Effect of Piperazine Derivatives on Hippocampal and Dopaminergic Neurotransmission

by

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Abstract

Designer Drugs are a group of psychoactive substances synthesized from chemicals to emulate the pharmacodynamic and pharmacokinetic actions of widely used substances of abuse. A minor modification of the chemical structure can alter the pharmacodynamic and pharmacokinetic profile. These designer drugs affect the neurotransmitters, endocrine and/or exocrine systems. Most of the pathologies associated with the central (CNS) and peripheral nervous system (PNS) occur due to the alteration in the content of a single neurotransmitter in a specific or multiple region of the brain or spinal cord. Moreover, these pathologies can occur due to the imbalance in the content of two or more neurotransmitters in a specific or multiple region of the brain. The change in neurotransmitter content occurs due to interference by designer drugs in the basic process of neurotransmission, which in turn can affect the synthesis (precursor, cofactors synthesizing enzymes), storage (vesicular), release, pre- and/or post-synaptic receptor action(s), as well as metabolism (degrading enzymes) and reuptake of the neurotransmitters. The designer drugs exert their actions by affecting various sites through single or multiple pharmacodynamic effects, which can increase or decrease the neurotransmission. Alterations of the neurotransmission (augmented or diminished) by the designer drugs are mostly detrimental to humans; however, there are certain mechanisms that can reduce the symptoms and provide beneficial effects in numerous pathologies. Currently, there is a substantial increase in the manufacture and usage of piperazine designer drugs worldwide. In the current study, we illuminate the site(s) and mechanism(s) of action of the piperazine designer

drugs affecting the cholinergic, and dopaminergic neurotransmission. There are very limited studies on the hippocampal and dopaminergic neurotoxicity of 1-(3-trifluoromethylphenyl) piperazine (3-TFMPP) and its derivatives (2-TFMPP, 4-TFMPP). N27 rat dopaminergic cells are valid *in vitro* model to investigate the dopaminergic neurotoxic effects and establish the neurotoxic mechanisms of various substances associated with movement disorders. Furthermore, HT-22 mouse hippocampal cells have been used to study the cholinergic / glutamatergic neurotransmission as well as the etiopathology of various types of dementia (Alzheimers disease). Furthermore, in this study, we assessed the possible pharmacodynamic and pharmacokinetic effects of (2, 3 and 4-TFMPP) derivatives using receptor binding assay and QikProp software. Additionally, we established the mechanisms of hippocampal and dopaminergic neurotoxicity of TFMPP derivatives. TFMPP derivatives caused dose-dependent and time-dependent neurotoxicity and produced hippocampal and dopaminergic neuronal death. Furthermore, TFMPP derivatives altered dopaminergic and cholinergic neurotransmission through decreasing the synthesis and increasing the breakdown of dopamine and acetylcholine. Moreover, TFMPP derivatives instigated oxidative stress, mitochondrial dysfunction, inflammation and apoptosis. The usage and manufacture of designer drugs need to be strictly regulated in order to avoid numerous central and peripheral disorders leading to a liability to the current and future society.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

5-HT	Serotonin
6-OHDA	6-Hydroxydopamine
AChE	Acetyl Cholinesterase
ACh	Acetylcholine
AD	Alzheimer's Disease
ADHD	Attention-Deficit Hyperactivity Disorder
ADME	Absorption, Distribution, Metabolism, and Excretion
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
Aβ	Amyloid-β
BBB	Blood Brain Barrier
BH_4	Tetrahydrobiopterin
BSA	Bovine Serum Albumin
ChAT	Choline Acetyltransferase
CNS	Central Nervous System
COMT	catechol-O-methyltransferase
CYP	Cytochrome P
DA	Dopamine
DCFH	2`, 7-Dichlorofluorescindiacetate

DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DOPAC	3,4-Dihydroxyphenyl acetic acid
DTNB	5,5'-Dithiobis-2-Nitrobenzoic Acid
EOAD	Early-Onset AD
FBS	Fetal Bovine Serum
GSH	Glutathione
GSH-Px	Glutathione peroxidase
H_2O_2	Hydrogen Peroxide
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HD	Huntington's disease
HVA	Homovanillic acid
JNK	c-Jun N-Terminal Protein Kinase
L-DOPA	3-(3,4-Dihydroxyphenyl)-L-alanine
LOAD	Late-Onset AD
MAO	Monoamine Oxidase
MAP	Mitogen-Activated Protein
MDA	Malondialdehyde
MNPV	Trans-N-Methyl-4-(1-Napthyvinyl)-Pyridinium
MTT	Thiazolyl Blue Tetrazolium Bromide
MW	Molecular Weight
NADH	Nicotinamide Adenine Dinucleotide

NE	Norepinephrine
NFTs	Neuro Fibrillary Tangles
NO	Nitric Oxide
OPT	O-Phthalaldehyde
PBS	Phosphate Buffer Saline
PD	Parkinson's disease
PDSP	Psychoactive Drug Screening Program
PNS	Peripheral Nervous System
PSEN	Presenilin
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TBA	Thiobarbuturic Acid
TBARS	Thiobarbituric Acid-Reactive Substances
TCA	Trichloroacetic Acid
TFMPP	Trifluoromethylphenylpiperazine
VEGF	vascular endothelial growth factor
VFDF	Very Fast Death Factor
VMAT	Vesicular Monoamine Transporter

1. Literature Review

Introduction

Toxin (synonym = bane, poison, venom) is a poisonous material which can be produced endogenously by microbes, plants, animals or synthesized in the lab. If ingested or administered into the body, toxins can affect the structure and/or functions of cells, tissues, and organs resulting in reversible or irreversible impairment or induction of death. Toxins can cause a dose-dependent effect or dose-independent actions such as; affecting the immune functions in the body and inducing hypersensitivity reactions or antibody formations. There are numerous ways to classify a toxin. The classification can be based on the source or structure of the toxin, tissue/organ system affected, mechanism of action, symptoms produced, etc. Humans have used these substances for centuries for various purposes. The Greeks, Romans, Chinese, Arabs, and Indians used them as weapons, for procuring food, political assassinations, executions, and euthanasia. Animals (snakes, bees, spiders, and fish) have developed these neurotoxins as evolutionary protectants, adapted to inflict detrimental effects on other threatening organisms. These toxins, most commonly in the form of venom, are used by various organisms to paralyze or kill their prey. In this review, we will focus on past and recent neurotoxins as well as designer drugs and their relevant effect on neurotransmission, mechanisms of action, and their possible therapeutic implications.

