AMERICAN UNIVERSITY OF BEIRUT

COMPARATIVE STUDY ON BLOCKADE OF THE NOREPINEPHRINE TRANSPORTER, UPTAKE-1BY DRUGS OF DIFFERENT CHEMICAL CLASSES AND THE ROLE OF NITRIC OXIDE IN THE BLOCKADE.

by

CATHERINA NICOLAS EL-KHOURY

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Pharmacology and Toxicology
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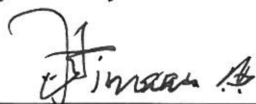

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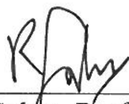

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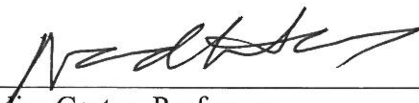

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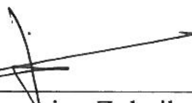

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AN ABSTRACT OF THE THESIS OF

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Title: Comparative study on blockade of the norepinephrine transporter, uptake-1 by drugs of different chemical classes and the role of nitric oxide in the blockade.

Evidence on the role of nitric oxide in modulating the activity of the norepinephrine transporter known as uptake-1 is scarce. The few studies available in the literature provide conflicting evidence on the subject. In preliminary studies in the laboratory showed that fresh synthesis of nitric oxide is needed for the transport activity of uptake-1, in postganglionic sympathetic nerve terminals, of two major substrates: norepinephrine, a direct agonist of the adrenergic receptors, and tyramine which is an indirectly acting sympathomimetic amine that enters across uptake-1 to the sympathetic nerve terminals to release norepinephrine from the adrenergic vesicles. The blocking effect of cocaine, atomoxetine and reboxetine on uptake-1 and its dependence on fresh synthesis of nitric oxide were also studied.

In this study, we explored the role of fresh synthesis of nitric oxide in the blockade of uptake-1 by methylphenidate (1.52±0.4 mg/kg) and imipramine (4± 0.7 mg/kg), the therapeutic counterparts of the experimental blocker of uptake-1 cocaine, in Sprague-Dawley rats in which mean arterial pressure was measured. Synthesis of nitric oxide was blocked by nitro-L-arginine (L-NNA) and the rise in mean arterial pressure it induces was restored to starting level by an infusion of nitroglycerin, a nitric oxide donor. Norepinephrine (0.05, 0.1, 0.2 µg) showed potentiation of its pressor effect after methylphenidate by 56± 11%, 40± 6% and 34 ± 6% respectively. Treatment with L-NNA and an infusion of nitroglycerin did not further change the pressor effect of norepinephrine. The pressor effect of tyramine (0.025, 0.05, 0.1 mg) was reduced by 85 ± 2%, 83 ± 2% and 78 ± 2% after methylphenidate and restored by 650 ± 106%, 472 ± 86% and 424 ± 129% after treatment with L-NNA and an infusion of nitroglycerin. The pressor effect of methoxamine, not a substrate of uptake-1, was not affected by either pretreatment with methylphenidate, or L-NNA and nitroglycerin. Norepinephrine showed potentiation of its pressor effect after imipramine by 86 ± 9%, 75 ± 6% and 60 ± 5% respectively. Treatment with L-NNA and an infusion of nitroglycerin did not further change the pressor effect of norepinephrine. The pressor effect of tyramine was reduced by 85 ± 4%, 84 ± 3% and 81 ± 3% respectively after imipramine and restored by 641 ± 206%, 409 ± 123% and 233 ± 44% respectively after treatment with L-NNA and an infusion of nitroglycerin.

It is concluded that the blocking effect of methylphenidate and imipramine on uptake-1 is confirmed and this blockade is significantly dependent on the fresh synthesis of nitric oxide.

CONTENTS

ACKNOWLEDGEMENTS…………………………………… v

ABSTRACT……………………………………………… vi

LIST OF TABLES………..……………………………… x

LIST OF FIGURES……………………………………… xi

Chapter

I. INTRODUCTION……………………………………….…… 1

A. The norepinephrine transporter (uptake-1)…………………………..….. 1

1. General overview………..………………………….……………….. 1
2. Transporter structure……….……………………….……………..... 2
3. Transport mechanism……..…………………….……………..……… 4
4. Transporter regulation……………………………………............. 5
5. Uptake-1 physiology &patho-physiology………...…………….... 6
6. Uptake-1 substrates & inhibitors……………..………………….... 7
    a. Substrates of uptake-1…………………………………… 7
    b. Inhibitors of uptake-1………………………………..... 7
        i.Desipramine…………….…………………......... 7
        ii. Reboxetine……………………………………. 8
        iii.Atomoxetine………………………………….... 9

B. Imipramine ……………………………………………………………….. 11

1. Historical overview…………………...……………………...... 11
2. Drug chemistry…………………………….…………………….. 12
3. Pharmacokinetics & drug interactions…………….…………… 12
4. Pharmacology…………………………….……………………… 14
5. Adverse effects…………………….…………………………….. 15
6. Therapeutic applications…………………………..……….. 18

a. Imipramine & Major Depressive Disorder (MDD)…..….. 18
i. Etiology of Major Depressive Disorder…….…………………….. 18
ii. Efficacy of imipramine ………....................................... 21
b. Other therapeutic applications…………………………… 23
C. Methylphenidate…………………..……………….….……….…… 24
1. Historical overview……………………………………………… 24
2. Drug chemistry………………………………………………….. 24
3. Pharmacokinetics……………………………………………….. 25
a. Immediate & extended-release forms………………………….. 27
b. Abuse potential…….…………………………………….. 28
4. Pharmacology…………………………………………………… 30
a. Methylphenidate & the dopamine transporter…..……….. 30
b. Methylphenidate & uptake-1…………………..……… 31
5. Adverse effects…………………………………………………... 32
6. Therapeutic Applications………………………………………... 33
a. Attention Deficit Hyperactivity Disorder………………… 33
b. Narcolepsy……………………………………………… 36
D. Nitric oxide……………………………………………………… 37
1. Historical overview…………………………………………..... 37
2. Synthesis and distribution……………………………………….. 38
3. Physiology of NO…………………..…………………………….. 41
a. NO & the vascular response to sympathetic stimulation… 41
b. NO & the cardiac response to sympathetic stimulation….. 42
4. Dysfunctions in the nitric oxide system…………………………. 45
a. NO deficiency & hypertension……………….….……. 45
b. NO deficiency & chronic kidney disease……….……….. 47
c. NO Deficiency & heart failure…………………………… 47
5. NO& uptake-1……………………………….…………...…….. 49

II. OBJECTIVES OF THE STUDY…………......…………... 50

III. MATERIALS AND METHODS…………………………. 52

IV. RESULTS……………………………………………. 55

V. DISCUSSION……………………………………….. 76

VI. BIBLIOGRAPHY…………………………………… 82

LIST OF TABLES

Table		Page
1.	The effect of increasing doses of norepinephrine on mean arterial pressure under control conditions, after methylphenidate, and after methylphenidate and nitro-L-arginine/nitroglycerin……………………	59
2.	The effect of increasing doses tyramine on mean arterial pressure under control conditions, after methylphenidate, and after methylphenidate and nitro-L-arginine/nitroglycerin…………………...	62
3.	The effect of increasing doses methoxamine on mean arterial pressure under control conditions, after methylphenidate, and after methylphenidate and nitro-L-arginine/nitroglycerin…………………...	65
4.	The effect of increasing doses of norepinephrine on mean arterial pressure under control conditions, after imipramine, and after imipramine and nitro-L-arginine/nitroglycerin………………………...	68
5.	The effect of increasing doses of tyramine on mean arterial pressure under control conditions, after imipramine, and after imipramine and nitro-L-arginine/nitroglycerin………………………........	71
6.	Potentiation of the pressor effect of norepinephrine after treatment with various blockers of uptake-1: methylphenidate, imipramine, reboxetine and atomoxetine..	74
7.	Maintainance of the potentiation of the pressor effect of norepinephrine with various blockers of uptake-1: methylphenidate, imipramine, reboxetine and atomoxetine after nitro-L-arginine and nitroglycerin……………………………………………………………	74
8.	Decrease of the pressor effect of tyramine after treatment with various blockers of uptake-1: methylphenidate, imipramine, reboxetine and atomoxetine………...	75
9.	Restoration of the pressor effect of tyramine with various blockers of uptake-1: methylphenidate, imipramine, reboxetine and atomoxetine after nitro-L-arginine and nitroglycerin……………………………….	75

LIST OF FIGURES

Figure Page

1. The effect of increasing doses of norepinephrine on mean arterial pressure under control conditions, after methylphenidate, and after methylphenidate and nitro-L-arginine/nitroglycerin…………………... 61

2. The effect of increasing doses tyramine on mean arterial pressure under control conditions, after methylphenidate, and after methylphenidate and nitro-L-arginine/nitroglycerin……..……………. 64

3. The effect of increasing doses methoxamine on mean arterial pressure under control conditions, after methylphenidate, and after methylphenidate and nitro-L-arginine/nitroglycerin………..…………. 67

4. The effect of increasing doses of norepinephrine on mean arterial pressure under control conditions, after imipramine, and after imipramine and nitro-L-arginine/nitroglycerin………………………... 70

5. The effect of increasing doses of tyramine on mean arterial pressure under control conditions, after imipramine, and after imipramine and nitro-L-arginine/nitroglycerin……………………............................ 73

CHAPTER I
INTRODUCTION

A. The norepinephrine transporter (uptake-1)

1. General Overview

Neurotransmitters in the central and peripheral nervous system are characterizedby the brevity of their actions at the post-synaptic level. This is due to the termination of their action by precise mechanisms such as enzymatic degradation or reuptake by membrane transport proteins. The activity of norepinephrine, the major neurotransmitter of the sympathetic nervous system, is inhibited by reuptake through the pre-synaptic membrane via an active sodium-dependent symporter known as the norepinephrine transporter, first described by Iversenin 1963[1] through his work on the isolated perfused heart. The discovery of this transporter came after thorough investigations on the accumulation of radio-labeled norepinephrine and epinephrine in sympathetically innervated organs such as the adrenal medulla, the spleen and the heart. It was also named uptake-1 to distinguish it from the non-neuronal low affinity transporter, uptake-2 [1]. This symporter is also responsible for the neuronal sequestration of other catecholamines like dopamine and epinephrine. Other than in the adrenergic synapses, uptake-1 is also localized in sympathetic ganglia, adrenal chromaffin cells [1] and catecholamine-secreting tumors. Uptake-1 is also localized in cell lines derived from these tumors for cloning purposes such as SK-N-SH cells[1] which are human neuroblastoma cells and in rat pheochromocytoma cell line known as PC-12 cells, first used by Bonische et al. in 1984 as a model to study transport of radio-labeled norepinephrine [2]. This transporter is of particular pharmacological importance

since it is a target to numerous agents used for treatment of some psychiatric disorders, including depression and attention deficit hyperactivity disorder (ADHD).

2. Transporter structure

It is fundamental to fully understand the structure and mechanism of substrate binding and transport to elucidate the molecular basis of blockade of the reversible and irreversible inhibitors of uptake-1. The inability of researchers to properly purify any mammalian uptake-1 from PC-12 cells[3] has rendered it impossible to perform direct sequencing of the transporter in order to identify the basic structure and sites of regulation. In 1991, Pacholczyk et al. adopted an indirect method for the determination of the primary amino acid sequence of uptake-1, which was the cloning approach. Human uptake-1 complementary DNA was isolated from SK-N-SH cells and transfected into a culture of HeLa cells [3]. To ensure whether the protein expressed in the membrane of the HeLa cells was uptake-1, binding experiments were done using a radio-labeled ligands in the presence and in the absence of desipramine, a potent and selective inhibitor of uptake-1 [3, 4]. These cloning experiments have lead to the identification of a membrane transporter of 617 amino acids [4] with 12 hydrophobic segments each comprising of 18 to 25 amino acids,forming 12 membrane spanning domains [5]. Homology studies with other transporters such as those of GABA, cloned by Guastella et al. in 1990 [3] almost at the same time as uptake-1, resulted in a high degree of sequence similarity. This lead to the classification of uptake-1 and the GABA transporters in the Solute Carrier 6 (SLC6) family of membrane sodium-chloride-dependent neurotransmitter transporters [6]. Site directed mutagenesis was initially used

to determine the role of each primary sequence of amino acids in the transporter, however it did not provide valuable insight to the tertiary structure of uptake-1 [7] which is essential to determine the mechanism of norepinephrine transport and the mode of action of selective blockers of uptake-1. The breakthrough came in 2005 when Yamashita et al. determined the X-ray crystallographic structure of the prokaryotic leucine transporter (LeuT), the prokaryotic homolog of the SLC6 transporters [6]. Although the LeuT was characterized by little sequence homology to uptake-1, it proved to be a very useful structural and functional template of uptake-1 [7]. Combining the results of the sequencing studies with site directed mutagenesis experiments and the use of the LeuT as a structural homolog we now have a somewhat clearer understanding of the structure, mechanism of binding and transport, and the site of action of blockers of the transporter. Uptake-1 is composed of 12 trans-membrane alpha-helical domains with cytosolic N and C termini, in addition to the presence of an extracellular loop between trans-membrane domains 3 and 4 which is said to be a target of N-glycosylation [4]. In reference to site-directed mutagenesis and using LeuT as a molecular model, two substrate-binding sites were determined so far by Yamashita et al. in 2005 and Singh et al. in 2008: the S1 binding pocket which is the main substrate-binding site, hypothesized to be between four trans-membrane domains 1,3, 6 and 8 and the S2 binding pocket [6] whose role is still controversial since Shi et al. suggested that binding to the S2 site is required for substrate translocation intra-cellularly while Zhou et al. suggested that it is a site of binding for uptake-1 inhibitors thereby inhibiting substrate translocation [6]. In addition, one sodium (Na^+) and one (Cl^-) binding sites have been determined as well[6]. The passive transport of sodium provides the energy

required for the conformational change allowing transport of norepinephrine actively, whereas the role of Cl^- in the transport is still poorly understood [6].

3. Transport mechanism

Many conceptual models were suggested to describe the transport mechanism in monoamine transporters, the most important of which was "the alternation access model" first described by Peter Mitchell and Walter Willbrandt in the late 1950s[6]. This model was later improved by Jardetzky in 1966 who gave it its current name. It states that the transporter exists in three conformations: the outward facing conformation where the S1 pocket is accessible to the extracellular medium and hence ready to bind the substrate, the occluded conformation where access to the pocket is blocked by either side and the inward facing conformation where the S1 pocket is open to the cytosol for substrate release[5]. Nonetheless, the LeuT which was used as template to elucidate the transporter structure and transport mechanisms does not exhibit the inward facing conformation [6]. Other models such as "the hinge model" proposed by Krishnamurthy et al. in 2009 and "the rocking bundle model" proposed by Forrest and Rudnick, followed the alternating access model [5]. In 2012, Krishnamurthy and Gouaux suggested that the transport of the substrate and ions is achieved by a hybrid mechanism involving the hinge and the rocking bundle model [5]. Therefore the transport mechanism in all monoamine and amino acid transporters including uptake-1 remains somewhat ambiguous.

4. Transporter regulation

Sequencing and analysis experiments on neurotransmitter transporters showed that numerous amino acids in these transporters are subjected to post-translational modifications by phosphatases, kinases, as well as by other proteins altering transporter function and cell surface availability.

Glycosylation is one of the essential regulatory post-transcriptional modifications. The three major glycosylation sites are in the extra-cellular loop located between trans-membrane domains 3 and 4. These sites bind sugar residues that form a coat that protects uptake-1 from proteolytic cleavage. Site directed mutagenesis performed by Melikian et al. in 1996 and Li et al. in 2004 or enzymatic de-glycosylation has shown that uptake-1 becomes unstable, consequently leading to a smaller number of surface transporters and as a result to a reduced uptake activity [6].

