607	0.95443	1.88552
2	21	1.83460	4.25572	0.77733	1.43317
	22	1.53177	5.97227	1.28894	2.85971
	23	0.93003	3.64708	0.65618	1.58730
	24	2.00692	5.92145	1.02773	2.03456
	25	2.17727	4.26883	1.21221	2.22138
	26	2.84966	7.66433	2.20867	4.13162
	27	1.66044	9.15168	2.09559	4.25769
	28	1.34839	7.25979	1.64732	4.08878
3	29	1.63273	5.21322	0.91644	1.92794
	30	1.68785	4.74029	1.03780	1.70575
	32	2.35039	9.90847	1.92776	4.63517
	33	2.26743	8.54207	1.22068	2.58501
	34	2.18670	9.72168	1.90551	4.51503
	35	1.87858	4.88836	1.07113	1.69752
	36	1.86390	9.80614	1.72053	4.25382
4	37	1.30312	4.95992	0.90160	1.66537
	38	1.43368	4.13687	1.07278	1.86197
	39	1.93540	7.98363	1.36109	3.14048
	40	2.27370	6.64148	1.57467	1.63698
	41	2.47891	9.53362	1.87188	4.06373
	42	1.97522	4.36921	0.71230	1.14977
	43	1.50050	5.05812	1.06458	2.29367

Table A - 18 Brain Monoamine Levels (continued).

Concentrations (ng / g wet mass)						
Group	Rabbit	Wet mass (g)	DOPAC	5HIAA	DA	5-HT
1	13	0.03877	2452.351	4353.812	353.897	1104.620
	14	0.03914	1908.142	4269.539	312.551	810.781
	15	0.01839	2293.602	2262.725	351.300	716.281
	18	0.06786	2238.901	4820.153	218.973	660.926
	19	0.05461	1978.358	4292.132	198.482	733.756
	20	0.06795	982.940	2600.926	185.556	563.446
2	21	0.04267	1703.929	3549.878	235.539	686.486
	22	0.05112	1177.003	4191.732	341.166	1127.841
	23	0.03588	982.565	3600.981	231.339	901.809
	24	0.04560	1750.905	4658.375	299.721	904.590
	25	0.05879	1478.046	2584.672	277.803	764.821
	26	0.07698	1490.430	3587.861	399.134	1077.472
	27	0.09490	690.158	3483.828	306.554	900.425
	28	0.08137	646.136	3212.381	277.953	1008.873
3	29	0.04385	1467.469	4253.314	275.024	892.348
	30	0.05545	1201.637	3051.463	249.103	626.003
	32	0.09257	1016.116	3870.650	288.083	1004.163
	33	0.06283	1442.416	4907.047	261.886	830.713
	34	0.08448	1033.198	4160.407	311.865	1072.051
	35	0.05419	1375.298	3222.393	263.779	637.538
	36	0.09108	811.590	3892.859	259.930	937.345
4	37	0.03589	1412.701	4938.417	330.049	944.805
	38	0.04548	1233.672	3234.932	314.821	831.529
	39	0.07330	1048.841	3927.376	252.148	862.722
	40	0.04755	1911.392	5021.399	453.576	701.254
	41	0.07987	1244.308	4314.389	323.784	1021.584
	42	0.03919	2003.793	3971.060	232.484	603.669
	43	0.05965	986.990	3031.566	238.049	777.890

Average Concentrations (ng / g wet mass) and Standard Deviations

		DOPAC	5HIAA	DA	5-HT
Average	1	2174.2707	3766.5477	270.1264	764.9684
	2	1430.4798	3608.7134	296.1511	921.5399
	3	1192.5320	3908.3045	272.8101	857.1660
	4	1475.7845	4062.7344	306.4159	820.4932
Standard deviation	1	226.3581	1058.7124	77.8565	185.6032
	2	299.3931	617.2511	55.1557	149.0137
	3	249.3810	630.6585	21.1829	172.1543
	4	391.6647	766.4290	77.1058	141.9165
Relative standard deviation	1	10.4108	28.1083	28.8222	24.2629
	2	20.9296	17.1045	18.6242	16.1701
	3	20.9119	16.1364	7.7647	20.0841
	4	26.5394	18.8649	25.1638	17.2965

5.3. Weight

Table A - 19 The weight and percentage variation in weight (g) from day 1 to day 31.