Neurotoxins are substances that are considered poisonous to the cells in the central and peripheral nervous system. The neurotoxins can be exogenous, synthetic, natural, or endogenous substances. Their accumulation in the body can alter the physiology, function of the nervous system, by reversibly or irreversibly destroying neurons and glia. Currently, there are approximately 1000 known potential neurotoxic compounds. Historically, the most common exogenous neurotoxins include carbon monoxide, ethanol, methanol, arsenic, pesticides, insecticides, and metals such as lead. On the other hand, endogenous neurotoxins include nitric oxide (NO), glutamate, and hydrogen peroxide (H_2O_2). NO, glutamate, and H_2O_2 are critical components of cell signaling in the nervous system. However, they can exert toxic effects when produced in high concentrations. Other endogenous substances include vitamin A and vitamin B_6, both of which are important for development and normal functioning of the nervous system. Yet, in large doses they become neurotoxic and have detrimental effect on the nervous system. Tau proteins have also been associated with blocking postsynaptic signaling that promotes learning and memory. Tau is located inside the neuron and when hyperphosphorylation occurs the protein becomes aggregated. Once aggregated, tau has the potential to inhibit postsynaptic AMPA-type glutamate receptors and promote the loss of dendritic spines.

Numerous characteristic features of the cells in the nervous system make them vulnerable to the effects of toxins. These characteristics include:

- ✓ Large surface area of neurons and nerve cells, which provide a large absorption area and amplify harm caused by chemicals
- ✓ High lipid constituent with a dry weight that is made up of approximately 50% lipids, resulting in higher absorption of lipophilic toxins

- ✓ Continuance of neurons and nerve cells through life, which results in accumulation of damage over years
- ✓ High blood flow to the nervous system, which results in increased exposure to toxins.

Since, the nervous system is vulnerable to harm because of its properties, it has evolved a defensive barrier, in higher organisms, to protect it from internal and external harm. This barrier is referred to as the blood-brain barrier (BBB). The blood-brain barrier is a unique anatomical system composed of astrocytes surrounding capillaries in the brain forming a compact hydrophobic layer. This layer allows the entry of nutrients and the removal of waste through the high blood flow that circulates through the nervous system. At the same time, it prevents the passage of large or hydrophilic compounds that cannot be actively transported into the CNS. Another defensive layer that the nervous system has against toxins is the choroid plexus. The choroid plexus consists of ependymal cells that synthesize cerebrospinal fluid (CSF) which selectively allows the transition of ions and nutrients to the nervous system and captures heavy metals. However, a number of neurotoxins are small in size or/and lipophilic which allows them to cross these barriers to the nervous system, where they can cause damage, resulting in neuronal impairment. The United States Environmental Protection Agency (EPA) was founded at 1998 in order to evaluate the neurotoxic effects of compounds. The EPA established several protocols and guidelines that aim to reduce the contaminants that have neurotoxic properties from environment and food industry. Moreover, in vitro and in vivo systems have been developed in order to provide better understanding of the toxic effect of different chemical substances. Neurotoxicity is a crucial pathology because it can impair cognitive, sensory and motor

functions. It has a major impact on drug discovery and development, as approximately 30% of drugs fail in phase I and II clinical trials due to neurotoxicity.

For centuries, humans have been associated with neurotoxins. Some relevant historical examples include:

(i) Deadly nightshade (Belladonna), used in Scotland (Macbeth to kill Danes) and Roman empire (to kill the emperor Claudius).

(ii) Snake venom used to kill the Egyptian, Cleopatra

(iii) Poison hemlock (*Conium maculatum*) used to kill the great Greek philosopher Socrates

(iv) Strychnine used by Dr. Thomas Neil Cream to kill his patients

(v) Arsenic used to poison Napoleon Bonaparte, George III of England, and Simon Bolivar.

(vi) Curare used by native Americans to fight European- settlers

(vii) Polonium used to eliminate the spy Alexander Litvinenko

(viii) Lead exposure to the masses in the Roman empire due to the wide construction of plumbing networks and canals and the tradition of boiling vinegar wine in lead pans producing lead acetate recognized as (lead sugar).

(ix) Stimulants and designer drugs more recently have become an epidemic which is slowly but steadily compromising the health of millions.

Exposure to neurotoxin arises from different sources such as plants, animal bites, bacteria, polluted water and food and mainly causes improper transmission of signals from nerves to:

- ✓ Muscles (skeletal, cardiac or smooth) leading to contraction or relaxation of muscles, resulting in paralysis or atrophy
- ✓ Glands (exocrine or endocrine) leading to increase or decrease of glandular secretions

The next section examines the effect of various neurotoxins and designer drugs on the processes of neurotransmission regarding the major neurotransmitters: acetylcholine, dopamine/norepinephrine, serotonin, GABA, glutamate. This section details how neurotoxins can affect receptor affinity, precursor availability, cofactor function, as well as neurotransmitter synthesis, storage, release, reuptake, and metabolism.

Effect of Neurotoxins and Designer Drug-TFMPP on Cholinergic Neurotransmission

Neurotoxins can modulate (increase or decrease) the actions of acetylcholine or cholinergic neurotransmission by interacting with the muscarinic and nicotinic receptors, enzymes of biosynthesis (choline acetyltransferase-synthesizing and degradation (acetylcholinesterase-degrading), storage (vesicles), and release. Acetylcholine is a small molecular weight and structurally a simple ester (no aromatic rings and long sidechain). It is readily and immediately metabolized by acetylcholinesterase and its process of termination by reuptake is not significant. However, there are toxins that affect the uptake of acetylcholine's biosynthetic precursor, choline. Hemicholinium (Figure 1.1) binds to the high-affinity choline transporter in the pre-synaptic neuronal terminal and is a potent inhibitor of the uptake of choline and thus acts as a cholinolytic toxin because it decreases the synthesis of acetylcholine[1,2]. Hemicholinium has no therapeutic value but is used as a tool for drug design research in cholinergic neurotransmission. Tetrodotoxin is a naturally occurring inhibitory neurotoxin present in the liver and sex organs of pufferfish, globefish, and toadfish. Tetrodotoxin inhibits the voltage-gated Na^+ channel in a highly potent and selective manner without effecting any other receptor or ion channel systems[3]. Choline acetyltransferase is the biosynthetic for acetylcholine and organic mercurial compounds[4,5] and trans-N-methyl-4-(1-napthyvinyl)-Pyridinium (MNPV) have been shown to inhibit this enzyme and decrease the cholinergic component of parasympathetic neurotransmission in the PNS[6]. Black Widow spider venom and botulinum toxin affect the