Various protein kinases are implicated in the regulation of cell membrane availability of all monoamine and amino acid transporters including uptake-1, the most important of which being protein kinase C (pKC). This particular kinase has numerous intra-cellular consensus sites [1] especially in the N and C termini as well as in intra-cellular loops although older data showed that pKC does not directly reduce the activity of uptake-1 but rather decreases its cell surface expression. Apparsundaram et al. performed in vitro phosphorylation experiments using pKC activators and inhibitors on SK-N-SH cells and showed that pKC modulated uptake-1 membrane trafficking. Applying pKC activators such as β-PMA, they observed reduced [^{3}H]-norepinephrine transport and reduced density of [^{3}H]-nisoxetine binding sites[8]. In 2004, Jayanthi et al.treated transfected cells as well as tissues expressing uptake-1 with pKC activators

such as phorbol esters and monitored the effect of pKC activation on uptake-1 trafficking and concluded that phosphorylation of the consensus sites by pKC reduces transport activity (V_{max}) of uptake-1 due to membrane internalization [9]. Nevertheless, this must not be confused with transporter down-regulation since truncation of the dopamine transporter N-terminus blunts the pKC binding sites and no phosphorylation is further detected, yet the dopamine transporter down-regulation remains unaltered by this truncation and the loss of pKC binding sites [6].

5. Uptake-1 physiology and patho-physiology

As we have previously mentioned uptake-1 is an essential modulator of norepinephrine activity and its function was understood by simple knock out (KO) experiments done by Xu et al. in 2000. As expected, knocking out the uptake-1 gene resulted in high extra-cellular levels of norepinephrine since its synthesis remained unaltered or even increases in the absence of a functional transporter [10]. The outward diffusion of the vesicular content results in the depletion of the intra-cellular stores which probably increases norepinephrine synthesis. Uptake-1 KO mice showed marked behavioral changes such as decreased locomotor activity and extravagant response to psycho-stimulants [10]. Also uptake-1 KO mice exhibited the same behavioral criteria as anti-depressant-treated animals which is understandable since knocking out the uptake-1 gene should result in the same effect observed with blockers of uptake-1 like cocaine, imipramine and reboxetine[11]. In addition, cardiovascular changes such as marked tachycardia and hypertension under conditions of sympathetic activation were seen in uptake-1 KO mice, while these effects were not observed at rest probably due to

the prevalence of the parasympathetic activation [12]. Furthermore, polymorphisms in the uptake-1 gene are implicated in many disorders such as anorexia nervosa, mood disorders, attention deficit hyperactivity disorder (ADHD), depression, as well as orthostatic intolerance [13].

6.Uptake-1 substrates and inhibitors

a. Substrates of uptake-1

Other than norepinephrine,human uptake-1 (hNET) binds dopamine with the highest affinity, and epinephrine with the lowest affinity according to Apparsundaram et al. [14]. This further confirms the role of uptake-1 in dopaminergic dysfunctions and establishes it as target of central nervous system drugs.

b. Inhibitors of uptake1

In addition to the classical non-selective blocker of uptake-1 cocaine which has been extensively studied for its rewarding and addictive effects, other uptake1 inhibitors are currently in use for therapeutic purposes.

i. Desipramine

Desipramine is the secondary amine congener and active metabolite of one of the oldest used tricyclic anti-depressants, imipramine. It is characterized by its predominant blockade of uptake-1, a selectivity not observed with imipramine that

normally acts as a norepinephrine and serotonin reuptake inhibitor [15] . Since dopamine is also a substrate of uptake-1, desipramine can promote dopaminergic stimulation indirectly whichcontributes to its success as an anti-depressant. Another advantage rendering desipramine more favorable to imipramine is its milder adverse effects. Nowadays desipramine is being used as alternative for treatment of ADHD in patients responding poorly or intolerant to the drugs of choice for the disorder such as methylphenidate [15], which will be extensively discussed in another section.

ii.Reboxetine

Reboxetine is a selective norepinephrine reuptake inhibitor clinically used for the treatment of depression [15].Its efficacy was thoroughly investigated. In an 8-week, double-blind, random assignment, multicenter trial conducted on 381 patients assigned to reboxetine, 8–10 mg/day, fluoxetine, 20–40 mg/day, or placebo, reboxetine and fluoxetine produced a statistically significant higher response and remission rate than placebo [16]. Multiple comparative studies between reboxetine and serotonin selective reuptake inhibitors(SSRIs) such as fluoxetine have proven the superiority of reboxetine in terms of patient compliance. For example, in 1999 Massana et al. conducted a double blind trial on 168 patients with major depressive disorder and observed that reboxetine is more effective in treatment of severe depression and more tolerable as well [17]. A randomized double blind study conducted by Claytona et al. in 2002 on 450 patients with major depressive disorder showed a relative absence of sexual dysfunction especially in females treated with reboxetine versus patients treated with fluoxetine [18]. Reboxetine was first approved as an antidepressant in the United Kingdom in 1997, shortly followed by the approval of the European Union [19]. In 2001, the FDA

rejected reboxetine due to lack of evidence on its efficacy as an anti-depressant since in many studies it was no more effective than placebo [19], knowing that by then it was approved by more than 50 countries. Berzewski et al. [20] and Katona et al. [21]compared the efficacy and tolerability of this drug to the classical tricyclic antidepressant imipramine,and showed that reboxetine is of comparable efficacy to imipramine but of better tolerability since patients showed reduced cardiovascular, atropine-like effects, and sedative effects. This is the result of high selectivity of the drug to uptake-1 and its low affinity to H_1histaminergic, α_1 adrenergic and muscarinic receptors. Hence the available data on the therapeutic use of reboxetineas an anti-depressant are somewhat perplexing. Furthermore, similar to desipramine, reboxetine was tested for treatment of attention deficit hyperactivity disorder in children and adults in whom methylphenidate, the conventional drug of choice, was discontinued because of toxicity or lack of response [22, 23]. In both groups of patients, reboxetine has proven to be a possible alternative for long term treatment, yet further investigation and more trials are necessary to confirm reboxetine as a first line drug of choice for ADHD.

iii.Atomoxetine:

Atomoxetine is also a selective blocker of uptake-1. It was approved by the FDA in November 2002 for treatment of ADHD in children, adolescents and adults [24] . It is the first non-stimulant drug approved for treatment of ADHD and the first drug to be approved for treatment of adult ADHD [24]. Trials testing the efficacy and toxicity of atomoxetine in various age groups mostly showed improvement in the symptoms of ADHD [25] with mild to moderate adverse effects including dyspepsia, nausea, vomiting and weight loss in children and adolescents, as well as dry mouth, insomnia,

decreased appetite, constipation, urinary retention, and sexual dysfunction in adults [26]. The efficacy of atomoxetine is by no means greater than that of methylphenidate, the first line drug for treatment of ADHD, and the few clinical trials comparing the two drugs provided contradictory results concerning the efficacy of atomoxetine versus methylphenidate. A trial conducted by Kratochvil et al. on American children showed that atomoxetine is equi-efficacious to methylphenidate in treating symptoms of the disorder [27]. On the other hand, Starr and Kemner conducted an open label study on African American children and observed that methylphenidate is superior to atomoxetine in terms of symptom improvement [28]. In 2006, Wang et al. conducted a double blind comparison trial on Korean, Mexican, and Chinese children producing similar observations as those of Kratochvil et al [29]. As for the tolerability, both drugs exhibited similar tolerability in all three trials with atomoxetine producing additional cardiovascular effects associated with increased sympathetic tone leading to increase in heart rate and blood pressure, but these effects were modest and asyptomatic[29]. Also the metabolism of atomoxetine is affected by poor CYP2D6 (cytochrome P450 enzyme) metabolizers or CYP2D6 inhibitors such as the SSRI paroxetine [30] so drug interactions with atomoxetine are an important consideration. In 2008 Newcorn et al. performed a comparative study on atomoxetine versus the extended-release formulation of methylphenidate (OROS® Methylphenidate) and the findings showed that the OROS methylphenidate was superior in efficacy to atomoxetine[31]. Data from the study also suggested that in almost a third of the patients there was a differential response to the two treatments. This is consistent with the recent practice guidelines in the treatment of ADHD, whereby if a patient does not respond or exhibits tolerance to a treatment, it is

recommended to change the treatment to a different class of drugs (stimulant to non-stimulant and vice versa) [32].

Finally, in patients susceptible to drug abuse, or with a past history of drug abuse, prescription of a non-stimulant medication such as atomoxetine may be of particular benefit, in addition to patients suffering from nervous disorders such as facial tics and spasms [32], who are susceptible to stimulants.

B. Imipramine

1. Historical overview

Imipramine was discovered by serendipity. At the end of the 1940s, Hafliger and Schindler synthesized a group of compounds for potential use as antihistamines, sedatives, analgesics and anti-parkinsonism drugs [15]. One of these compounds was imipramine, a chemical analogue of the phenothiazine anti-psychotics. The success of chlorpromazine in the 1950s prompted the testing of imipramine [33], a molecule with an analogous three-ringed center, for sedative and hypnotic properties. When Kuhn studied imipramine in 1958, he noticed that it was ineffective in reducing psychosis but it improved depressive overlay in schizophrenic patients [34]. It was then introduced in the market as an anti-depressant by Geigy Pharmaceuticals (now Novartis) under the trade name of Tofranil[34] and since that time it has been used for the treatment of major or unipolar depression. It was introduced at the same time as iproniazid, the monoaminooxidase inhibitor (MAOI) whose anti-depressive properties were also accidentally discovered as it was intended to be a drug for treatment of tuberculosis [34]. The adverse effects of imipramine and other tricyclic anti-depressants (TCAs)

rendered them less popular in therapy over the years. Consequently they were rapidly replaced by the more selective serotonin reuptake inhibitors (SSRIs) in the 1970s. Nonetheless, imipramine currently remains a first line drug for treatment of major depression and other disorders.

2. Drug chemistry

Imipramine is a dibenzazepine compound derived from phenothiazines by replacement of the sulfur in the central ring with an ethylene bridge resulting in a seven-membered central ring analogous to the anti-psychotic agent benzazepine[34]. Due to its characteristic tertiary amino group, it was classified as a tertiary tricyclic anti-depressant with other compounds such amytryptiline[15]. The variation in the chemical structure of the tertiary TCAs versus their secondary congeners immensely affected their pharmacological effects as well as their adverse effects such that the secondary TCAs are characterized by more specificity and fewer adverse effects.

3. Pharmacokinetics and drug interactions

All TCAs are rapidly absorbed after oral administration and extensively bind to plasma albumin (90-95%) at therapeutic plasma concentrations [35]. They are lipophylic compounds that usually bind to extra-vascular tissues which accounts for their high volume of distribution [15, 35]. Imipramine is metabolized by the microsomal cytochrome P450 enzymes: CYP1A2 and CYP2C19 and to a lesser extent by CYP2D6 [15] . The hepatic N-demethylation of imipramine produces its secondary amine

congener, desipramine. Desipramine undergoes hydroxylation and glucouronidation (conjugation) and is renally excreted [35]. Desipramine is a pharmacologically active metabolite that can accumulate systemically. Pharmacokinetic variations would result as well as it would take longer to eliminate imipramine systemically, thus prolonging parameters such as the half life ($t_{1/2}$), clearance (CL) as well as area under the curve (AUC) [15].

Recent findings suggest that TCAs are less problematic than SSRIs with respect to drug interactions, yet these interactions could be clinically significant [35]. CYP enzymes are substrates to countless drugs and interactions become inevitable if a patient is receiving more than one drug simultaneously. Administration of imipramine with other agents that are CYP inhibitors or inducers alters the effectiveness of the imipramine dose. Paradoxically, a common drug interaction of TCAs is with SSRIs which is critical since in many depressive states TCAs are co-administered with SSRIs to achieve a rapid therapeutic effect or for treatment-resistant patients [15]. It was documented that some combinations of TCAs with SSRIs could be lethal and should be prohibited such as dotheipine with fluvoxamine or clomipramine with fluvoxamine, where fluvoxamine is said to raise the plasma concentration of clomipramine above 1200 ng/ml (lethal concentration) by inhibiting its metabolizing enzymes [35]. In addition, binding of imipramine to plasma albumins with high affinity can affect the plasma concentration of co-administered drugs. So far, no significant drug interaction with imipramine has been documented it remains a possibility.

In addition to drug interactions, genetic factors can alter the effectiveness of an administered imipramine dose. Polymorphisms in the genes coding for imipramine metabolizing enzymes can render patients either fast or slow metabolizers. For example,

18% of the Swedish population are rapid CYP2C19 metabolizers versus 5% that are slow metabolizers [35]. These polymorphisms are critical factors in determining individual plasma concentrations such that a therapeutic imipramine dose produces a sub-therapeutic plasma concentration with rapid metabolizers, whereas it produces a high plasma concentration in slow metabolizers, resulting in toxicity. In both cases the patient would not be receiving the therapeutic benefits of imipramine. Studies underlined the importance of genetic testing on the basis of which individual imipramine dosing is done as a requirement for adequate therapy [36]. In the United States, the FDA recently approved the use of 'The Amplichip CYP 450 Test' to assess CYP2C19 and CYP2D6 genotypes [35]. In addition, monitoring of the serum concentration would also aid in dose adjustment to enhance effectiveness and reduce toxicity [36]. However, clinical application of genetic testing and plasma concentration monitoring in the course of treatment remain limited.

4. Pharmacology

The pharmacological effects of imipramine were first tested by Sulser in 1962, who observed that low doses of imipramine prevented the depressive state induced by reserpine in rodents [34]. Subsequent series of animal experiments showed that imipramine enhanced levels of norepinephrine, serotonin and to a lesser extent dopamine centrally and peripherally neither by binding to their receptors nor by promoting their release but by inhibiting the reuptake of these neurotransmitters [33].

Imipramine is a potent inhibitor of uptake-1 elevating extracellular levels of norepinephrine and serotonin (5-hydroxytryptamine) in cortical regions of the brain

[15]. The effect of its metabolite desipramine is restricted to blockade of uptake-1, which enhances the blocking effect of imipramine on uptake-1.According to Potter et al., the extensive metabolism of imipramine to desipramine blurs the drug's specificity of action [37]. Imipramine also blocks the reuptake of dopamine, although to a lesser extent, in an indirect and non-specific manner due to its blockade of uptake-1 [15]. In spite of minimal distribution of dopaminergic neurons in the cortical regions of the brain, increased extracellular levels of cortical dopamine could be essential in depression since they may improve the cognitive and affective dysfunctions occurring in depressive states [38]. Also the blockade of the serotonin 5-$HT_{2A/2C}$ receptors may contribute to the benefits of imipramine according to Jenck et al. [39]. Imipramine, like all TCAs, is characterized by its slow onset of action, although the blockade of reuptake of norepinephrine and/or serotonin is immediate. This is due to pharmacodynamic long term changes initiated upon imipramine binding to its substrates such as receptor desensitization, alterations in signal transduction cascades and gene expression as well as modifications in synaptic architecture and signaling [38].

5. Adverse effects

Uptake-1 and the serotonin transporters are not the only targets of imipramine. In fact it interacts with receptors centrally and peripherally, the muscarinic, α_1 adrenergic and H_1histaminergic receptors. These interactions are responsible for the toxicity profile of imipramine that decreased its popularity over the years [15]despite its documented therapeutic efficacy:

- The anti-muscarinic effects include dry mouth, sour and metallic taste, blurred vision, constipation, dizziness, tachycardia and urinary retention[34]. Epileptic seizures are possible due to the central anti-cholinergic effect of imipramine[37].
- The blockade of α_1 adrenergic receptors disrupts cardiovascular homeostasis through triggering hypotension. A high-dose infusion of imipramine produced a remarkable drop in blood pressure in anesthetized dogs [40] accompanied by negative chronotropic effects on the heart which triggers sympathetic outflow [40]. These observations are in accordance with older findings on the isolated guinea pig atria and anesthetized guinea pigs [40]. In humans, postural hypotension is common early in therapy leading to syncope in some patients [41]. According to Shrivastava et al., this phenomenon is a common cause of discontinuation of therapy although it tends to attenuate with time [41]. Tachycardia is probably the result of the increased sympathetic activation following hypotension combined with the anti-muscarinic effect on the heart. Shrivstava et al. reported that treatment with imipramine for a year enhanced patients' heart rates about 5 to 10 beats per minute [41]. TCAs are also known to delay conduction through the atrio-ventricular (AV) node prolonging the P-R interval [41]. This was observed with in vivo canine infusion of a high imipramine dose as well as on rat, bovine and guinea pig ventricular myocytes in vitro [40]. Prolongation of the Q-T interval causing ventricular arrhythmias like tosardes de points was also documented [40, 42] with low-dose imipramine infusions, suggesting that imipramine is a highly pro-arrhythmic drug, although these effects may only be associated with high plasma concentrations due to

high dosing or reduced metabolism [41]. However, depressed patients with conduction problems can experience arrhythmias even when the plasma concentration of imipramine is normal [41]. Thus the cardio-toxicity of imipramine necessitates the use of other classes of anti-depressants in patients recovering from myocardial infarction or with congestive heart failure [43] with the aim of reducing the risk of any cardiovascular accidents. A study done on guinea pigs comparing the arrhythmogenicity of the SSRI fluvoxamine versus that of imipramine reported that fluvoxamine is fivefold less arrhythmogenic than imipramine when both drugs are given at therapeutic and sub therapeutic doses [42].