Rabbit	Day 1	Day 27		Day 31	
13	2065	2663	(+28.96 %)	2681	(+0.68 %)
14	2071	2521	(+21.73 %)	2577	(+2.22 %)
15	2104	2415	(+14.78 %)	2507	(+3.81 %)
16	1825	2432	(+33.26 %)	2462	(+1.23 %)
17	2112	2328	(+10.21 %)	2536	(+8.93 %)
18	1491	1677	(+12.51 %)	1814	(+8.15 %)
19	1460	1562	(+6.99 %)	1751	(+12.12 %)
20	1877	2158	(+14.97 %)	2425	(+12.37 %)
				Average 6.19 %	
21	1343	1996	(+48.60 %)	2015	(+0.95 %)
22	1717	2280	(+32.83 %)	2247	(-1.45 %)
23	1515	2262	(+49.35 %)	2309	(+2.06 %)
24	1422	2002	(+40.79 %)	2048	(+2.26 %)
25	1566	2281	(+45.62 %)	2334	(+2.34 %)
26	1728	2149	(+24.38 %)	2203	(+2.52 %)
27	1881	2355	(+25.19 %)	2333	(-0.92 %)
28	1345	2076	(+54.29 %)	2129	(+2.57 %)
				Average 1.29 %	
29	1926	2270	(+17.89 %)	2397	(+5.61 %)
30	2282	2600	(+13.96 %)	2614	(+0.55 %)
31	1812	2398	(+32.35 %)	2511	(+4.73 %)
32	1688	2294	(+35.86 %)	2360	(+2.87 %)
33	2232	2505	(+12.23 %)	2740	(+9.38 %)
34	2223	2634	(+18.49 %)	2619	(-0.59 %)
35	2311	2641	(+14.29 %)	2702	(+2.31 %)
36	2251	2659	(+18.16 %)	2794	(+5.06 %)
Average 26.15 %				Average 3.74 %	
38	2219	2783	(+25.42 %)	2641	(-5.09 %)
39	2306	2805	(+21.66 %)	2838	(+1.17 %)
40	1697	2302	(+35.68 %)	2211	(-3.97 %)
41	1882	2538	(+34.84 %)	2489	(-1.95 %)
42	2124	2602	(+22.52 %)	2306	(-11.36 %)
43	1814	2349	(+29.47 %)	2362	(+0.55 %)
Average 28.27 %				Average -3.44 %	

5.4. Competitive inhibition of MAO in Baboon Liver Mitochondria by NAD

Table A - 20 Calibration Curve.

[Aldehyde] μM	Peak Height
6.25	17.022
12.5	28.95
25	71.32
50	107.58
100	242.75

Table A - 21 MAO inhibition by NAD in baboon liver mitochondria.

[Inhibitor] μM	[Substrate] μM	Peak Height	[Aldehyde] nmol / 500 μL	Rate nmol / min-mg	1 / V	1 / [S]
0 μM	125	18.02	3.46	5.76	0.174	0.0080
	250	29.06	5.78	9.64	0.104	0.0040
	500	43.99	8.93	14.89	0.067	0.0020
	750	50.87	10.38	17.30	0.058	0.0013
100 μM	125	19.72	3.81	6.36	0.157	0.0080
	250	30.13	6.01	10.02	0.100	0.0040
	500	49.077	10.00	16.67	0.060	0.0020
	750	52.71	10.77	17.95	0.056	0.0013
200 μM	125	17.73	3.40	5.66	0.177	0.0080
	250	31.02	6.20	10.33	0.097	0.0040
	500	45.97	9.35	15.58	0.064	0.0020
	750	57.32	11.74	19.57	0.051	0.0013
400 μM	125	17.16	3.28	5.46	0.183	0.0080
	250	28.04	5.57	9.28	0.108	0.0040
	500	41.15	8.33	13.89	0.072	0.0020
	750	53.66	10.97	18.28	0.055	0.0013

5.5. Bovine Liver Mitochondria Calibration Curve

Table A - 22 Calibration Curve.

[Aldehyde] μM	Peak Height
6.25	28739
12.5	55474
25	109088
50	215337

Table A - 23 MAO inhibition by NAD in bovine liver mitochondria.

[Inhibitor] μM	[Substrate] μM	Peak Height	[Aldehyde] nmol / 500 μL	Rate nmol / min·mg	1 / V	1 / [S]
0	125	81918	7.902546	13.17091	0.075925	0.008
	250	110713	10.76135	17.93558	0.055755	0.004
	500	143279	13.99454	23.32423	0.042874	0.002
	750	157976	15.45368	25.75613	0.038826	0.001
1000	125	76166	7.33148	12.21913	0.081839	0.008
	250	68877	6.607819	11.01303	0.090802	0.004
	500	150807	14.74193	24.56988	0.0407	0.002
	750	161544	15.80791	26.34652	0.037956	0.001
2000	125	72027	6.920555	11.53426	0.086698	0.008
	250	105564	10.25015	17.08358	0.058536	0.004
	500	150274	14.68901	24.48169	0.040847	0.002
	750	161068	15.76065	26.26775	0.038069	0.001
4000	125	70516	6.770541	11.28424	0.088619	0.008
	250	107598	10.45209	17.42014	0.057405	0.004
	500	130263	12.7023	21.17049	0.047236	0.002
	750	149245	14.58685	24.31142	0.041133	0.001