release of acetylcholine and there by act as a cholinomimetic or cholinolytics. The venom of the Black Widow spider releases acetylcholine from the vesicles and increases the cholinergic neurotoxin while botulin inhibits the release and decrease of cholinergic neurotransmission. Volatile and lipophilic organophosphate (sarin, tabun) have been shown to irreversibly inhibit the acetylcholine degrading enzyme, acetylcholinesterase and increase the cholinergic neurotransmission; leading to immediate death[7–9]. These toxins have mainly been used in bioterrorism. Anatoxin-a came into the limelight in 1961 after the death of cows that had been drinking from a lake containing an algal bloom in Canada. Anatoxin-a is a cyanotoxin produced by cyanobacteria and is responsible for rapidly progressive toxic effects such as twitching, convulsions, and rapid death by respiratory paralysis[10,11]. Humans were also affected by the contaminated drinking-water which resulted in death within minutes to a few hours. Anatoxin-a acts as an antagonist of muscular and neuronal nicotinic receptors. It is a structural analogue to acetylcholine (Figure 1.1.) and acts as a competitive antagonist by binding to the nicotinic (ion-channel) receptor with a higher affinity than acetylcholine. It acts as a postsynaptic depolarizing blocking agent that causes skeletal muscle overstimulation. When anatoxin-a was first discovered, it was called the Very Fast Death Factor (VFDF) due to this rapidly induced paralysis and death. Later, in 1977, the structure of VFDF was determined to be a secondary, bicyclic amine alkaloid, and the cyanotoxin was renamed anatoxin-a (Figure 1.1.).

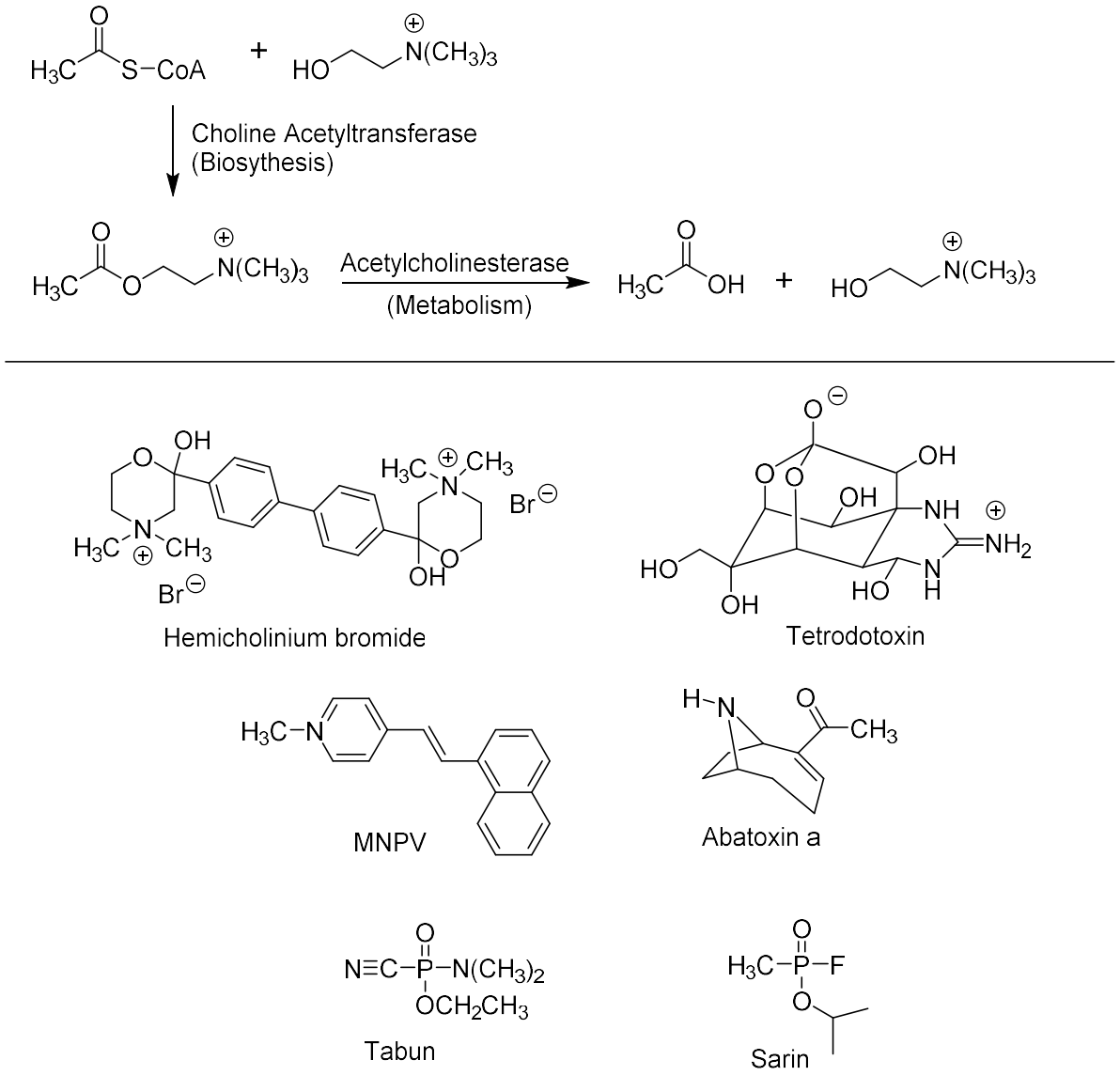

Figure 1.1. Acetylcholine and drugs affecting cholinergic neurotransmission

The word atropine was coined from Greek folklore, specifically the stories of Atropos, a Moirai goddesses, who ascertained the fate of every human. *Atropa belladonna* (belladonna or deadly nightshade) is a perennial herbaceous plant, belonging to the family Solanaceae, that produces atropine (Figure 1.2.). Belladonna alkaloids act as antagonists at muscarinic receptors (G-protein coupled receptor) and are toxic; although, some alkaloids have therapeutic utility at lower doses. At these lower doses alkaloids can induce headache, dizziness, weakness; sleep problems drowsiness; blurred vision, xerostomia; increase body temperature (atropine fever) due to decreased sweating or urination[12–14].