- Inhibition of the central H_1 receptors inducessedation, weakness and fatigue and elicits obesity [38]. The sedative effect can be particularly beneficial in depressions with an associated anxiety component [43].

- TCAs have a narrow therapeutic index and an intake of five to six times of the maximal daily dose is lethal especially that depressed patients are potentially suicidal. This necessitates providing patients with minimal quantities to avoid morbidity [37].

- In a systematic review by Gijsman et al., patients treated for major depression with psychosis using TCAs showed increased risk of exacerbated psychosis which can worsen disease prognosis [44]. This observation was also supported by a systematic review by Kantrowitz et al. in 2008 but a number of limitations such as the lack of placebo controlled trials can affect the credibility of these findings [44].

Preskorn et al. repetitively highlighted the importance of therapeutic drug monitoring during TCA therapy whereby periodic monitoring of the TCA plasma concentration can help avoid toxicity as well as sub-optimal drug levels [45]. This is beneficial with imipramine due to its narrow therapeutic index. The incidence of TCA-induced seizures was reduced to about 0.4% [45] in a group of patients subjected to drug monitoring. This method ensures that the patients receive adequate therapy by maintaining optimal plasma concentration of imipramine [36].

The search for more selective agents that are equi-effective to TCAs but less toxic was fundamental in order to achieve better patient compliance especially that treatment is long term. The first of these agents were the SSRIs that have relatively mild adverse effects that typically disappear as treatment is continued.

6. Therapeutic applications

a. Imipramine and Major Depressive Disorder (MDD)

i. Etiology of Major Depressive Disorder

Major or unipolar depression is a serious prevalent disorder resulting from complex interplay of multiple genetic, developmental and environmental factors [38]. Depression is associated with a substantial social and economic burden in terms of loss of productivity, the need for sustained medical care, early morbidity due to suicide and vulnerability to other serious disorders [38].In 2001 the World Health Organization (WHO) acknowledged depression and cardiovascular disease as the leading causes of disability on a global scale [38]. They predicted that by 2020, depression would become the second largest cause of global disease burden, ischemic heart disease being the first

[33]. Its major diagnostic symptoms are: depressed mood (sadness) and a complete and long-lasting inability to experience pleasure (anhedonia) in addition to a wide range of symptoms ranging from poor concentration to recurrent thoughts of suicide [38]. Sleep disorders, weight loss and severe fatigue are also common accompanying symptoms [38]. The heterogeneity of the disorder is further exemplified by its co-morbidity with other disorders such that depression can co-exist with other states such as general anxiety disorder or Parkinsonism [38]. It can also accompany somatic disorders such as cardiovascular disease. According to the Statistical Manual of Mental Disorders *(DSM-IV)*, the diagnosis of major depression requires the long term appearance of anhedonia and sadness in addition to two or three of the criteria listed by the manual [38, 43]. Hence the appearance of five major symptoms consistently in a period of no less than two weeks is required for diagnosis [43].

The initiation of proper and effective pharmacotherapy in major depression requires a clear understanding of the etiology of the disorder which remains poorly understood. This explains why major depressive states often remains undertreated, or inappropriately treated [38]. Animal and human experiments from the 1950s showed that reserpine, a drug that treats hypertension by reducing sympathetic activity, induced depression which indicated that monoamine depletion is a cause of the appearance of depression [33, 34, 43]. Furthermore, the MAO inhibitor iproniazid alleviated symptoms of depression by inhibiting monoamine metabolism and administration of imipramine to reserpine pre-treated animals by Sulser in 1962 abolished the symptoms of depression [34]. Thus these experiments showed that the appearance of depressive symptoms is probably caused by a deficiency in norepinephrine, serotonin and dopamine in certain areas of the brain and imipramine reversed this effect to a certain

extent [34]. In 1965, Schildkraut proposed the “norepinephrine hypothesis” that stated that some forms of depression are associated with norepinephrine deficiency [43]. The serotonin hypothesis was added in 1972 by Copen since he argued that reserpine depleted 5-HT [33, 34] as well. Likewise, administration of MAOIs with the 5-HT precursor tryptophan elevated the mood of control patients and potentiated the anti-depressant effect of MAO inhibitors [33]. Conversely tryptophan depletion in patients treated with SSRIs resulted in relapse whereas control patients were unaffected [33]. Hence a deficiency of 5-HT seems to be associated with the appearance of depression as well.The proposed hypotheses were tested by injecting precursors of the deficient neurotransmitters in the brain as well as measurement of monoamine levels post-mortem in depressed suicides, the variations of the norepinephrine and serotonin levels and their metabolites were equivocal to the proposed theories [33]. Hertting et al. were able to demonstrate that imipramine inhibited uptake of norepinephrine in peripheral tissues which enabled it to fit in the norepinephrine hypothesis [33]. Moreover, the appearance of SSRIs as anti-depressive agents verified the serotonin theory as well. Studies on SSRIs showed that improvement of serotonergic signaling in the brain of depressed patients is mediated by enhancing extra-cellular serotonin levels increasing post-synaptic 5-HT receptor activation by promoting the inhibition of the pre-synaptic 5-HT_{1A} auto-receptors and the desensitization of these receptors is partly responsible for the time lag in current anti-depressants [33]. So far evidence implicates norepinephrine and serotonin in the etiology of depression although a role for dopamine is possible [34] in spite of disappointing responses to treatment with the dopamine precursor, L-DOPA [34]. Nonetheless, dopamine deficiency could be implicated in depression since drugs that enhance extracellular levels of dopamine such as cocaine or amphetamine alleviate

mood [33] and anti-depressants enhance dopamine levels indirectly through the blockade of uptake-1.

Vigorous effort was placed over the years to elucidate the molecular basis of depression but so far discrepancies characterize all the proposed theories on the etiology of depression due to accumulated contradictory findings. Hence, the molecular basis of the depressive state remains questionable which is probably why epidemiologic studies show that about 30% of depressed patients do not benefit from present drugs [33]. In addition, it was recently established that 5-HT auto-receptors and other neurotransmitters such as GABA and glutamate as well as other systems such as the hypothalamo-pituitary axis are involved in the pathogenesis of major depression [46].

ii. Efficacy of imipramine

Clinical trials compared the efficacy of imipramine versus newer anti-depressants such as SSRIs or SNRIs. In a meta-analysis by Anderson that incorporated 17 studies comparing TCAs with SSRIs, TCAs were more efficacious in the treatment of symptoms of depression than SSRIs, but the majority of TCA-treated patients discontinued their treatment due to the known adverse effects [47]. Another meta-analysis by the same author that included 102 randomized control trials on patients with major depression reported that TCAs and SSRIs are of comparable efficacy in treatment but TCAs were more effective in inpatient subgroups [48]. In addition, Anderson compared treatment discontinuation in 95 studies and reported that only patients treated with TCAs discontinued their treatment [48]. In a randomized double-blind study on 122 patients diagnosed with unipolar psychotic depression there was no difference in

the response and remission rates between imipramine and the selective serotonin and norepinephrine reuptake inhibitor (SNRI), venlafaxine [49]. The variability of the response to imipramine in psychotic versus non-psychotic patients was documented as well. Birkenhager et al. tested imipramine on a sample of 112 psychotic and non-psychotic patients and compared the changes in the Hamilton Rating Scale for Depression (HAMD) [50]. They found that imipramine was more effective in treatment of psychotic depression [50]. A more recent randomized double blind control trial conducted by Vermeiden et al. on 85 patients with severe major depression comparing the efficacy of imipramine to that of venlafaxine showed that the latter produced a higher rate of remission in depressed patients without psychosis whereas the remission rate was greater for imipramine in depressed patients with psychosis [51]. This study failed to show any superiority of venlafaxine to imipramine in treatment of symptoms of severe depression [51]. Similarly a meta-analysis done by van den Broek et al. in 2009 showed no significant difference in treatment effects with low-dose imipramine versus low-dose venlafaxine [52].

A randomized double blind trial conducted by van den Broek et al. in 2004 comparing the efficacy of imipramine to that of the SSRI fluvoxamine in 141 depressed patients showed that the efficacy of imipramine exceeded that of fluvoxamine [53]. These observations contradicted all previous clinical trials testing the two drugs that showed comparable efficacies of the two anti-depressants. Moreover, according to Birkenhager et al. the previous demonstrations are probably due to improper dosing of imipramine such that the given dose achieves sub-therapeutic plasma levels [54]. Hence dose enhancement would probably render imipramine superior to SSRIs [54]. They tested their hypothesis through a double blind trial using fluvoxamine and imipramine.

Patients received a 248 mg dose of imipramine to achieve a plasma concentration equal 200 ng/ml (therapeutic). The trial showed that imipramine could be more effective than fluvoxamine in treatment of depression upon proper dosing [54]. Improvement of many factors in the study increased its power such as: optimal dosing with the use of target concentrations, exclusion of non-compliant patients and a low drop-out rate [54].

The heterogeneity in findings from the large cluster of available data does not give a conclusive superiority to the first or second generation anti-depressants, although a good number of studies in the literature showed superiority of TCAs. Trials testing the efficacy of imipramine with other anti-depressants are sometimes contradictory but none of them reported imipramine to be inferior to the second generation drugs.

b. Other therapeutic applications

Anti-depressants are extensively used for treatment of other central nervous system disorders such as: bipolar depression, obsessive compulsive disorder and panic disorder [15]. However imipramine is not usually a drug of choice as it was replaced by the more novel selective reuptake inhibitors [15]. It remains to be useful for treatment of some disorders such as anorexia nervosa and nocturnal enuresis [55]. In addition, imipramine like desipramine is used as alternative treatment for ADHD in patients who are intolerant or respond poorly to methylphenidate [15].

C. Methylphenidate

1. Historical overview

The therapeutic application of central nervous system stimulants is not recent, in fact it began centuries ago with the Andean Indians who chewed the coca plant which they claimed, gave them contentment and which they also considered as a source of energy [15]. In 1860 Neiman isolated cocaine, an alkaloid, as the active ingredient of *Erythroxylon coca,* and towards the end of the 19th century the clinical use of cocaine as a local anesthetic started to prevail after the discovery of its physical and chemical properties [15]. Later on, the pharmacological properties of amphetamine were discovered and were found to be similar to those of cocaine and it was clinically introduced as a drug capable of diminishing fatigue and enhancing physical and mental performance [56]. The year 1937 marked the first use of stimulants for what was known as "hyperactivity". It was a racemic mixture of amphetamine, known as benzedrine[57]. Methylphenidate, another related compound was first synthesized in the 1940s. Initially it was used for treatment of a number of conditions such as fatigue, lethargy, depression, as well as psychosis associated with depression and narcolepsy [56].

2. Drug chemistry

Methylphenidate is a piperazine-substituted phenylisopropylamine structurally resembling amphetamine. It was first synthesized in 1944 by Ciba-Geigy Pharmaceuticals (now Novartis) under the trade name Ritalin[56]. It is currently sold as oral tablets of 10 mg of methylphenidate hydrochloride. Methylphenidate has two

centers of chirality and can hence exist as four possible stereo-isomers, but it is administered to patients as a racemic mixture of the *threo*dia-stereomers (Ritalin) since the *erythro* isomers have exhibited minimal central stimulatory effects and were hence excluded from the drug formulation [56]. In addition, both *threo*and *erythro*racemates exhibit similar hypertensive, monoamine oxidase inhibitory and toxic effects according to Szporny and Gorog[56]. In vivo and in vitro assessment of the efficacy of the D and L enantiomers of the *threo*racemate in rats showed that the predominant pharmacological effect resides in D-*threo* methylphenidate where the D-isomer exhibited greater induction of motor activity, and greater inhibition of reuptake of dopamine and norepinephrine in synaptosomes of the striatum and the hypothalamus of rats respectively [58], the main mechanism of action of methylphenidate. In 1998 Thai et al. also investigated the stereo-specificity of methylphenidate and confirmed that the D-*threo*isomer is a more potent blocker of synaptosomal [^{3}H]-norepinephine reuptake than the L-*threo*isomer in prefrontal cortical and striatal rat brain tissues [59].

3. Pharmacokinetics

Methylphenidate is administered orally and is readily absorbed. It reaches its peak plasma concentration [C_{max}] 1 to 3 hours after administration, significantly varying between individuals [56]. Itshalf life ($t_{1/2}$) is about 2.6 to 3 hours and it is hepatically metabolized. The major metabolite which accounts for 80% of the dose is ritalinic acid, an inactive ester which is excreted by the kidneys in the urine [60-62]. Investigators tackled the enantio-selectivity exhibited by methylphenidate pharmaco-kinetically as well. In 1993,Srinivas et al. evaluated pharmacokinetic parameters such as the clearance

(CL), half life ($t_{1/2}$), volume of distribution (V_b), area under the curve (AUC) etc. Upon administering methylphenidate orally to 11 healthy volunteers they observed a significant variation in the D to L enatiomer ratio. This was not observed when the drug was given intravenously (IV) where the D to L ratio variation started to appear more than 1.5 hrs after administration [63] . Moreover the absolute bioavailability of the orally administered D-methylphenidate was 23% whereas that of the L-isomer was 5% suggesting that the enantio-selectivity is at the pre-systemic level upon oral administration where the L-methylphenidate is metabolized to L-ritalinic acid [63]. Accordingly, bioavailability in the rat and monkey upon oral administration was measured to be 20% [61]. In children bioavailability was measured to be between 11% and 53% [64]. This is said to be due to the action of tissue and/or plasma esterases and pre-systemic metabolism [61]. In fact peak plasma levels of ritalinic acid are achieved immediately after or at same time as peak levels of methylphenidate, appearing as if both compounds have been administered simultaneously [56]. This accounts for the wide range of bioavailability observed experimentally which leads to an inter-individual variation in drug response, hence obtaining optimum drug effect requires determining the appropriate dosage range for each patient [56]. In conclusion, methylphenidate is a drug with high intrinsic clearance where tissue and/or plasma enzymes as well as pre-systemic pathways [61] metabolize a large fraction of the dose before it reaches the conventional routes of elimination, the liver and the kidneys.

a. Immediate and extended-release forms

Due to its short half life methylphenidate has been synthesized as three forms: the classical immediate-release form, the slow or sustained-release form and the Osmotic Controlled Release Oral Delivery System (OROS Methylphenidate Hydrochloridesold under the name of Concerta) [32] that has recently gained a good amount of attention where the methylphenidate dose was formulated such that it is absorbed by osmotic pressure at a constant rate via an oral formulation [65] . It delivers methylphenidate in a unique pattern starting with a small bolus (immediate release) followed by an ascending delivery (extended release)[66]. All forms are equally absorbed, but differ in their pattern of absorption and duration of action where the effect of the immediate-release form lasts for about 4 hours whereas that of the osmotic-release form lasts up to 12 hours [65].The prolonged duration of action provided by the latter allows better compliance, fewer missed doses [65, 67] and reduces abuse liability. In fact the sustained-release forms were synthesized in order to avoid the noontime dosing which is usually missed by children in school and by adults as well [65]. It is noteworthy that although the three forms vary in terms of duration of action, they all show similar plasma concentrations as a function of time profiles and have comparable relative bioavailability but the OROS form shows less fluctuations and greater stability in terms of the plasma concentration versus time profiles [68]. Clinical trials testing the efficacy of the OROS methylphenidate versus placebo in adults have shown tremendous and rapid improvement in symptoms and most importantly maintained improvement over time [69, 70] which is fundamental since therapy is usually long term.

b. Abuse potential

As is the case with most psycho-stimulants, the abuse potential of methylphenidate is a problem that must be taken into account especially when given to adults. Animal [71] and human comparative studies [72] suggest that methylphenidate, being structurally related to amphetamine, exhibits similar reinforcing effects. Nonetheless its reinforcing effects seem to be relatively less according to Chait et al. who conducted a study on 35 adults provided with a single oral dose of methylphenidate. Methylphenidate produced somewhat different subjective effects and is probably less reinforcing than amphetamine [73] in comparison to a previous study by the same author. In 1995, Volkow et al. compared the binding and effects of [^{11}C]-methylphenidate to those of cocaine (from a previous study) in adults [74]. They observed via Positron Emission Tomography (PET), that although both drugs are of comparable distribution in the striatum and extent of dopamine transporter blockade, they differ pharmaco-kinetically mainly in the duration of the peak plasma concentration and the clearance, both being shorter for cocaine [74]. This is predicted to be the cause of the greater reinforcing effect of cocaine versus methylphenidate. In 1998, Volkow et al. examined the relationship between the blockade of the dopamine transporter by methylphenidate and abuse potential in 7 adult subjects[75]. They observed that following a dose of methylphenidate that blocks 60% of dopamine transporters(DATs) in the brain (therapeutic dose) the reinforcing effects were not seen. These findings allow us to infer that it is not the percentage of dopamine transporters blocked, nor the duration of the blockade that determines the reinforcing effects of methylphenidate but rather the speed of the blockade, clinically manifested as the time between the intake of methylphenidate and the time of onset of effects [56].

Consequently, due to the oral intake of methylphenidate, the time needed for the appearance of the effects is longer than that that of cocaine thus a lesser reinforcing effect is expected. Spencer et al. also proposed that the reason behind the abuse liability of methylphenidate could be pharmacokinetic and therefore the form of methylphenidate administered is of the essence here [76]. They used PET and altropane, a diagnostic dopamine transporter blocker to compare the binding to the dopamine transporter and study the pharmacokinetics of the immediate-release form versus the OROS methylphenidate in 12 healthy adults. They found that it is not the plasma concentration (C_{max} being greater for the OROS form) nor the percentage of blockade of the dopamine transporters (70% vs. 50%) that is relevant with the reinforcing effects but it is the pharmacokinetics where the slower rate of rise of plasma methylphenidate in the OROS form versus the sharper rate of rise in the immediate-release form (time to reach C_{max} for OROS is 7.5 hours versus 2.2 hours for immediate release) is the major contributor to misuse of the immediate-release form of methylphenidate [76]. Furthermore epidemiological data suggested that abuse of immediate-release forms may be greater than that of extended-release forms [77, 78]. In addition, two recent trials were conducted by Parasrampuria et al. in 2007 comparing the abuse potential of the two forms of methylphenidate versus placebo in adults with ADHD and have confirmed the previous findings [79, 80]. Therefore, the literature provides vast evidence on the necessity of proper dose adjustment upon treatment with methylphenidate which is critical in adults to avoid dependence.

4. Pharmacology

a. Methylphenidate and the dopamine transporter

In 1985, a study by Janowski et al. was the first to suggest that methylphenidate blocks the dopamine transporter. They found that radio-labeled methylphenidate binds to dopaminergic neurons in the striatum [56]. These findings were supported the same year by Unis et al. who also did in vitro receptor autoradiography experiments with radio-labeled methylphenidate on rat brains. In 1998, Volkow et al. quantified the blockade of dopamine transporters by a clinical dose of methylphenidate (0.5mg/kg) as 60% [75]. Another study done on normal adult rats using [^{3}H]-methylphenidate showed that methylphenidate blocks the dopamine transporters in the striatum, prefrontal cortex, nucleus accubens and increases norepinephrine levels in the prefrontal cortex only [81]. These studies corroborated the findings of an older investigation by Lin et al. who studied the binding of psycho-stimulants such as methylphenidate, modafinil and amphetamine in cats by studying the expression of the *c-fos* gene. This is an immune-cytochemical technique used to observe activation of gene expression in the target neurons following drug administration. Methylphenidate evoked a dense *c-fos* expression in the cortex and the striatum [56] confirming that both regions are targets of methylphenidate. Extra-neuronal accumulation of dopamine and norepinephrine induced by methylphenidate gave rise to numerous hypotheses regarding the consequences of this accumulation. Andrews and Lavin studied the effects of methylphenidate on infant rats and concluded that increasing the extra-cellular levels of norepinephrine by methylphenidate enhances cortical pyramidal neuronal excitability [82] which confirms its cognitive role. Seeman and Madaras suggested that the extra-cellular accumulation of dopamine activates the

pre-synaptic D_2 auto-receptors and reduces the impulse-induced release of dopamine reducing the activation of the postsynaptic D_1 and D_2 receptors resulting in decreased hyperactivity [83]. Volkow et al. used PET to study the binding of a radio-labeled D_2 receptor antagonist, $[^{11}C]$- raclopride in 11 healthy subjects and observed that in the presence of methylphenidate the binding of the radio-ligand was significantly reduced presumably due to the competition of the accumulated dopamine with raclopride which markedly reduced D_2 receptor availability for the binding of raclopride[84]. It appears that the dopamine transporter is not the only target of methylphenidate since novel evidence is proposing that its therapeutic effect may also be mediated by D_1 and α_2 adrenergic receptors. Administering low oral doses of methylphenidate (1-2 mg/kg) to rats producing similar plasma concentration as in children, improved performance during a spatial working memory task that requires the prefrontal cortex [83]. This effect was abolished upon the application of D_1 and α_2 antagonists[83]. In the previously mentioned study by Andrews and Lavin the cortical excitability achieved by methylphenidate was lost upon administering an α_2 antagonist [82]. Hence all the findings combined have lead to the development of a model displaying the activity of methylphenidate as a dopamine agonist via multiple mechanisms, first the increase in extra-cellular dopamine, second the disinhibition of the pre-synaptic D_2 auto-receptors, and third the activation of the postsynaptic D_1 receptors [83].

b. Methylphenidate and uptake-1

Uptake-1 has been implicated as a target of methylphenidate after the dopamine transporter, and atomoxetine, a selective uptake-1 inhibitor is now approved

for treatment of ADHD. Kuczenski and Segal showed using micro-dialysis experiments that methylphenidate inhibits the reuptake of both norepinephrine and dopamine but not of serotonin in particular regions of rat brains [85]. Kim et al suggested in 2006 that a polymorphism of uptake-1 may be a "risk inducing" allele for ADHD [83]. In the literature the effects of methylphenidate on uptake-1 are not very well studied but Berridge et al. suggest that low doses of methylphenidate given to rats exert a dual effect on cognitive function and norepinephrine levels [86]. Hahn and Gu also demonstrated that methylphenidate potently inhibits uptake-1 in humans and in mice [83]. Also in 2004 Yang et al. suggested an association between the uptake-1 polymorphisms in the Chinese populations and responsiveness to methylphenidate [83]. Micro-dialysis experiments on male Sprague-Dawley rats in the presence of methylphenidate show a significant rise in norepinephrine levels particularly in the prefrontal cortex [87]. All these findings demonstrate that the pharmacological effects of methylphenidate are partially mediated by its blockade of uptake-1.

5. Adverse effects

The safety profile of methylphenidate was tested by clinical trials on adults with ADHD and suggested common symptoms such as insomnia, decreased appetite, nausea, dry mouth, rise in blood pressure, as well as weight loss but these symptoms were of mild to moderate severity [69, 70] . The need for switching medications and dose adjustment [56] depends on the individual tolerability to methylphenidate in all age groups.

6. Therapeutic applications

a. Attention Deficit Hyperactivity Disorder (ADHD):

ADHD is the most common behavioral disorder in children and adolescents. It is said to affect 3 to 7% of children [88] and can persist into adulthood if ignored. The American Psychiatric Association defined ADHD in 2000 as a debilitating disorder diagnosed on the basis of persistent and developmentally inappropriate levels of over-activity, poor behavioral organization, inability to sustain attention, and impulsivity [89]. This neurological disorder gives rise to great concerns since it affects a child's academic achievement and performance of simple tasks. ADHD was first observed around 1902 [57] as a neurological disorder occurring in boys and was called "hyperactivity" or "hyperkinetic syndrome of children" according to *DSM-II* (Diagnostic and Statistical Manual of Mental Disorders). It was found to be caused by a disturbance at the neuro-physiological level and not the result of bad parenting [88]. This recognition was followed by a subsequent focus on inattention linked to the hyperactivity giving it its modern name ADHD. As in all psychiatric and somatic disorders, numerous hypotheses arose to describe the patho-physiology of ADHD which is an integral pre-requisite for determining treatment requirements. Investigators tried to describe the molecular basis of ADHD via neuro-imaging techniques mainly, and one consistent finding between them was a reduction in total cerebral volume that persists into adolescence [90]and a reduction in the dimension of many areas of the brain such as the caudate nucleus, prefrontal cortex white matter, corpus callosum, striatum (basal ganglia) and the cerebellar vermis[91, 92]. In fact, the literature emphasized the complex etiology of ADHD since multiple genetic as well as environmental factors are implicated that can trigger the modifications seen in the various brain regions [88]. The

genetic factors are essential contributors to ADHD due to hereditability of around 80%. According to twin and family studies, these factors are chiefly polymorphisms in genes coding for dopamine transporters and receptors involved in maintenance of dopamine homeostasis in the brain, specifically the dopamine transporter 1 (DAT_1), D_4[93, 94], and D_5 receptor polymorphisms. The DAT_1 and D_4 receptor polymorphisms together were consistently reported in patients with ADHD [89], as well as polymorphisms in uptake-1 as Kim et al. observed[83]. Also environmental factors have been equally considered as possible triggers for ADHD such as prenatal exposure to nicotine [95], alcohol intake [96], as well as exposure to toxins such as lead [97]. Malnutrition, dietary deficiencies of certain fatty acids [98] and iron [99] are currently under study as potential risk factors. Many studies in the literature acknowledge the interaction of the genetic and the environmental factors in the etiology of the disorder. For instance, in 2003 Kahn et al. found that prenatal exposure to nicotine in children homozygous for a DAT_1 polymorphism increases the chance of appearance of ADHD symptoms more than either factor alone [89]. A similar interaction between DAT_1 polymorphisms and alcohol was determined by Brooks et al. in 2006 [100]. Yet further elucidation of these interactions is required by new studies that link these factors to the pathological manifestations at the cellular level in ADHD [89].The Statistical Manual of Mental Disorders, in its fourth edition *DSM-IV*, enumerates three subtypes of ADHD: 1) the predominantly hyperactive type, 2) the predominantly inattentive/impulsive type and 3) the combined type [89]. The *DSM-IV* has defined the criteria of diagnosis as being six or more of a list of nine symptoms for each type in each domain or of both in case of the combined form, suggesting a high degree of heterogeneity and complexity in the diagnosis [89].

At the molecular level, patients diagnosed with ADHD exhibit a clear dysfunction in the dopaminergic system which results in the characteristic symptoms of impulsivity, hyperactivity, and inattention.

The molecular cause of this dysfunction is not very well understood. Studies on the DAT_1 gene polymorphisms show that certain polymorphisms (in the 3' un-translated region in the DAT_1 gene) affect the cell surface expression of DAT_1 but not its affinity to methylphenidate, where an enhanced expression of DAT_1 can result in increased surface density of DAT_1 causing a higher degree of reuptake of dopamine [101]. This results in a reduced dopaminergic signaling at the postsynaptic level, hence a reduced effect of dopamine on the regulation of motor activity and cognitive function. According to Tripp and Wickens, patients diagnosed with ADHD do not necessarily have dopamine deficiency yet it remains a possibility since the PET imaging does not usually allow the measurement of basal dopamine level [89].

Currently two stimulant drugs are approved for treatment of ADHD: methylphenidate and dextro-amphetamine (which also promotes the release of dopamine and norepinephrine). Both drugs are equally effective in reducing visible ADHD symptoms in addition to the non-stimulant atomoxetine. These agents enhance the extracellular levels of dopamine and norepinephrine by different mechanisms. Rosa Neto et al. compared [^{11}C]-raclopride binding through PET scanning in 9 adolescent subjects diagnosed with ADHD before and after treatment with a dose of methylphenidate[102]. They found that the binding of methylphenidate to the high density of dopamine transporters resulted in minimal raclopride binding confirming that the high extracellular dopamine levels is behind the improvement of ADHD symptoms[102].Volkow et al. also used PET to show that methylphenidate enhances

dopamine levels in the basal ganglia of the human brain, which is probably the cause of improved attention and reduced distractibility [103]. However, treatment of ADHD is not simple since it is characterized by individual variability where the response and tolerability to the drugs approved for treatment can vary between patients which explains why switching treatments is a common phenomenon in ADHD pharmacotherapy [56].

Finally, clinical experts have underlined the importance of integrating psychotherapy as a necessity for sustained improvement. According to Curatolo et al., a period of six months of psychotherapy is followed by an assessment of the patient's improvement before the physician initiates any form of pharmacotherapy [88].

b. Narcolepsy

Narcolepsy is a disorder of disturbance in the sleep-wake cycle, characterized by a brisk daytime rapid eye movement (REM) sleep onset, cataplexy, and fragmented nighttime sleep [104]. The etiology of narcolepsy is said to be autoimmune, characterized by the loss of hypocretin-secreting neurons in the hypothalamus, causing a deficiency in hypocretin, a neuropeptide essential for the regulation of sleep and arousal states [105]. Hence measuring hypocretin levels in the cerebrospinal fluid (CSF) allows definitive diagnosis of the disorder.

The most recent edition of the International Classification of Sleep Disorders (ICSD-2) recognized three forms of narcolepsy: narcolepsy with cataplexy, narcolepsy without cataplexy, the most prevalent form (10 to 50% of narcoleptics) [106]and narcolepsy due to a medical condition [104].

Pharmacotherapy of narcolepsy targets the three main symptoms: excessive daytime sleep, cataplexy which is the sudden loss of muscle tone while awake causing limpness as well as inability to move and fragmented sleep [104]

Stimulants are the most commonly used drugs for treatment of excessive daytime sleep which include amphetamine-like agents such as dextro-amphetamine and methylphenidate [107]. In the United States dextro-amphetamine is the FDA approved stimulants for excessive daytime sleep in narcolepsy according to the 2005 Physicians' Desk Reference, while methylphenidate is often used as off-label [104]. While in Europe, only methylphenidate is approved by EMEA for excessive daytime sleep in narcolepsy [108]. Although this group of drugs is effective in reducing the sudden onset of sleep, side effects such as rebound hypersomnia can decrease tolerability and the abuse potential always remains a possibility [104]. Now modafinil and its R enanatiomerarmodafinil are used as first line agents in treatment of excessive daytime sleep since as opposed to other stimulants they are characterized by less abuse liability, better tolerability and absence of rebound hypersomnia as clinical trials have demonstrated [109]. In spite of the preference for modafinil in this disorder, under certain conditions such as reduced effectiveness, intolerable adverse effects, and cost, the classical stimulants such as methylphenidate are preferred. In child narcolepsy, methylphenidate is a drug of choice since modafinil is not approved in children [104]

D. Nitric Oxide

1. Historical overview

Nitric oxide (NO) was first discovered in 1772 by Joseph Priestly who described it as a colorless clear gas with a lifespan of about 6 to 10 seconds in vivo

[110]. The physiological role of NO was thoroughly studied starting at the end of the 1970s. In 1979 Gruetter et al. delivered NO in an organ bath containing pre-contracted strips of bovine coronary arteries and observed relaxation of the arteries hence discovering the smooth muscle relaxant property of NO [110]. A year later,Furchgott and Zawadzki discovered that acetylcholine produces relaxation in isolated perfused carotid artery strips only when the endothelium is intact [111]. They named the substance released by the strips endothelium derived relaxing factor (EDRF). In 1987, Moncada and Ignarro[112]proved that EDRF is identical to NO [113]. A year later, Moncada showed that NO is synthesized by the oxidation of the guanidine nitrogen of the amino acid L-arginine and what is now known as the L-arginine-NO pathway was elucidated[113]. Although clinical prescription of NO donors such as sodium nitroprusside and nitroglycerin began in 1857[15], little was known about their mechanism of action until Murad discovered that these drugs stimulate solubleguanylate cyclase (sGS) to form cGMP in smooth muscle fibers resulting in their relaxation [114]. In 1992, NO was declared "molecule of the year" by Science magazine [115] and 6 years later Ignarro, Moncada and Murad were recognized for their discoveries on NO by a Nobel Prize in physiology and medicine [110]. That same year, Pfizer released sildenafil (Viagra), a drug for erectile dysfunction which inhibits phosphodiesterase 5 (PDE5), thus promoting the concentration of cGMP and exaggerating its vasodialatory effect [110].