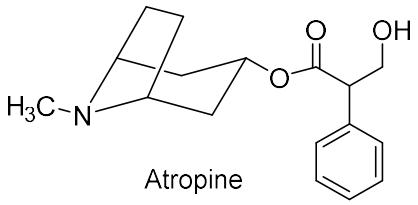

Figure 1.2. Atropine chemical structure

Curare (tubocurarine) was mainly used as an arrow poison by native Americans in Central and South America (Figure 1.3.). These alkaloids competitively and reversibly antagonize the nicotinic receptor leading to skeletal muscle paralysis [15]. Similarly, nicotine (Figure 1.3) addiction, as mentioned by C. Everett Koop Statement (U.S. Surgeon General) is the "chief, single, avoidable cause of death in our society and the most important public health issue of our time in the United States". Environmental Protection Agency classifies cigarette smoke as a Class A carcinogen [16]. Per the National Institute of Drug Abuse (NIDA-NIH), it is one of the most commonly abused drugs. Cigarette smoking is the leading known cause of preventable death. Nicotine has psychoactive effects and is used in a highly controlled or compulsive

manner. It exerts its effect by binding to nicotinic receptors in the central, autonomic, and somatic nervous systems. The pharmacologic and behavioral processes induced by nicotine are similar to heroin and cocaine although significantly milder. Nicotine readily crosses the blood brain barrier and immediately penetrates the CNS. It is estimated that nicotine reaches the brain within seconds after inhalation. At low doses, nicotine causes arousal, relaxation, and promotes involvement in attention, learning, reaction time, and problem solving, as well as a certain degree of euphoria and tolerance. It has a biphasic effect, initial stimulation followed by depression. It can depress the vital medullary respiratory center and cause central respiratory paralysis and hypotension due to medullary paralysis.

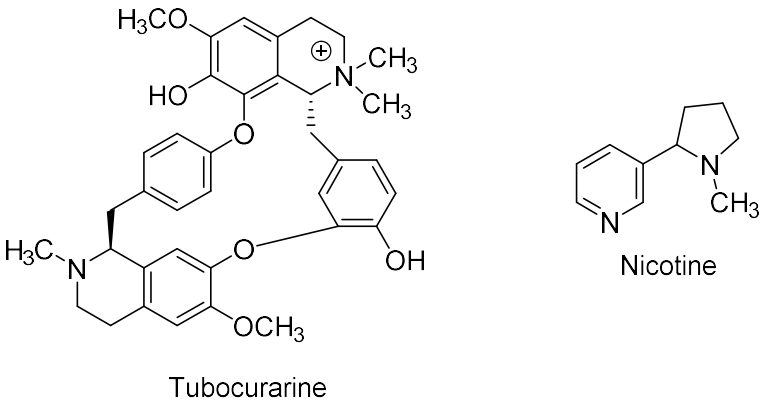

Figure 1.3. Tubocurarine and Nicotine chemical structures

Moreover, chemotherapeutic agents, such as methotrexate and 5-fluorouracil, and the antipsychotic agents, such as haloperidol, can potentially induce cholinergic neurotoxicity. Nevertheless, these agents are still used in modern day therapy because their therapeutic advantages are usually greater than their risks. It is important to differentiate between cytotoxicity and neurotoxicity produced by cytotoxic chemotherapeutic agents; in vitro axonal or

dendritic neuronal growth was suggested as one differentiating prospective. In addition, biochemical determination of the ability of chemicals to affect cellular mechanism such as the inhibition of acetylcholinesterase, have been suggested for neurotoxin identification and evaluation[17–19].

Neurotoxins play a major role in neurodegenerative disorders and have been linked to a variety of ailments including; Alzheimer's disease (AD), Parkinson's disease (PD), neurodevelopmental disorders, and many psychiatric disturbances. Neurotoxins most commonly have a negative impact on the brain's learning and memory function. Almost all neurodegenerative diseases are characterized by the production of aggregated misfolded proteins. In Alzheimer's the specific peptide is Beta-amyloid (A-beta). When enough A-beta proteins aggregate tightly a plaque is formed in the brain. This can eventually cause healthy brain cells to die. Tau is also another protein that aggregates in the neuron when hyperphosphorylated. Tau binds to microtubules which causes the synapse to lose function and changes neurotransmitter production and release. In ADA-beta and tau proteins are considered neurotoxic. The A-beta plaques can cause removal of acetylcholine (ACh) due to binding of the plaque to the neuronal cell. Eventually the production and release of ACh is diminished. This loss in cholinergic activity correlates with disruption of memory and an increase in severity of Alzheimer's disease. The loss of neuron function in AD cannot be reversed, but the rapid degeneration can be slowed down by the use of acetylcholinesterase inhibitors such as donepezil and rivastigmine (Figure 1.4.).

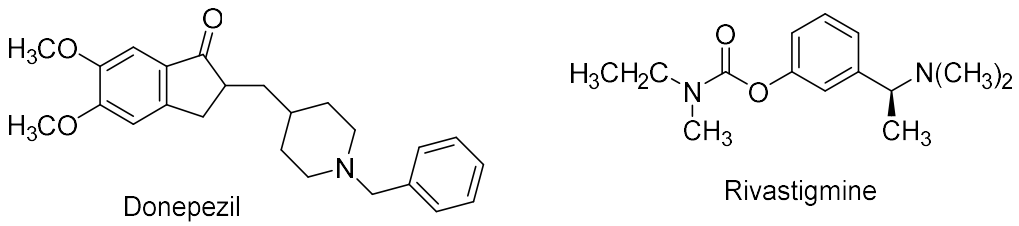

Figure 1.4. Acetylcholinesterase inhibitors

Neurotoxins Classification

Based on their varying sites of the action in the neuron, neurotoxins exert their effect and can be classified as axonal neurotoxins, presynaptic neurotoxins and postsynaptic neurotoxins. Neurotoxins can also be classified based on their target of action in the neuron; presynaptic or postsynaptic neourotoxins[1].