2. Synthesis and distribution

NO is an intra-cellular and paracrine second messenger. It is an autacoid formed by the oxidation of L-arginine by the enzyme nitric oxide synthase (NOS) that oxidizes the guanidine nitrogen, releasing NO and citrulline as end products [15]. Being

a gaseous lipophylic compound, NO diffuses into nearby smooth muscle fibers to activate solubleguanylate cyclase resulting in an increase in cGMP concentration and smooth muscle relaxation [110].

NO synthase is activated by a variety of endogenous vasodilators, the first known being acetylcholine in addition to thrombin, adenosine diphosphate (ADP), serotonin, bradykinin, histamine and shear stress [116]. NO is a ubiquitous molecule since NO synthase is widely distributed in the body and not restricted to the endothelium. Three isoforms of the enzyme are known to date and molecular cloning has shown considerable sequence similarity between them [117] yet they are the product of the expression of three distinct genes localized on three distinct chromosomes [117]. The neuronal NO synthase (nNOS, NOS1 gene derivative) is localized in the brain and in the non-adrenergic non-cholinergic nerves (NANC), known as the nitrergic nerves that innervate the esophagus, gastrointestinal tract and certain blood vessels [118]. Endothelial NO synthase (eNOS, NOS3 gene derivative), initially discovered in endothelial cells is also present in platelets, myocardium and the endocardium [119]. Inducible NO synthase (iNOS, NOS2 gene derivative) is mainly found in macrophages and other tissues and is induced by immune stimulation [119]. The first two isoforms are said to be "constitutive" since they are expressed under basal conditions and are activated by a rise in $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$>0.5μM) [117]. The iNOS is absent under physiological conditions since its expression is activated by cytokines in a state of inflammation [118]. Recent evidence shows that iNOS is expressed constitutively under physiological conditions [120]. The activation of iNOS is calcium-independent, unlike the first two isoforms [121]. Furthermore, all isoforms utilize L-arginine as a substrate and have many cofactors such as 5,6,7,8 tetrahydrobiopterin (BH4), nicotinaminde-

adenine-dinucleotide phosphate (NADPH), flavin-adenine dinucleotide (FAD), flavinmononeucleotide (FMN) and calmodulin (CaM), the protein that binds calcium leading to the enzymatic activation [118]. Although the inducible form of NOS does not require calcium for its activation, yet it binds calmodulin but with much less affinity [117] . Also caveolin1, the essential coat protein for the endothelial caveolae, serves as a regulator of eNOS function since *cav1* gene knock-out mice are characterized by disrupted NO synthesis [122]. Caveolin is known to have a negative modulatory effect on NO formation and was shown to be over-expressed in rats with ischemic renal tubular injury [123].

The NO signal transduction pathway has been gradually clarified following the discovery of NO and its activation of the soluble guanylate cyclase. First messengers, such as acetylcholine, bind to a G_q protein which in turn activates via its α subunit the membrane-bound phospholipase C enzyme, which converts membrane phosphoinositol diphosphate (PIP2) into the second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 in turn opens the endoplasmic reticulum calcium channel causing an efflux of calcium from the endoplasminc reticulum store into the cytosol. Calcium activates calmodulin, a calcium binding protein and a cofactor of NOS resulting in the activation of NOS, leading to the synthesis of NO from L-arginine. NO diffuses into nearby smooth muscle fibers and activates the soluble guanylate cyclase, a heterodimeric enzyme consisting of α and β subunits [117]. Activation involves binding of NO to soluble guanylate cyclase through its receptor, a prosthetic heme group [124]. This results in a rise in $[cGMP]_i$which activates protein kinase G (pKG) that decreases $[Ca^{2+}]_i$, causing smooth muscle relaxation. The effect of NO is terminated by

phosphodiesterase which hydrolyses cGMP [116]. The activation of NOS is also mediated by another pathway via a G_i protein (inhibitory G protein) activated mainly by serotonin and thrombin [125].

Although NO is the most essential EDRF, it is not the only one. It was confirmed that the inhibition of the L-arginine-NO pathway does not inhibit all smooth muscle relaxation dependent on an intact endothelium[118]. Nagao and Vanhoutte observed that endothelial dependent relaxation to bradykinin took place even with blockade of nitric oxide synthesis with nitro-L-arginine [126]. Hence another factor exists producing the same effect as NO. It has not been identified yet but it was given the name endothelium derived hyperpolarizing factor (EDHF) [118]. It is said to produce its effect by opening K^+ channels [126]. However its precise mechanism of action leading to smooth muscle relaxation has not been identified.

3. Physiology of NO

a. NO and the vascular response to sympathetic stimulation

The physiological role of NO and how it affects variable physiological parameters were first determined by simple experiments in which analogues of L-arginine such as N-monomethyl-L-arginine (L-NMMA), N_w-L-arginine (L-NNA), and N-nitro-L-arginine methyl ester (L-NAME) were given intravenously to anesthetized rats to block NOS [127, 128] producing an increase in mean arterial pressure and inhibiting the hypotension caused by acetylcholine and bradykinin[128, 129]. Comparable outcomes were seen in preparations such as the rat anococcygeus[130], the dog mesenteric artery [131], the rat aorta [132] and pulmonary artery [133] .

Furthermore experiments performed by Vo et al. on rat caudal arteries confirmed that the inhibition of NO synthesis by L-NAME or by L-NNA enhances the vasoconstrictor responses to sympathetic nerve stimulation and norepinephrine[134]. Greenberg et al. found that administering exogenous NO in canine pulmonary vessels and in mesenteric arteries decreased the efflux of radio-labeled norepinephrine during trans-mural nerve stimulation [135]. In addition, endothelium-rubbed segments released a higher concentration of radio-labeled NE [135]. Another approach involving the blockade of other components of the NO pathway such as soluble guanylate cyclase by methylene blue or NO by hemoglobin[136], led to the same results which were absent in endothelium-denuded segments [134]. In humans, these observations were confirmed by administration of L-NMMA as an infusion in the brachial artery causing a reduced forearm blood flow [137]. Consequently all these findings confirm a role for NO in the modulation of blood pressure and blood flow.

b. NO and the cardiac response to sympathetic stimulation

Multiple clinical trials and reviews in the literature addressed the role of NO as a modulator in the autonomic nervous system. The first evidence suggesting a role for NO in neuronal transmission was by Garthwaite et al. in 1988 who demonstrated in vitro the release of NO in response to N-methyl D-aspartate (NMDA) receptor stimulation by the excitatory neurotransmitter glutamate in rat cerebellar cells [138]. Animal experiments demonstrated that NO blunts the baroreceptor-heart rate-reflex arc in many key sites, ranging from the baroreceptors to the centers of neuronal integration to the effectors [119]. Directly recorded sympathetic cardiac nerve activity showed a

15% increase upon IV administration of L-NMMA in baroreceptor-denervated rabbits, which was reversed by L-arginine administration [139]. Further evidence was provided by Jimbo et al. who observed that administration of L-arginine to stimulate endogenous NO synthesis in anesthetized rabbits caused a decrease in cervical sympathetic nerve activity and renal sympathetic nerve activity despite a decrease in blood pressure [140] indicating that nitric oxide modulates sympathetic activity not just in the afferent and efferent limbs of the reflex arc but also centrally. Furthermore, the role of nNOS in the central components of the baroreflex was studied by the administration of arginine analogues in the nucleus tractussolitarius, the initial recipient of the afferent impulse from the baroreceptors as well as the therostroventral medulla [119]. Here paradoxical results were obtained. Liu et al. observed that the gain of the baroreceptor reflex was increased by acute NOS inhibition with L-NNA in conscious rabbits, an effect reversed with L-arginine [141]. Matsumura et al. found that administering L-NAME into the cerebral ventricles of conscious rabbits also produced the same results while a NO donor attenuated the baroreflex gain [142]. On the other hand, experiments performed on anesthetized animals showed thatblockade of NO synthesis had no effect on the baroreflex gain. In vitro experiments with isolated neonatal and adult rat ventricular myocytes show that eNOS is responsible for attenuating the cardiac sympathetic stimulation. Balligand et al. showed that the response to isoproterenol in adult rat myocytes treated with L-NMMA was exaggerated, while the effect of carbachol was lost[136]. In vivo experiments done by Sears et al. show that the blockade of nNOS by 1-(2-trimethylphenyl) imidazole in cardiac sympathectomized and vagotomized anesthetized rabbits as well as intact guinea pig atria significantly enhanced the change

in the heart rate in response to sympathetic stimulation, an effect reversed with L-arginine [143].

The antagonistic effects of NO on sympathetic activation raise the assumption that it promotes parasympathetic stimulation. Sears and Paterson showed that in intact frog hearts, L-NMMA reduced the inhibitory effects of acetylcholine on the heart rate implicating NO as a promotor of the vagal reflex on the heart rate [119]. In eight closed-chested dogs, intracoronary L-NMMA infusion attenuated the inhibitory effects of the vagal (by bilateral stimulation of the vagus) response to dobutamine[144]. Administering L-arginine restored this attenuation which was manifested in the diminished dobutamine response [144]. Therefore NO is said to have a "direct effect" in promoting vagal cardiac regulation and an indirect effect by inhibiting to a certain degree sympathetic stimulation of the heart but both lead to the same end result which is the promotion of parasympathetic control.

Despite the frequent use of NO analogues such as L-NNA or L-NAME as pharmacological tools to determine the physiological role of NO, the lack of specificity of these analogues with respect to the isoforms they inhibit remains a limitation [145]. Moreover, they might trigger other pathways such as the rennin-angiotensin-aldesterone system (RAAS) which would affect the variations in parameters such as the heart rate or blood pressure [145].

4. Dysfunctions in the NO system

a. NO deficiency and hypertension

The mechanism of the development of hypertension is still unclear but some hypertensive states have been attributed to excessive sympathetic activity and to endothelial dysfunction. Animal experiments showed that inhibition of NO synthesis results in hypertension. A study byMcarthur et al. compared nerve-stimulated perfusion and norepinephrine overflow in mesenteric preparations of spontaneously hypertensive rats versus those of normotensive Wistar-Kyoto rats [146]. The study attributed oxidative stress as the cause of the increased norepinephrine overflow and reduced perfusion in the hypertensive rats. Administration of N-acetyl cysteine, an antixodant, restored the counterbalancing effect of NO on the effect of norepinephrine but the perfusion remained unaffected due to the release of neuropeptide-Y [146]. Furthermore the administration of L-NAME blocked the effects of N-acetyl cysteine indicating that N-acetyl cysteine acts via increasing the bioavailability of NO [146]. These findings are supported by many studies that showed that oxidative stress is a debilitating factor to the nitrergic system, promoting vascular damage leading to hypertension [147, 148]. Taddei et al. showed that in hypertensive humans, particularly in the forearm circulation, the vasodialatory response to acetylcholine is unaffected by the NOS inhibitor (L-NMMA) [149]indicating that nitric oxide deficiency is a contributor to the hypertensive state. Oxidative stress is a deactivator of NO [116] so it would be pertinent to link the NO deficiency caused by oxidative stress to hypertension. Treatment with antioxidants improves endothelial function and lowers blood pressure in experimental models of hypertension [150, 151]. For example, Frenneaux et al. administered the anti-oxidant α-tocopherol to spontaneously hypertensive and normotensive rats and

compared parameters such as LDL, VLDL and cholesterol. All the parameters that reflect oxidative stress were reduced in hypertensive rats compared to normotensive rats [151]. Also inducing oxidative stress by depleting glutathione (using a glutathione synthase inhibitor) in normotensive rats resulted in the development of hypertension [152]. Paradoxically all isoforms of NOS can resort to forming superoxide anions in conditions where L-arginine is deficient [117, 147].

Barri and Wilkox showed in 1998 that L-arginine decreases salt reabsorption through the proximal and distal tubules in humans by administering L-arginine infusions in eight human subjects and measuring salt excretion [153]. Rajapakse et al. and Kakoki et al. observed that spontaneously hypertensive rats have impaired L-arginine transport in the kidney, especially in the medulla where cationic amino acid transporters (CATs) are dense[154]. This can result in reduced renal perfusion thus causing hypertension [154]. Their study implicated L-arginine as a useful approach to enhance NO bioavailability. Measuring the forearm and peripheral L-arginine uptake using radio-labeled L-arginine showed that hypertensive and genetically predisposed normotensive subjects have impaired renal L-arginine transport [155]. In addition L-arginine supplements improved the response to acetylcholine and in subjects who are hypertensive and with a positive family history [155].

Hence, deficiency in one or more of the substrates involved in the nitric oxide pathway seems to be the cause of the appearance of hypertension.

b. NO deficiency and chronic kidney disease

Chronic inhibition of NOS in animals caused systemic and glomerular hypertension, glomerular ischemia, glomerulosclerosis, tubulo-interstitial injury, and proteinuria according toZatz and Baylis[156]. In 1992, Baylis et al. created experimental models of chronic NOS inhibition where rats were given L-NAME for eight week. Consequently, the rats developed of hypertension and glomerulosclerosis[156]. Ribiero et al. used a much higher dose of L-NAME and obtained a severe and progressive form of hypertension associated with glomerular ischemia, glomerulosclerosis and renal interstitial hypertension [156].

A marker of renal disease is asymmetric dimethyl arginine (ADMA), a naturally occurring byproduct of protein metabolism found in the plasma [157]. It is structurally similar to L-arginine and as an analogue it acts as an endogenous inhibitor of NOS. The infusion of exogenous ADMA to healthy subjects caused a dose dependent decrease in renal plasma flow and to an increase in renal vascular resistance [158]. Increased ADMA levels are observed in many pathologies, but mainly in kidney disease, and is a biomarker reflecting the progression of kidney disease and cardiovascular risk factor [159].

c. NO deficiency and heart failure

In-vitro experiments on rat ventricular myocytes proved that NO endogenously regulates the cardiac response to sympathetic stimulation. Hence it would be pertinent to link NO deficiency to heart failure [136]. Zucker et al. documented that the activity of all isoforms of NOS is reduced in chronic heart failure [160]. In vitro inhibition of

nNOS using L-NNA in cat carotid arteries leadto the loss of the inhibition in chemoreceptors resulting in enhanced firing and subsequent increase in sympathetic activity [161]. Li et al. showed that decreased synthesis of NO in the carotid body caused an exaggerated chemoreceptor reflex in rabbit models of heart failure [162]. Conversely the NO donor SNAP (S-nitroso-N-acetyl-penicillamine) inhibited the carotid body firing in these rabbits [162]. Therefore it is the absence of NO rather than the inability of the carotid to respond to it is the cause of excessive firing in this case. The absence of NO seems to be caused by the down-regulation in the expression of the NOS isoforms found in the carotid. The constitutive isoforms of NOS in the carotid body are suppressed in heart failure according to Ding et al. [163]. On the other hand when nNOS was overexpressed and elevates the NO levels, the overactivation of the carotid chemoreceptors in heart failure was reversed[162]. Drexler and Hornig attributed the downregulation of eNOS in the carotid body to increased oxidative stress and decreased shear stress leading to endothelial dysfunction occurring in heart failure [164]. An upregulation of the nNOS was observed post-myocardial infarction (MI) in animal models[165, 166]. Furthermore, nNOS knockout mice subjected to MI showed increased mortality compared to control mice as well as worse cardiac performance, large left ventricular diameter and more intense hypertrophy of cardiac myocytes[167]. In female mice, ischemic injury upregulatednNOS. This was associated with depression in the activity of the L-type calcium channels, leading to decreased calcium entry into the myocytes and protecting the heart from the calcium overload [168].

5. NO and uptake-1

A study by Kaye et al. showed that nitric oxide donor SNAP (S-nitroso-acetylpenicillamine) inhibits [^{3}H]-norepinephrine reuptake in PC-12 cells in a cGMP-independent mechanism probably mediated by S-nitrosylation[169]. They proved in a subsequent study that nitric oxide may have a role in regulating transporter trafficking since it S-nitrosylates a cysteine 351 residue on the seventh trans-membrane domain, thus leading to decreased norepinephrine reuptake [170]. These findings conflicted with those by Apparsundaram et al. who demonstrated that the reuptake of norepinephrine through uptake-1 increases in SK-N-SH cells treated with SNAP[171]. These findings remain controversial since in 2011, Simaan and Sabra found evidence implicating NO in promoting the uptake of pressor drugs such as norpeinephrine and tyramine via uptake-1 in vivo [172]. Thus, the role of NO and the mechanism by which it modulates the activity of uptake-1 remains unclear and requires a more thorough investigation retaining in the background the certainty of its role in modulating sympathetic activation.

CHAPTER II
OBJECTIVES OF THE STUDY

In previous studies in our laboratory, we showed that fresh synthesis of NO plays a fundamental role in regulating the transport of sympathomimetic amines across uptake-1, to the inside of the sympathetic nerve terminals. Blockade of nitric oxide synthesis with nitro-L-arginine, a nitric oxide synthase blocker, potentiated the pressor effect of norepinephrine, a direct stimulant if the adrenergic receptors, by decreasing its uptake across uptake-1, thus augmenting its concentration at the alpha-adrenoreceptors.

On the other hand, prevention of the synthesis of NO markedly reduced the pressor effect of tyramine, an indirectly acting sympathomimetic amine that enters across uptake-1 to the sympathetic nerve terminal to release norepinephrine from its site of storage in adrenergic vesicles. Furthermore, prevention of NO synthesis practically annulled the blocking effect of cocaine on uptake-1, its classical blocker. Thus the cocaine-induced potentiation of the pressor action of norepinephrine and its blockade of the indirectly-mediated pressor effect of tyramine were relieved after blocking the synthesis of NO. That the site of interaction between NO and the pharmacologic effect of norepinephrine, tyramine and cocaine was uptake-1, was confirmed by the observation that the pressor action of angiotensin II, which is not a substrate of uptake-1, was not modified by blockade of NO synthesis [172]. Further exploration of the role of NO in regulating the transport process in uptake-1 revealed that only the sympathomimetic drugs which are substrates of uptake-1 are influenced by fresh synthesis of NO but those that are not substrates of uptake-1, like methoxamine, are refractory. It was further shown that the tolerance in pressor effect after repeated

administration of some sympathomimetic amines, like the tolerance exhibited by mephentermine and ephedrine requires fresh synthesis of NO to be established. Further exploration showed that a variety of chemically unrelated blockers of uptake-1 that are useful clinically for the treatment of psychotic depression like reboxetine [173] or for attention deficit disorder in the younger age group like atomoxetine [174] require fresh synthesis of NO for their blocking effect. This thesis project is a comparative study of blockers including the classical antidepressant, imipramine to compare it with, the newer antidepressant reboxetine, and the classical drug for attention deficit disorder methylphenidate, to compare it with the newer drug for this treatment, atomoxetine. The blocking effect on uptake-1 was tested by potentiation of the pressor effect of norepinephrine and prevention of the pressor effect of tyramine in rat preparations before and after blockade of NO synthesis with nitro-L-arginine. Treatment with nitro-L-arginine always raises the blood pressure to a high level. The rise in pressure will be restored to the basal level by an infusion of nitroglycerin, a NO donor, to ensure that the comparison of the changes in blood pressure will always be from the same baseline and at the same basal concentration of NO. This will also confirm that the blockade of uptake-1is dependent on fresh synthesis of NO and not on the prevailing basal concentration. The choice of measurement of blood pressure as a main parameter of study in these experiments is justified by the objective of determining the functional relevance of the findings in an intact preparation, with one limitation, that the preparations are under the effect of general anesthesia.

CHAPTER III
MATERIALS AND METHODS

The experiments were done on male Sprague-Dawley rats of average weight of 450 grams. The rats were anesthetized with phenobarbital hydrochloride (80 mg/kg/4 ml) intra-peritoneally and were placed supine on a plate with soft padding. The animals were subjected to tracheostomy and were allowed to breathe spontaneously. The carotid artery was cannulated and connected to a Gould P23XL pressure transducer for measurement of mean arterial pressure. The left and right jugular veins were also cannulated one for single drug injections and the other for drug infusions. Recordings were made on a Gould Ta11 recorder.

Experiments were done on 19 rats divided into 2 series:

Series 1: This series consisted of 8 rats. It was designed to study the effect of single increasing doses of norepinephrine (0.05, 0.1, and 0.2 µg) tyramine (0.025, 0.05, and 0.1 mg) and methoxamine (5, 10, and 25 µg) on mean arterial pressure under control conditions, following administration of repeated doses of methylphenidate to produce complete blockade of uptake-1, following blockade of NO synthesis with nitro-L-arginine (L-NNA), and an infusion of nitroglycerin, a NO donor to restore the rise in blood pressure induced by L-NNA to starting level.

Series 2: This series consisted of 11 rats. It was designed to study the effects of the same single increasing doses of norepinephrine and tyramine on mean arterial pressure under control conditions, following the administration of repeated doses of imipramine

to produce complete blockade of uptake-1, after blockade of NO synthesis with L-NNA, and an infusion of nitroglycerin that restores the rise in mean arterial pressure induced by L-NNA back to starting level.

The drugs used in these experiments were either dissolved in acidic saline or in normal saline.

The drugs dissolved in acidic saline are:

Norepinephrine: ([-]- norepinephrine) bitartrate salt (Sigma)

Tyramine: tyramine hydrochloride (ICN BiomedicalsInc)

Methoxamine: methoxamine hydrochloride (Sigma)

The drugs dissolved in normal saline are:

Nitro-L-arginine: N_{ω}-Nitro-L-arginine (Sigma)

Nitroglycerin: Lenitrol

Imipramine: imipramine hydrochloride (Sigma).

The drugs dissolved in water:

Methylphenidate: methylphenidate hydrochloride (Novartis).

Drugs were prepared in the following concentrations: norepinephrine (1 µg/ml), tyramine (0.5 mg/ml), methoxamine (50 µg/ml), methylphenidate (1mg/ml), imipramine (1 mg/ml), nitro-L-arginine (2.5 mg/ml), nitroglycerin (200 µg/ml)

All dilutions were prepared on the day of the experiment.

Statistical analysis.

Data are expressed by mean ± standard error of the mean. Data in the same series were compared using the Student t-test for paired comparisons, a P value less than 0.05 was considered significant.

CHAPTER IV

RESULTS

1. The effect of increasing doses of norepinephrine on mean arterial pressure under control conditions, after repeated doses of methylphenidate to produce maximal potentiation in the pressor effect of norepinephrine, after methylphenidate and L-NNA, and after methylphenidate, L-NNA and an infusion of nitroglycerin.

Table 1 and figure 1 show that single doses of norepinephrine (0.05, 0.1, 0.2 μg) increased mean arterial pressure by 27± 2, 38± 2 and 47± 3 mmHg respectively ($P<0.000$). Treatment with methylphenidate (1.52± 0.4 mg/kg) potentiated the pressor effect of the three doses to 42± 3, 53± 3 and 62± 3 mmHg (56±11%, 40±6%, 34±6%, $P<0.000$). Administration of the three doses of norepinephrine after treatment with L-NNA (15 mg/kg) resulted in a loss of the potentiation for the two higher doses by 21± 8% and 26± 5% ($P<0.05$, $P<0.002$). Repetition of the three doses of norepinephrine during an infusion of nitroglycerin (25± 3μg/kg/min) that restored the rise in pressure induced by L-NNA back to starting levels produced a rise in pressure by 39± 4, 49± 4 and 56± 4 mmHg (23± 8%, 33± 12%, 31± 12%, $P<0.02$, $P<0.03$, $P<0.03$).These variations are not significantly different from respective values after methylphenidate ($P<0.649$, $P<0.476$, $P<0.116$).

2. The effect of increasing doses of tyramine on mean arterial pressure under control conditions, after repeated doses of methylphenidate to produce maximal

decrease in the pressor effect oftyramine, after methylphenidate and L-NNA, and after methylphenidate, L-NNA and an infusion of nitroglycerin.

Table 2 and figure 2 show that single doses of tyramine (0.025, 0.05, 0.1 mg) increased mean arterial pressure by 25± 2, 35± 3, and 47± 4 mmHg respectively(P<0.000). Treatment with methylphenidate (1.52± 0.4 mg/kg) reduced the pressor effect of the three doses to 4± 0.4, 6± 1 and 10± 1 mmHg respectively (85± 2%, 83± 2%, 78± 2%, P<0.000). Repetition of the three doses of tyramine after treatment with L-NNA (15 mg/kg) restored the pressor effect of tyramine by 19±2, 28±4, and 35±5 mmHg respectively (13±1%, 20±3%, 25±4%, P<0.000, P<0.001, P<0.001) representing a difference of 598±160%, 457±114%, and 348±149% respectively as compared to the pressor effect of tyramine after methylphenidate. Repetition of the three doses of tyramine during an infusion of nitroglycerin (25± 3µg/kg/min) that restored the rise in arterial pressure induced by L-NNA back to starting level, did not modify the pressor response to the first two doses, but increased that of the highest dose by 36±13% (P<0.04).

3. The effect of increasing doses of methoxamine on mean arterial pressure under control conditions, after repeated doses of methylphenidate to produce maximal decrease in the pressor effect of tyramine, after methylphenidate and L-NNA, and after methylphenidate, L-NNA and an infusion of nitroglycerin.

Table 3 and figure 3 show that single doses of methoxamine (5, 10, 20 µg) produced a rise in mean arterial pressure by 17± 2, 23± 1, and 34± 3 mmHg respectively (P<0.000). Treatment with either methylphenidate (1.52 ± 0.4 mg/kg) or L-NNA (15

mg/kg) after methylphenidate and an infusion of nitroglycerin (25 ±3 µg/kg/min) did not further modify the pressor effect of methoxamine.

4.The effect of increasing doses of norepinephrineon mean arterial pressure under control conditions, after repeated doses of imipramine to produce maximal potentiation in the pressor effect of norepinephrine, after imipramine and L-NNA and after imipramine, L-NNA, and an infusion of nitroglycerin.

Table 4 and figure 4 show that single doses of norepinephrine (0.05, 0.1, 0.2 µg) increased mean arterial pressure by 20± 1, 26± 2 and 32±2 mmHg respectively (P<0.000). Treatment with imipramine (4±0.7 mg/kg) potentiated the pressor effect of the three doses of norepinephrine to 36± 3, 46± 3 and 51± 3 mmHg (86±9%, 75±6%, 60±5%, P<0.000). Administration of the three doses of norepinephrine after treatment with L-NNA (15 mg/kg) resulted in a loss of the potentiation by 40± 6%, 44± 6% and 43± 6% respectively (P<0.000). Repetition of the three doses of norepinephrine during an infusion of nitroglycerin (44± 5 µg/kg/min) that restored the rise in pressure induced by L-NNA back to starting level, increased mean arterial pressure by 32± 3, 40± 4 and 46± 4 mmHg (32±3%, 40±4%, 46±4%,P<0.000). These variations are not significantly different from respective values after imipramine (P<0.127, P<0.124, P<0.210).

5. The effect of increasing doses of tyramine on mean arterial pressure under control conditions, after repeated doses of imipramine to produce maximal decrease in the pressor effect of tyramine, after imipramine and L-NNA, and after imipramine, L-NNA, and an infusion of nitroglycerin.

Table 5 and figure 5 show that single doses of tyramine (0.025, 0.05, 0.1 mg) increased mean arterial pressure by 19±1, 27± 2 and 40± 2 mmHg respectively ($P<0.000$). Treatment with imipramine (4 ± 0.7 mg/kg) reduced the pressor effect of the three doses of tyramine to 2± 1, 4± 1 and 7± 1 mmHg (85± 4%, 84±3%, 81±3%, $P<0.000$). Repetition of the three doses of tyramine after treatment with L-NNA (15 mg/kg) restored the pressor effect of tyramine to 6±1, 11±2, and 18±3 mmHg respectively (5±1%, 9±2%, 15±2%, $P<0.000$) representing a difference of 296±113%, 240±67% and 192±54% respectively as compared to the pressor effect of tyramine after imipramine. Repetition of the three doses of tyramine during an infusion of nitroglycerin (44± 5µg/kg/min) that restored the rise in pressure induced by L-NNA back to starting level, did not modify the pressor response of the two higher doses, but increased that of the lowest dose by 114± 37% ($P<0.006$).

Table 1:Change in mean arterial pressure (ΔMAP ± SEM) in response to single doses of norepinephrine (NE) under control, norepinephrine after repeated doses of methylphenidate to produce maximal potentiation in the pressor effect of norepinephrine (MP 1.52 ± 0.4 mg/kg), norepinephrine after methylphenidate and L-NNA (15 mg/kg) and norepinephrine after methylphenidate, L-NNA and an infusion of nitroglycerin (NG 25± 3 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level. S: starting pressure: P: peak Pressure; D: difference; NS: not statistically significant, N=8.