Axonal Neurotoxins

An axon is a thin elongated projection of a nerve cell, that facilitates the transport of action potentials. Generally, axons are considered transportation routes of the nervous system, forming nerves when they cluster. Axons can be classified based on the presence of myelin sheaths, formed by glial cells, as either myelinated and unmyelinated axons. Axon malfunction is one of the main reasons for several neurological disorders, that impact neurons including multiple sclerosis and schizophrenia. Axons can be affected by neurotoxins. For example, Tetrodotoxin, has shown to inhibit depolarization by inhibiting flow of sodium ions resulting in the blockade of action potentials. Another example of an axonal neurotoxin is batrachotoxin, which is produced in *Phyllobates aurotaenia* frogs. Batrachotoxin exerts its toxic effect by irreversibly enhancing a

neuron's permeability to sodium (Na+). Any toxins affecting the sodium channel can affect the neurotransmission in nicotinic receptors and affect cholinergic neurotransmission.

Presynaptic Neurotoxins

Presynaptic neurotoxins are the most common type of toxins found in neurodegenerative disorders. They target the terminal axon of the neuromuscular junction (NMJ). Similar to axonal neurotoxins, presynaptic neurotoxins increase sodium permeability resulting in depolarization of nerve and muscle membranes. Some presynaptic neurotoxins are modified phospholipase A2 toxins. They cause a dumping effect of neurotransmitters then cause damage to the axon. This interferes with neurotransmitter production and release as well as irreversible damage to the neurons. These types of neurotoxins can be produced by bacterium, various scorpions, black widow spiders, and species of snakes. Interestingly, the most potent presynaptic neurotoxin and the most toxic biological compound known is botulinus toxin produced by Clostridium botulinum. Clostridium botulinum and Clostridium tetani neurotoxins, are the causes of two fatal neurologic disorders, botulism and tetanus. Botulism is transmitted by the ingestion of contaminated food and it is usually accompanied by blurred or double vision, facial weakness, paralysis, difficulty breathing, swallowing or speaking. The symptoms of tetanus include stiffness or rigidity of skeletal muscles, irritability, and convulsive spasms. Multiple studies have demonstrated that botulinus toxin exerts its toxic effects through inhibition of the release of several neurotransmitters such as dopamine, acetylcholine, glutamate, GABA, and serotonin. The inhibition of the secretion of these neurotransmitters in the nervous system can result in

several neurologic disorders including AD and PD. However, botulinum toxin in low doses and a controlled medical environment can have beneficial effects. The FDA approved Botulinum toxin A (onabotulinumtoxinA) (Botox®) to be used for temporary improvement of frown lines on a patient's face and for treatment of chronic migraines. Botulinum toxin Type A has a high affinity toward cholinergic receptors located on the nerve terminals. It enzymatically cleaves a protein called SNAP-25 which is vital for the regulation of the release of neurotransmitters (especially acetylcholine) from presynaptic vesicles at the nerve endings. SNAP-25 facilitates the fusion of the vesicles with the cell membrane. Its cleavage results in the inhibition of neuromuscular transmission by inhibiting acetylcholine release at the neuromuscular junction. It has also been found that botulinum toxin Type A not only inhibits but can alter the release of neurotransmitters. It does so by blocking peripheral sensitization which in turn will indirectly block central sensitization. Several studies suggested this as the mechanism and the reason behind botulinum toxin Type A effectiveness in treating chronic pain.

Postsynaptic Neurotoxins

Postsynaptic neurotoxins also target the neuromuscular junction but are typically polypeptides of various sizes. Beta-amyloid peptides represent one example of postsynaptic neurotoxins. These neurotoxins specifically bind to the acetylcholine receptors on the muscle-end plate blocking the neurotransmitter from binding. This type of damage is more rapid, but reversible in some cases.[1] Postsynaptic neurotoxins such as the toxins from the snake families *Elapidae* and *Hydrophidae* are usually much less toxic than the presynaptic snake neurotoxins. Toxins from these snake

families can be classified as small toxins, which have 60 to 62 amino acids and four disulfide bridges (Ca 7000 molecular weight). Sea snake neurotoxins in addition to most of cobra species neurotoxins, are considered large toxins that contain 71 to 74 amino acids and five disulfides (Ca 8000 molecular weight). Large toxins have higher affinity at ACh receptors than small toxins. Additionally, small postsynaptic snake toxins have higher vulnerability toward chemical degradation.

Designer drugs such as MDMA and TFMPP mainly exert their actions by targeting the monoaminergic neurotransmission and have minimal effect on acetylcholine[20]. A recent study that evaluated the *in vitro* effects of a wide vareity of illicit drugs and new psychoactive substances (cathinones-MDPV, α-PVP, mephedrone, 4-MEC, pentedrone, methylone, cannabinoids-JWH-018, hallucinogenics-phenethylamines, 4-fluoroamphetamine, benzofurans-5-APB, 6-APB, 2C-B, NBOMes-25B-NBOMe, 25C-NBOMe, 25I-NBOMe, arylcyclohexylamines-methoxetamine, and the piperazine derivatives-mCPP, TFMPP, BZP) further validated that these drugs have minimal effects on cholinergic neurotransmission [21].