NE (µg)	0.05	0.1	0.2
Control			
S	119 ± 3	118 ± 3	118 ± 3
P	146 ± 4	156 ± 3	165 ± 3
D1	27 ± 2	38 ± 2	47 ± 3
%	23 ± 2	32 ± 2	40 ± 3
	P<0.000	P<0.000	P<0.000
MP: S 115 ± 3; P 125 ± 3; D 10 ± 1		P<0.000	
NE after MP			
S	114 ± 2	114 ± 2	113 ± 2
P	156 ± 4	167 ± 3	175 ± 3
D2	42 ± 3	53 ± 3	62 ± 3
%	36 ± 2	46 ± 2	55 ± 3
	P<0.000	P<0.000	P<0.000
D2-D1	14 ± 3	15 ± 2	15 ± 2
%	56 ± 11	40 ± 6	34 ± 6
	P<0.000	P<0.000	P<0.000
L-NNA:S 112 ± 2; P 143 ± 3; D 31±3 P<0.000			
NE after MP and L-NNA			
S	139 ± 3	139 ± 3	137 ± 3
P	174 ± 5	179 ± 5	183 ± 5
D3	34 ± 5	40 ± 5	46 ± 5
%	25 ± 4	29 ± 4	34 ± 4
	P<0.001	P<0.000	P<0.000
D3-D2	-4 ± 5	-11 ± 4	-16 ± 3
%	-10 ± 14	-21 ± 8	-26 ± 5
	NS	P<0.05	P<0.002
D3-D1	7 ± 4	3 ± 4	1 ± 4
%	28 ± 16	9 ± 11	4 ± 9
	NS	NS	NS

NG: S 135 ± 4; P 108 ± 2; D -27 ± 4 P<0.002

NE after MP, L-NNA and NG

S	**114 ± 2**	**113 ± 2**	**111 ± 2**
P	**153 ± 2**	**162 ± 3**	**167 ± 3**
D4	**39 ± 4**	**49 ± 4**	**56 ± 4**
%	**35 ± 4**	**44 ± 4**	**51 ± 4**
	P<0.000	**P<0.000**	**P<0.000**
D4-D3	**7 ± 2**	**11 ± 4**	**13 ± 4**
%	**23 ± 8**	**33 ± 12**	**31 ± 12**
	P<0.02	**P<0.03**	**P<0.03**
D4-D2	**-2 ± 5**	**-4 ± 5**	**-6 ± 3**
%	**-2 ± 13**	**-5 ± 9**	**-9 ± 5**
	P<0.649	**P<0.476**	**P<0.116**
D4-D1	**12 ± 4**	**11 ± 4**	**9 ± 5**
%	**49 ± 16**	**33 ± 13**	**23 ± 10**
	P<0.002	**P<0.004**	**P<0.08**

Figure 1. Change in mean arterial pressure (ΔMAP ± SEM mmHg) in response to single doses of norepinephrine (NE) under control, norepinephrine after repeated doses of methylphenidate (MP) to produce maximal potentiation in the pressor effect of norepinephrine (MP 1.52 ± 0.4 mg/kg), norepinephrine after MP and nitro-L-arginine (L-NNA 15mg/kg) and norepinephrine after MP,L-NNA and an infusion of nitroglycerin (NG 25 + 3 μg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level, N=8. Statistical comparison between NE-Control and NE-MP: a; between NE-MP and NE/MP/L-NNA: b, P<0.000**, P<0.002***, P<0.03**, P<0.05*.**

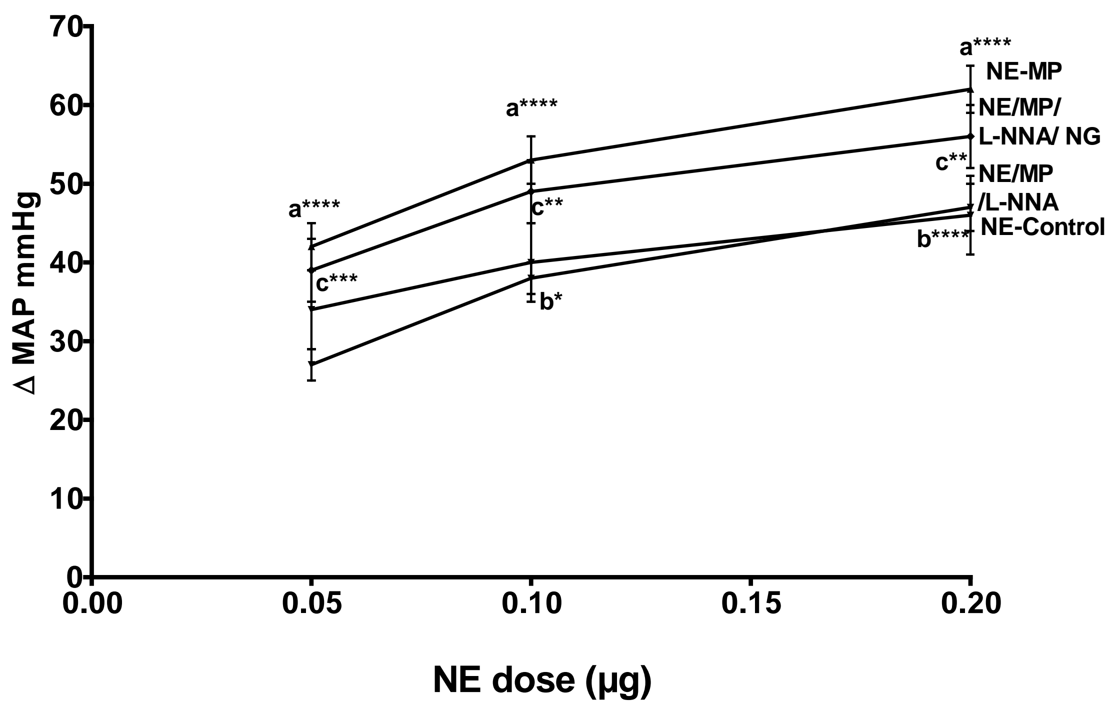

Table 2 :Change in mean arterial pressure (ΔMAP ± SEM) in response to single doses of tyramine (T) under control, tyramine after repeated doses of methylphenidate to produce maximal decrease in the pressor effect of tyramine (MP 1.52 ± 0.4 mg/kg), tyramine after methylphenidate and L-NNA (15 mg/kg) and tyramine after methylphenidate, L-NNA and an infusion of nitroglycerin (NG 25± 3 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level. S: starting pressure: P: peak Pressure; D: difference; NS: not statistically significant, N=8.

T (mg)	0.025	0.05	0.1
Control			
S	119 ± 3	118 ± 3	118 ± 3
P	143 ± 3	154 ± 4	166 ± 4
D1	25 ± 2	35 ± 3	47 ± 4
%	21 ± 2	30 ± 3	40 ± 3
	P<0.000	P<0.000	P<0.000
MP: S 115 ± 3; P 125 ± 3; D 10 ± 1 P<0.000			
T after MP			
S	112 ± 3	113 ± 2	113 ± 2
P	116 ± 3	119 ± 3	124 ± 3
D2	4 ± 0.4	6 ± 1	10 ± 1
%	3 ± 0.4	5 ± 1	9 ± 1
	P<0.000	P<0.000	P<0.000
D2-D1	-21 ± 2	-30 ± 3	-37 ± 3
%	-85 ± 2	-83 ± 2	-78 ± 2
	P<0.000	P<0.000	P<0.000
L-NNA: S 112 ± 2; P 143 ± 3;D 31±3 P<0.000			
T after MP and L-NNA			
S	141 ± 3	141 ± 3	142 ± 3
P	160 ± 5	169 ± 5	177 ± 6
D3	19 ± 2	28 ± 4	35 ± 5
%	13 ± 1	20 ± 3	25 ± 4
	P<0.000	P<0.001	P<0.001
D3-D2	16 ± 3	22 ± 4	26 ± 5
%	598 ± 160	457 ± 114	348 ± 149
	P<0.002	P<0.001	P<0.005

NG: S135 ± 4; P 108 ± 2; D -27 ± 4 P<0.002

T after MP, L-NNA and NG

S	**112 ± 2**	**113 ± 3**	**113 ± 3**
P	**135 ± 3**	**144 ± 3**	**157 ± 2**
D4	**23 ± 2**	**31 ± 2**	**44 ± 3**
%	**21 ± 1**	**28 ± 2**	**40 ± 4**
	P<0.000	**P<0.000**	**P<0.000**
D4-D3	**2 ± 3**	**2 ± 2**	**10 ± 3**
%	**20 ± 14**	**12 ± 8**	**36 ± 13**
	NS	**NS**	**0.04**
D4-D2	**20 ± 2**	**25 ± 2**	**34 ± 3**
%	**650 ± 106**	**472 ± 86**	**424 ± 129**
	P<0.000	**P<0.000**	**P<0.000**
D4-D1	**-2 + 3**	**-4 + 3**	**-3 + 4**
%	**-2 + 12**	**-10 + 8**	**-4 + 7**
	P<0.610	**P<0.190**	**P<0.443**

Figure 2. Change in mean arterial pressure (ΔMAP ± SEM mmHg) in response to single doses of tyramine (T) under control, tyramine after repeated doses of methylphenidate (MP) to produce maximal decrease in the pressor effect of tyramine (MP 1.52 ± 0.4 mg/kg), tyramine after methylphenidate and L-NNA(15 mg/kg) and tyramine after methylphenidate, L-NNA and an infusion of nitroglycerin (NG 25 ± 3 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level, N=8. Statistical comparison between T -Control and T-MP: a; between T-MP and T/MP/L-NNA: b; between T/MP/L-NNA and T/MP/L-NNA/NG: c, P<0.000***, P<0.001****, P<0.002***, P<0.005**, P<0.04*.**

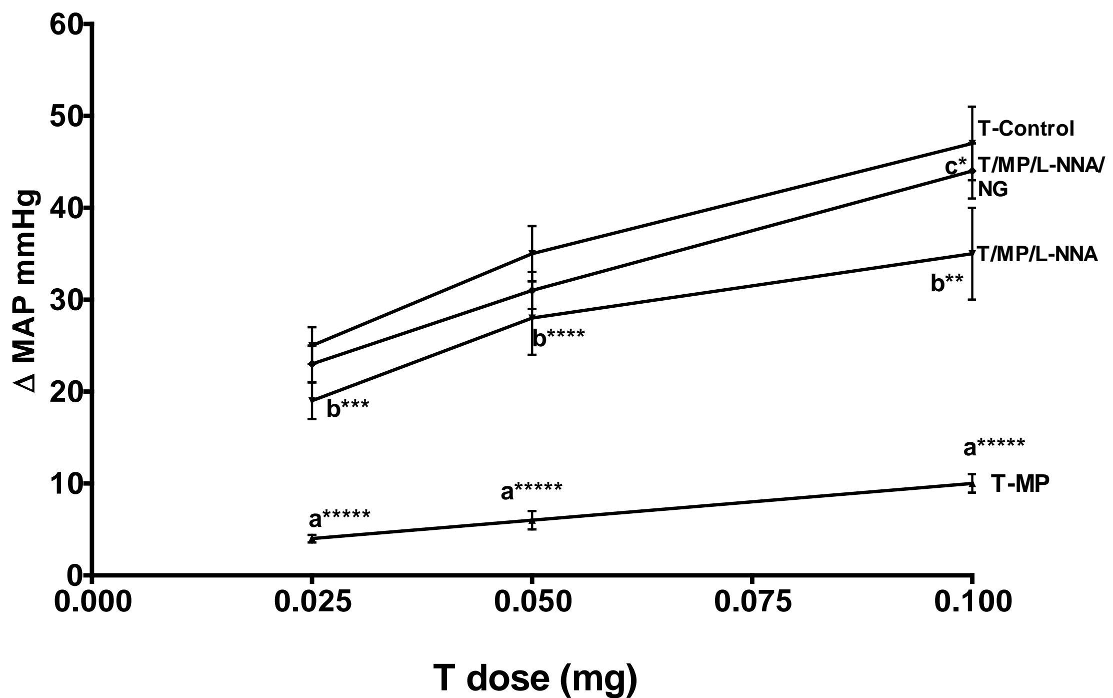

Table 3: Change in mean arterial pressure (ΔMAP ± SEM) in response to single doses of methoxamine (M) under control, methoxamine after repeated doses of methylphenidate to produce maximal decrease in the pressor effect of tyramine (MP 1.52 ± 0.4 mg/kg), methoxamine after methylphenidate and L-NNA (15 mg/kg) and mehoxamine after methylphenidate, L-NNA and an infusion of nitroglycerin (NG 25± 3 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level. S: starting pressure: P: peak Pressure; D: difference; NS: not statistically significant, N=8.

M (µg)	5	10	20
Control			
S	117 ± 3	118 ± 3	119 ± 3
P	134 ± 4	141 ± 3	152 ± 3
D1	17 ± 2	23 ± 1	34 ± 3
%	14 ± 1	20 ± 1	29 ± 3
	P<0.000	P<0.000	P<0.000
MP: S 115 ± 3; P 125 ± 3; D 10 ± 1		**P<0.000**	
M after MP			
S	114 ± 2	112 ± 2	114 ± 2
P	130 ± 3	135 ± 3	150 ± 4
D2	17 ± 2	23 ± 1	36 ± 2
%	15 ± 1	20 ± 1	32 ± 2
	P<0.000	P<0.000	P<0.000
D2-D1	0 ± 0.4	0 ± 1	2 ± 1
%	0 ± 3	0 ± 4	8 ± 4
	NS	NS	NS
L-NNA: S 112 ± 2; P 143 ± 3;D 31±3 P<0.000			
M after MP and L-NNA			
S	136 ± 3	136 ± 3	134 ± 3
P	154 ± 4	159 ± 5	166 ± 6
D3	18 ± 2	23 ± 2	33 ± 3
%	13 ± 2	16 ± 2	24 ± 2
	P<0.000	P<0.000	P<0.000
D3-D2	1 ± 1	-1 ± 1	-4 ± 2
%	4 ± 4	-3 ± 4	-10 ± 5
	NS	NS	NS
NG: S 135 ± 4; P 108 ± 2; D -27 ± 4 P<0.002			

M after MP, L-NNA and NG

S	**111**	**±**	**2**	**110**	**±**	**2**	**111**	**±**	**2**
P	**127**	**±**	**2**	**134**	**±**	**2**	**144**	**±**	**3**
D4	**17**	**±**	**2**	**24**	**±**	**2**	**33**	**±**	**2**
%	**15**	**±**	**1**	**22**	**±**	**2**	**30**	**±**	**2**
	P<0.000			**P<0.000**			**P<0.000**		
D4-D3	**-1**	**±**	**1**	**2**	**±**	**3**	**1**	**±**	**1**
%	**-3**	**±**	**5**	**12**	**±**	**11**	**4**	**±**	**4**
	NS			**NS**			**NS**		

Figure 3. Change in mean arterial pressure (ΔMAP ± SEM mmHg) in response to single doses of methoxamine (M) under control, methoxamine after repeated doses of methylphenidate (MP) to produce maximal decrease in the pressor effect of tyramine (MP 1.5 ± 0.4 mg/kg), methoxamine after methyphenidate and L-NNA (15mg/kg) and methoxamine after methylphenidate, L-NNA and an infusion of nitroglycerin (NG 25 ± 3 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level, N=8. Changes in the pressor effect of methoxamine under control and after different treatments are not significantly different at any dose level.

Δ MAP (mmHg)

60

Table 4: Change in mean arterial pressure (ΔMAP ± SEM) in response to single doses of norepinephrine (NE) under control, norepinephrine after repeated doses of imipramine to produce maximal potentiation in the pressor effect of norepinephrine (I 4 ± 0.7 mg/kg), norepinephrine after imipramine and L-NNA (15 mg/kg) and norepinephrine after imipramine, L-NNA and an infusion of nitroglycerin (NG 44 ± 5 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level. S: starting pressure: P: peak Pressure; D: difference; NS: not statistically significant, N=11

NE (µg)	0.05	0.10	0.2
Control			
S	108 ± 2	108 ± 2	107 ± 2
P	127 ± 2	134 ± 2	139 ± 2
D1	20 ± 1	26 ± 2	32 ± 2
%	18 ± 1	25 ± 2	30 ± 2
	P<0.000	P<0.000	P<0.000
Imipramine(4 ± 0.7 mg/kg) S101±3; P 100±2; D -2±1 P<0.205			
NE after imipramine			
S	99 ± 2	100 ± 2	100 ± 2
P	135 ± 2	146 ± 2	151 ± 2
D2	36 ± 3	46 ± 3	51 ± 3
%	37 ± 3	47 ± 4	52 ± 4
	P<0.000	P<0.000	P<0.000
D2 - D1	17 ± 2	20 ± 2	19 ± 2
%	86 ± 9	75 ± 6	60 ± 5
	P<0.000	P<0.000	P<0.000
L-NNA (15 mg/kg) S 100±2; P 131±3; D 31±2, P<0.000			
NE after imipramine and L-NNA			
S	132 ± 3	132 ± 3	131 ± 3
P	154 ± 3	157 ± 3	160 ± 3
D3	22 ± 3	25 ± 3	29 ± 3
%	17 ± 2	19 ± 2	22 ± 3
	P<0.000	P<0.000	P<0.000

D3-D2	**-14 ± 3**	**-21 ± 3**	**-22 ± 3**
%	**-40 ± 6**	**-44 ± 6**	**-43 ± 6**
	P<0.000	**P<0.000**	**P<0.000**
D3-D1	**2 ± 3**	**-1 ± 3**	**-3 ± 3**
%	**14 ± 15**	**-2 ± 11**	**-10 ± 9**
	P<0.464	**P<0.750**	**P<0.283**

NG (44±5 µg/kg/min) S 132±3; P 101±2; D -31±1 P<0.000

NE after imipramine, L-NNA and NG

S	**101 ± 2**	**101 ± 2**	**101 ± 2**
P	**133 ± 3**	**141 ± 3**	**147 ± 3**
D4	**32 ± 3**	**40 ± 4**	**46 ± 4**
%	**32 ± 3**	**41 ± 4**	**46 ± 4**
	P<0.000	**P<0.000**	**P<0.000**
D4-D3	**10 ± 3**	**15 ± 4**	**17 ± 4**
%	**78 ± 33**	**78 ± 26**	**83 ± 27**
	P<0.014	**P<0.003**	**P<0.001**
D4-D2	**-4 ± 3**	**-5 ± 3**	**-5 ± 4**
%	**-10 ± 8**	**-11 ± 7**	**-8 ± 7**
	P<0.127	**P<0.124**	**P<0.210**
D4-D1	**12 ± 3**	**14 ± 3**	**14 ± 3**
%	**68 ± 16**	**55 ± 12**	**45 ± 10**
	P<0.001	**P<0.001**	**P<0.001**

Figure 4: Change in mean arterial pressure (ΔMAP ± SEM mmHg) in response to single doses of norepinephrine (NE) under control, norepinephrine after repeated doses of imipramine to produce maximal potentiation in the pressor effect of norepinephrine (I 4± 0.7 mg/kg), norepinephrine after imipramine and L-NNA (15 mg/kg) and norepinephrine after imipramine, L-NNA an infusion of nitroglycerin (NG 44± 5 mg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level, N=11. Statistical comparison between NE-Control and NE-I: a; between NE-I and NE/I/L-NNA: b; between NE/I/L-NNA and NE/I/L-NNA/NG: c, P<0.000**, P<0.001***, P<0.003**, P<0.01***

ΔMAP (mmHg)

Table 5 :Change in mean arterial pressure (ΔMAP ± SEM) in response to single doses of tyramine (T) under control, tyramine after repeated doses of imipramine to produce maximal decrease in the pressor effect of tyramine (I 4 ± 0.7 mg/kg), tyramine after imipramine and L-NNA (15 mg/kg) and tyramine after imipramine, L-NNA and an infusion of nitroglycerin (NG 44 ± 5 µg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level. S: starting pressure: P: peak pressure; D: difference; NS: not statistically significant, N=11

T (mg)	0.025	0.05	0.1
Control			
S	105 ± 2	106 ± 2	105 ± 2
P	124 ± 2	133 ± 2	144 ± 2
D1	19 ± 1	27 ± 2	40 ± 2
%	18 ± 2	26 ± 2	38 ± 2
	P<0.000	P<0.000	P<0.000
Imipramine (4 ± 0.7 mg/kg) S:101±3; P:100±2; D:-2±1 P<0.205			
T after imipramine			
S	101 ± 2	101 ± 2	101 ± 2
P	104 ± 2	105 ± 2	108 ± 3
D2	2 ± 1	4 ± 1	7 ± 1
%	2 ± 1	4 ± 1	7 ± 1
	P<0.000	P<0.000	P<0.000
D2 - D1	-16 ± 2	-23 ± 3	-32 ± 2
%	-85 ± 4	-84 ± 3	-81 ± 3
	P<0.000	P<0.000	P<0.000
L-NNA (15 mg/kg) S:100±2; P:131±3; D:31±2 P<0.000			
T after imipramine and L-NNA			
S	127 ± 3	126 ± 4	128 ± 4
P	134 ± 4	137 ± 4	146 ± 4
D3	6 ± 1	11 ± 2	18 ± 3
%	5 ± 1	9 ± 2	15 ± 2
	P<0.000	P<0.000	P<0.000

D3-D2	**4 ± 1**	**7 ± 2**	**11 ± 2**
%	**296 ± 113**	**240 ± 67**	**192 ± 54**
	P<0.004	**P<0.002**	**P<0.000**
D3-D1	**-12 ± 2**	**-16 ± 3**	**-21 ± 4**
%	**-64 ± 6**	**-54 ± 9**	**-51 ± 7**
	P<0.000	**P<0.001**	**P<0.000**

NG (44±5µg/kg/min) S:132±3; P:101±2; D:-31±1 P<0.000

T after imipramine, L-NNA and NG

S	**101 ± 2**	**100 ± 2**	**100 ± 2**
P	**112 ± 3**	**114 ± 3**	**121 ± 3**
D4	**11 ± 2**	**15 ± 2**	**21 ± 2**
%	**11 ± 2**	**15 ± 2**	**21 ± 2**
	P<0.000	**P<0.000**	**P<0.000**
D4-D3	**5 ± 1**	**3 ± 3**	**3 ± 2**
%	**114 ± 37**	**69 ± 40**	**37 ± 23**
	P<0.006	**P<0.218**	**P<0.286**
D4-D2	**9 ± 2**	**11 ± 2**	**14 ± 2**
%	**641 ± 206**	**409 ± 123**	**233 ± 44**
	P<0.000	**P<0.000**	**P<0.000**
D4-D1	**-7 ± 2**	**-13 ± 2**	**-19 ± 3**
%	**-37 ± 8**	**-46 ± 6**	**-46 ± 6**
	P<0.004	**P<0.000**	**P<0.000**

Figure 5: Change in mean arterial pressure (ΔMAP ± SEM mmHg) in response to single of tyramine (T) under control, tyramine after repeated doses of imipramine to produce maximal decrease in the pressor effect of tyramine (I 4± 0.7 mg/kg), tyramine after imipramine, and L-NNA (15 mg/kg) and tyramine after imipramine, L-NNA and an infusion of nitroglycerin (NG 44± 5 mg/kg/min) to restore the rise in MAP induced by L-NNA back to starting level, N=11. Statistical comparison between T-control and T-I: a; between T-I and T/I/L-NNA: b; between T/I/L-NNA and T/I/L-NNA/NG, P<0.000*, P<0.002**, P<0.004*,P<0.006*.**

Δ MAP mmHg

Table 6: Potentiation of the pressor effect (ΔMAP ± SEM mmHg) of the 3 doses of norepinephrine (NE)after treatment with various blockers of uptake-1: methylphenidate (MP), imipramine (I), atomoxetine(A) and reboxetine(R).

NE (µg)	0.05		0.1		0.2	
MP	+14 ± 3	P<0.000	+ 15 ± 2	P<0.000	+ 15 ± 2	P<0.000
I	+ 17 ± 2	P<0.000	+ 20 ± 2	P<0.000	+ 19 ± 2	P<0.000
A	+ 15 ± 3	P<0.000	+ 14 ± 3	P<0.000	+ 13 ± 4	P<0.000
R	+ 13 ± 2	P<0.000	+ 15 ± 2	P<0.000	+ 13 ± 2	P<0.000

Table 7: Maintenance of potentiation of the pressor effect (ΔMAP ± SEM mmHg) of the 3 doses of norepinephrine (NE) with various blockers of uptake-1: methylphenidate (MP), imipramine (I), atomoxetine (A) and reboxetine (R). after L-NNA and an infusion of nitroglycerin.

NE (µg)	0.05		0.1		0.2	
MP	-2 ± 5	NS	-4 ± 5	NS	-6 ± 3	NS
I	-4 ± 3	NS	-5 ± 3	NS	-5 ± 4	NS
A	+3 ± 1	NS	+3 ± 2	NS	+2 ± 3	NS
R	+5 ± 4	NS	+4 ± 5	NS	+5 ± 3	NS

Table 8: Decrease in the pressor effect (ΔMAP ± SEM mmHg) of the 3 doses of tyramine (T) in the presence of various blockers of uptake-1: methylphenidate (MP), imipramine (I), atomoxetine (A), and reboxetine (R).

T (mg)	0.025		0.05		0.1	
MP	-21 ± 2	P<0.000	-30 ± 3	P<0.000	-37 ± 3	P<0.000
I	-16 ± 2	P<0.000	-23 ± 3	P<0.000	-32 ± 2	P<0.000
A	-14 ± 1	P<0.000	-20 ± 2	P<0.000	-29 ± 2	P<0.000
R	-17 ± 2	P<0.000	-31 ± 3	P<0.000	-51 ± 3	P<0.000

Table 9: Restoration of the pressor effect (ΔMAP ± SEM mmHg) of tyramine in the presence of various blockers of uptake-1: methylphenidate (MP), imipramine (I), atomoxetine (A), and reboxetine (R), after L-NNA and nitroglycerin.

T (mg)	0.025		0.05		0.1	
MP	+20 ± 2	P<0.000	+25 ± 2	P<0.000	+34 ± 3	P<0.000
I	+9 ± 2	P<0.000	+11 ± 2	P<0.000	+14 ± 2	P<0.000
A	+7 ± 1	P<0.001	+11 ± 1	P<0.000	+17 ± 3	P<0.002
R	+9 ± 1	P<0.000	+14 ± 3	P<0.001	+15 ± 4	P<0.002

CHAPTER V
DISCUSSION

The literature provides extensive information on the norepinephrine transporter localized in sympathetic nerve terminals, also referred to as uptake-1. Its chemical identity and its modulation through glycosylation, phosphorylation and dephosphorylation by protein kinases and phosphatases have been extensively studied. On the other hand, studies on the role of nitric oxide in the regulation of uptake-1 are scarce. Kaye et al. were the first to show that nitric oxide S-nitrosylates uptake-1 on a specific cysteine residue, decreasing its transport activity in PC-12 cells, via a direct mechanism independent of cyclic GMP [169, 170].While Apparsundaram et al showed that administering SNAP, a nitric oxide donor, enhances the transport of radio-labeled norepinephrine in SK-N-SH cells[171]. In 2011, Sabra and Simaan were the first to study the role of NO in the transport activity of uptake-1 in vivo and found that NO regulates the transport of norepinephrine and other substrates across uptake-1 by studying the pressor response to exogenous norepinephrine, tyramine and angiotensin II under control conditions and after blockade of NO synthesis in intact rat preparations, the main physiological parameter under study being the mean arterial pressure. These observations were further extended to show that blockade of uptake-1 by its classical blocker, cocaine, depends on fresh synthesis of NO since blockade of NO synthesis was found to decrease the blocking effect of cocaine on uptake-1. Other blockers of uptake-1 such as atomoxetine and reboxetine were studied and their blocking activity was found to depend on fresh synthesis of NO, reboxetine representing a novel drug for the

treatment of psychic depression and atomoxetine, a novel drug for treatment of attention deficit hyperactivity disorder.

The aim of this study was to explore the modulatory role of nitric oxide on blockade of uptake-1 by the classical drug for the treatment of psychotic depression, imipramine and the classical drug for the treatment of attention deficit disorder, methylphenidate, both drugs being chemically and pharmacologically different from the newer drugs already studied. Emphasis was placed on comparing the degree of blockade of uptake-1 by newer drugs as compared to that of the classical drugs and the involvement of nitric oxide in this blockade. Again in this study, like in previous studies our important experimental objective was to demonstrate the functional relevance of the changes emanating from different experimental interventions, hence the choice of intact rats for exploration and measurement of arterial pressure as a main parameter.

The test drugs used to explore the degree of blockade of uptake-1 and the influence of NO in the blockade were norepinephrine, tyramine and methoxamine. Norepinephrine as a purely directly-acting drug on adrenergic receptors that is taken up by uptake-1 to be sequestered in the sympathetic nerve terminals is expected to produce a potentiated pressor effect after blockade of uptake-1. Tyramine, as a purely indirectly-acting sympathomimetic drug, enters across uptake-1 to the adrenergic vesicles in postganglionic sympathetic nerve terminals to liberate norepinephrine, hence blockade of uptake-1 is expected to reduce or depress its pressor effect. Methoxamine is not a substrate of uptake-1 and hence its pressor effect is expected to be unchanged by blockade of uptake-1.

NO synthesis was blocked by nitro-L-arginine which always induced a rise in mean arterial pressure, implying that NO is an important determinant of basal arterial pressure. The rise in mean arterial pressure was restored back to the starting level with an infusion of nitroglycerin, a NO donor. This ensured that the rise in pressure induced by the test drugs under control conditions and experimentally always started from the same baseline pressure as well as the same basal level of NO so that any anticipated changes are attributed to fresh synthesis of NO.

The results of this study show that the pressor effect of the three doses of norepinephrine was potentiated over a range of 42 to 62 mmHg after methylphenidate and by a range of 36 to 51 mmHg after imipramine, the values for the three respective doses not being significantly different. Repetition of the three doses of norepinephrine following blockade of NO synthesis and restoration of the rise in mean arterial pressure to starting level, did not modify the rise in pressure further. This does not imply that the blockade of NO synthesis was without a modulating effect on the pressor action of norepinephrine after blockade of uptake-1 with either methylphenidate or imipramine. In a previous study [172], absence of fresh synthesis of NO was found to depress uptake of norepinephrine through uptake-1 and potentiated its effect. On the other hand, absence of NO decreased the blocking effect of cocaine on uptake-1. It is conceivable that the absence of nitric oxide by blocking its fresh synthesis produces both a potentiation of norepinephrine by depressing its uptake and at the same time decreasing the blocking effect of both methylphenidate and imipramine, thus enhancing the uptake of norepinephrine, the net algebraic sum being maintenance of the potentiation. This further shows that the role of NO in the modulation of uptake of norepinephrine and in

enhancement of blockade of uptake-1 by various blockers is equally effective with no apparent dominance of effect at either site.

The pressor effect of the three doses of tyramine was reduced over a range of 21 to 37 mmHg after methylphenidate and 16 to 32 mmHg after imipramine, the values for the three respective doses not being significantly different, implying that the maximal blocking effect of methylphenidate and imipramine on uptake-1 as tested by the potentiation of the pressor effect of norepinephrine and reduction of the pressor effect of tyramine, is comparable. Blockade of NO synthesis and the restoration of the basal level of mean arterial pressure with nitroglycerin restored the pressor effect of the three doses of tyramine by 20 to 34 mmHg after methylphenidate and by 9 to 14 mmHg after imipramine, the values of the three respective doses of tyramine not being significantly different. It is clear from this data that the absence of NO by preventing its fresh synthesis relieved the blocking effect of both methylphenidate and imipramine to the same extent. One should not exclude the fact that the absence of NO by blocking its fresh synthesis decreased uptake of tyramine across uptake-1 to release norepinephrine [172]. However, the balance between the two opposing effects is in the direction of the enhanced uptake of tyramine and restoring its pressor effect to a highly significant degree. This confirms the role of fresh synthesis of MO in mediating the blockade of uptake-1 by methylphenidate and imipramine.

The three doses of methoxamine increased mean arterial pressure over a range of 17 to 34 mmHg prior to methylphenidate administration. The pressor effect did not change further after blockade of nitric oxide synthesis, implying that only the sympathomimetic drugs which are substrates of uptake-1 are influenced by blockade of NO synthesis.

Blockade of uptake-1 with either methylphenidate or atomoxetine resulted in potentiation of the pressor effect of norepinephrine (Table 6) and reduction of that of tyramine (Table 8) to a comparable degree. Similar potentiation in the pressor effect of norepinephrine (Table 6) and decrease of that of tyramine was observed between imipramine and reboxetine (Table 8). The observed potentiation was maintained after treatment with L-NNA and an infusion of nitroglycerin with all the blockers (Table 7) while the pressor effect of tyramine was restored to a similar extent with the four blockers (Table 9). This comparison establishes that the blocking effect of the four blockers on uptake-1 is to a comparable degree and this blockade is equally dependent on fresh synthesis of NO.

In conclusion, this study confirms the role of fresh synthesis of NO in the transport activity of uptake-1and in the blockade of uptake-1 by drugs of various classes. These findings provide valuable insights concerning an additional mechanism by which NO produces its vasodilatory effect counteracting sympathetic stimulation. These findings are of major patho-physiological relevance since a dysfunction in the nitrergic system can promote an excessive and prolonged effect by norepinephrine at the postganglionic adrenergic synapses which is translated as a rise in mean arterial pressure and a reduction in blood flow in a particular tissue or organ. This is expected to trigger the development or progression of cardiovascular disorders. Furthermore, this study also provides insight on a possible interaction between the intake of drugs that act on the central nervous system and the coexistence of cardiovascular disorders. Nonetheless, an important question remains unanswered, namely the mechanism which stimulates fresh synthesis of NO and the chemical pathways involved in the

enhancement of uptake of sympathomimetic drugs across uptake-1as well as blockade of uptake-1 by various blockers.

CHAPTER VI

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