Effect of Neurotoxins and Designer Drugs on Monoaminergic (Dopaminergic, Adrenergic and Serotonergic) Neurotransmission

Tyrosine (derived from the Greek word tyros = cheese, 4-hydroxyphenylalanine), a non-essential amino acid, is the precursor for dopamine, norepinephrine and epinephrine biosynthesis. In nerves, tyrosine is converted to levodopa (L-DOPA) by the enzyme tyrosine hydroxylase and this is the rate limiting enzyme in the formation of L-DOPA, which is the intermediate metabolite for dopamine, norepinephrine and epinephrine. Tyrosine hydroxylase requires the presence of divalent iron to accomplish its catalysis. In adrenergic neurons, L-DOPA is converted to dopamine which is then converted to norepinephrine. There are various endogenous and exogenous neurotoxins that inhibit the activity of tyrosine hydroxylase. N-methyl-norsalsolinol (2-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; 2-MDTIQ) (Figure 1.5.) is present in the brain and cerebrospinal fluid and can inhibit tyrosine hydroxylase activity and increases the risk for nigral dopaminergic neurodegeneration leading to PD[22]. Salsolinol derivatives (N-methyl-norsalsolinol, salsolinol and N-methyl-salsolinol) are synthesized endogenously by condensation reaction (non-enzymatic reaction) between dopamine and aldehydes or pyruvic acid. Other synthetic drugs such as demser and metyrosine (Figure 1.5.) also inhibit tyrosine hydroxylase and have been approved for the treatment of velocardiofacial syndrome associated psychosis and pheochromocytoma. Other metabolites and adducts such as H_2O_2, formed from dopamine metabolism, can also inhibit the activity of

tyrosine hydroxylase[23]. Lipopolysaccharides (LPS) present in gram negative bacteria, functions as a toxin that affects tyrosine hydroxylase and is capable of inducing PD like symptoms[24]. Lipopolysaccharides are toxic to plants and humans. They are usually found in Gram-negative bacteria and chemically are made of lipid A (hydrophobic domain known-endotoxin) and a non-repeating "core" oligosaccharide and a distal polysaccharide (or O-antigen). Endotoxin lipopolysaccharides obtained from bacteria have been shown to induce inflammation in the nigrostriatal tract. Neuroinflammation occurs due to glial activation (astrocytes and microglia) which leads to a release of proinflammatory mediators such as cytokines, lipid metabolites, reactive oxygen species and reactive nitrogen species. These toxic mediators have shown to induce the death of dopaminergic neurons in the substantia nigra [25]. Beta-carbolines are an endogenous neurotoxin present in the CNS and CSF (cerebrospinal fluid). They are synthesized in the brain using tryptophan and its derivatives as the precursor. Beta-carbolines and their derivatives have also been shown to inhibit tyrosine hydroxylase[26] (Figure 1.5.).

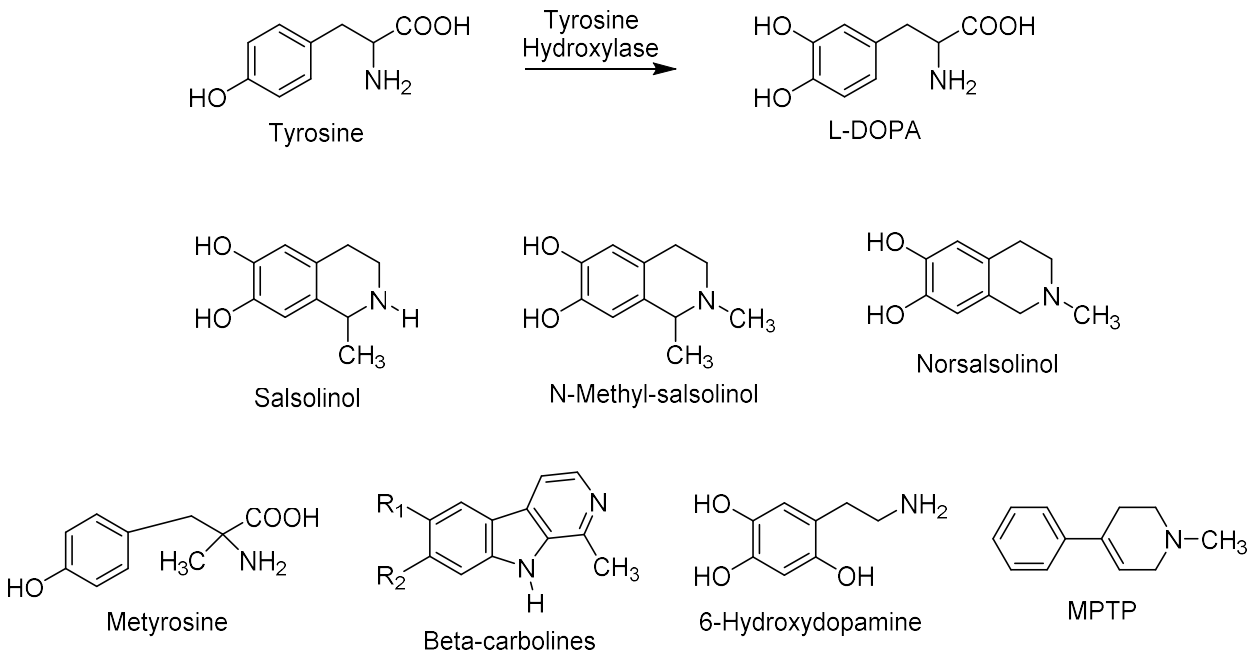

Figure 1.5. Tyrosine hydroxylase inhibitors

Another way tyrosine hydroxylase activity can be inhibited is through divalent metal accumulation or exogenous[27,28]. Exogenous neurotoxins 6-hydroxydopamine and MPTP have shown to inhibit tyrosine hydroxylase (Figure 1.5.). In the 1960s, 6-hydroxydopamine (6-OHDA) was the first selective dopaminergic neurotoxin to be discovered and its use aided in the understanding dopaminergic neurotransmission by serving as a valid animal model for drug discovery. 6-OHDA selectively destroys dopaminergic neurons in the substantia nigra, which results in dopamine depletion in the striatum. In the next few decades more selective neurotoxins were discovered with higher affinity toward the binding sites on nerves leading to neurotoxin accumulation on the nerves resulting in the interruption of crucial intraneuronal signaling, leading to cell death[29–32]. MPTP is a specific dopaminergic neurotoxin that causes nigrostriatal dopaminergic damage and induces behavioral and biochemical symptoms similar to PD.

Reserpine and tetrabenazine affect the storage of monoamines in the vesicles (Figure 1.6.). Reserpine is an alkaloid isolated from *Rawfolia serpentina* and has shown to deplete monoamines by irreversibly blocking vesicular monoamine transporter (VMAT). This botanical neurotoxin has been well documented in the ancient Indian literature (1000-800 B.C.) as Sarpagandha. However, Plumiers a French herbal expert, coined the word Rauwolfia to show his respect to the other botanist Leonard Rauwolf of Augsburg. *Rauvolfia serpentine* (Indian snakeroot, devil pepper) is a perennial undershrub mainly seen in the Indian subcontinent and belongs to the family, Apocynaceae. Stimulants (amphetamine, tyramine) and substances such as ephedrine from botanicals (*Ephedra sinica*) have shown to increase the release of monoamines (Figure 1.6.). The effect on the pre-synaptic stored monoamine is a dose-dependent effect. At low doses these stimulants increase the monoaminergic neurotransmission resulting in psychotic

symptoms. At high doses these stimulants deplete the monoamine, leading to decreased dopaminergic neurotransmission which can then lead to depression. Substances of abuse have also shown to affect the release of monoamines. Cocaine and other stimulants have shown to block the reuptake of monoamines. Monoaminergic reuptake is an important process in the neurotransmission of monoamine. When monoamines are not subject to reuptake, the excess monoamine in the synaptic cleft is taken by the dopamine transporters (DATs) into the presynaptic neuron. 6-hydroxydopamine or dopamine antagonists have shown to increase the dopamine (D_2) receptor density of in the striatum leading to tardive dyskinesia (movement disorder).

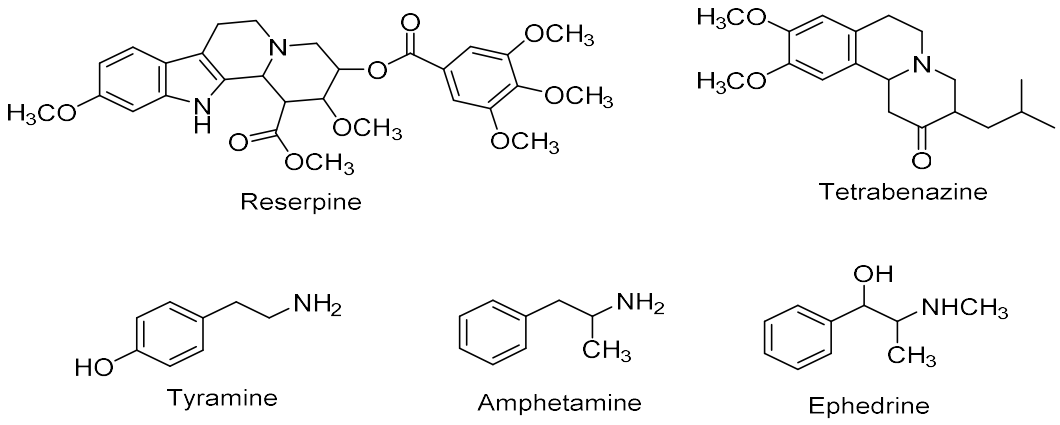

Figure 1.6. Drugs altering monoamine release and reuptake

Monoamines are metabolized by catechol-o-methyl transferase (COMT) and monoamine oxidase (MAO). Monoamine oxidase A and B are the primary enzymes that break down monoamines by catalyzing the oxidation of dopamine, norepinephrine, serotonin. This is accomplished by adding oxygen to the carbon adjacent to the amine which results in a loss of the amine group, terminating the actions of these biogenic neurotransmitters. The MAO isozymes are found

bound to the outer membrane of mitochondria in most cell types of the body. Neurotoxins, stimulants and alcohol significantly affect the activity of monoamine oxidases. TFMPP derivatives also significantly affect the monoamine oxidase activity. The neurotoxic effect of MPTP is mainly due to monoamine oxidase-B, which converts MPTP to its active neurotoxic metabolite MPP+ (Figure 1.7.). Another selective neurotoxin that destroys dopaminergic nerves is rotenone, which is a mitochondrial complex I inhibitor that causes the accumulation of synuclein on the nerves which caused a symptomatic presentation of PD in animals. The importance of selective neurotoxins is the ability to use them in animal modeling of multiple neural disease including; PD, AD, attention-deficit hyperactivity disorder (ADHD), tardive dyskinesia, and schizophrenia.

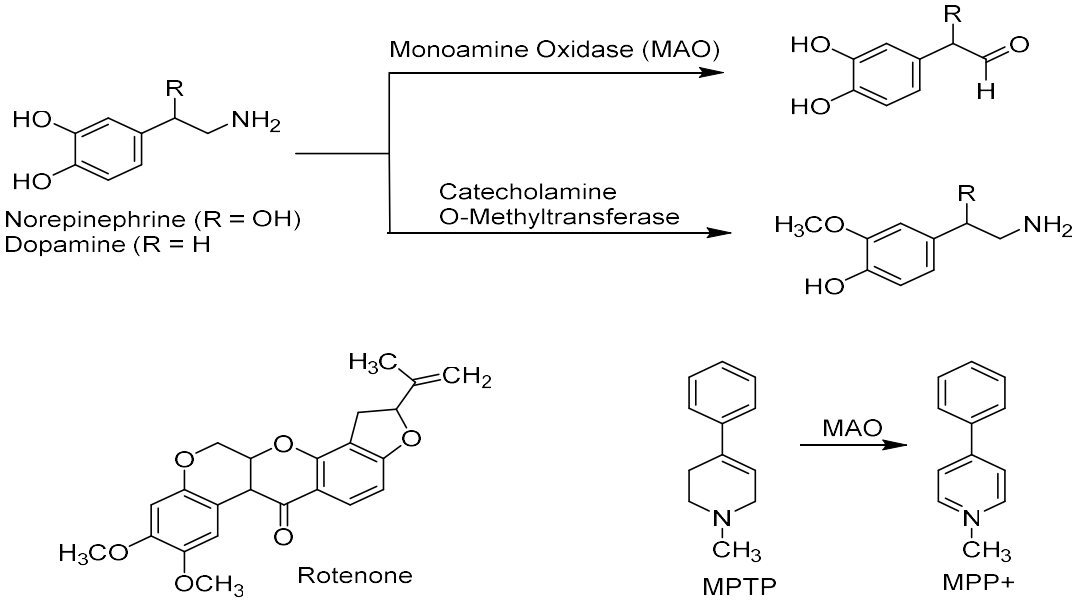

Figure 1.7. Drugs altering MAO activity

Toxins affecting aromatic amino acid processing in nerves are endotoxins. MPP+ and the drug phenelzine are examples of these endotoxins[33–35]. With regard to the dopamine beta-hydroxylase, the colchicine and 6-OHDA but not cytochalasin B, affect the axonal transport of

dopamine-beta-hydroxylase in the brain[36]. N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) is a specific adrenergic neurotoxin with long duration of action (Figure 1.8.). DSP-4 selectively and reversibly inhibits dopamine-beta-hydroxylase (DBH) and therefore has no effect on dopamine and serotonin[37,38]. LY134046 and 2,3,4,5-Tetrahydro-1H-2-benzazepine (THBA; 1) have shown to inhibit Phenylethanolamine-N-Methyl Transferase[39,40].

3-Nitropropionic acid and methamphetamine inhibit the ability to decarboxylate 5-HT in different regions of the brain[41] (Figure 1.8.). 5,7-dihydroxytryptamine (5,7-DHT), parachlorophenylalanine, and cobratoxin have been shown to significantly inhibit 5-hydroxytryptophan and deplete serotonin [42,43]. Neuronal inflammation can induce degradation of tryptophan[44,45]. Endogenously, dopamine has also shown to inhibit tryptophan hydroxylase[46]. Exogenously, infection (Pneumococcal meningitis, Herpes) can lead to tryptophan degradation[47]. Stimulants / Drugs of abuse have shown to inhibit the activity of tryptophan hydroxylase[48]. Furthermore, monoaminergic transporters are also affected by MDMA and amphetamine & its derivatives[49,50].

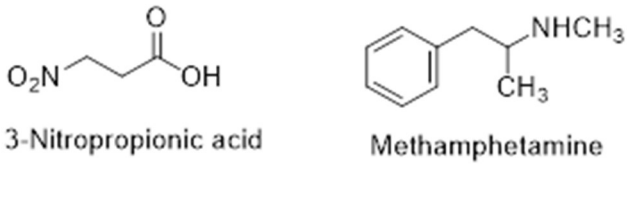

Figure 1.8. Drugs that alter hydroxylase activity

Effect of Neurotoxins and Designer Drugs on Gabaergic Neurotransmission

The major inhibitory neurotransmitter in the CNSis GABA (γ-aminobutyric acid). Stimulation of the post synaptic ionotropic GABA-A receptor exhibits inhibitory neuronal activity. One of the most common neurotoxins that modulates GABA-A receptor is one of the most abused substances, alcohol (ethanol). Ethanol's ability to affect the development of fetal brain is well established. In the 1970s, scientist discovered fetal alcohol syndrome which is caused by alcohol ingestion of mothers during pregnancy and is characterized by brain damage and developmental delays in children. While the outward symptoms of ethanol are well known, internal actions are less obvious, and include affects to our neurotransmitter systems, such as inhibition of excitatory glutamate receptors and facilitation of the inhibition of GABA receptors. It is because of these inhibitions that binge drinking symptoms such as tremors, hallucinations, seizures, constricted alertness, and autonomic instability occur. When this behavior is repeated, it is possible to cause not only early abstinence symptoms, but also glutamate-induced excitotoxicity and permanent neuronal damage, which can contribute to longer lasting neurological disorders like cognitive impairment, dependence, and increasing the risk for dementia. Ethanol also increases the fluidity of the neuronal membrane by increasing the amount of saturated fats in the membrane and changing the ratio of unsaturated to saturated fats [51–53]. This change in the membrane structure affects transport processes across the cell surface involving calcium and other electrolytes and the active transport of neurotransmitters such as GABA.

Tetanus toxin (Tetx) appears to have no significant effect on the L-glutamic acid decarboxylase (GAD) activity and gamma-aminobutyric acid (GABA) content in the brain[54]. However, Anisatin, a pure toxic substance isolated from the seeds of a Japanese plant (Illicium anisatum) acts like picrotoxin, and exhibits a non-competitive GABA antagonist effect[55]. There are various other neurotoxins such as muscimol, gaboxadol (4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol), beta-carboline derivative-harmane[56], (α-bungarotoxin (Snake neurotoxin), alpha-conotoxin [57], and bicuculline that affects the GABA neurotransmission by acting at the GABA-A and GABA-B receptors (Figure 1.9.). Interestingly the insecticides dieldrin and fipronil have also been shown to target the GABA-A receptor[58].

The abuse of the use of the common anxiolytics have increased in the recent years[59,60]. Benzodiazepines and barbiturates bind to the GABA-A receptor and increase the chloride influx leading to hyperpolarization. The most noteworthy aspect is that barbiturates do not require endogenous GABA to exert its action, unlike benzodiazepines. However, both these drugs have shown to increase cognitive impairment, fall risk, and weight gain. Flumazenil is an antagonist of the GABA(A) receptors and has been used to overcome the barbiturates and benzodiazepines induced toxicity.

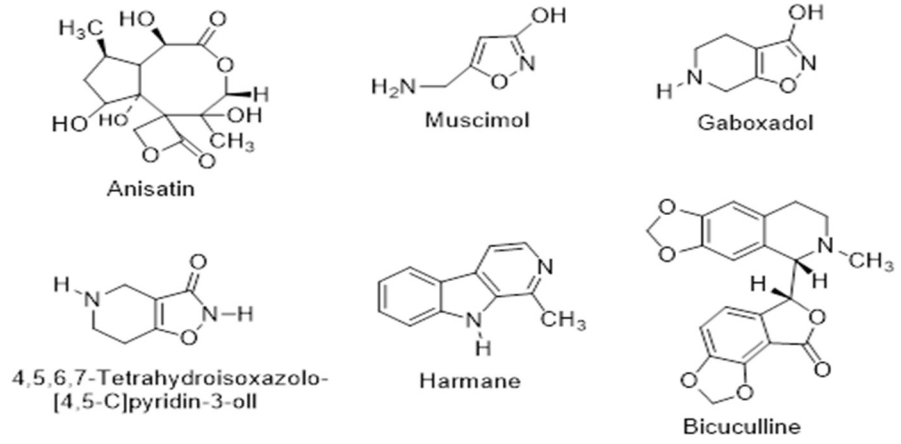

Figure 1.9. Glutamatergic neurotoxins

Miscellaneous Neurotoxins

Neurotoxins have also been found to inhibit membrane depolarization by deterring neuron control over ion concentrations across the cell membrane through altering Na+ or K+ permeabilities[61]. These toxins can also inhibit interneuron signaling by hindering communication between neurons across the synapse through altering the release of neurotransmitter or altering the receptors[62,63]. Oher neurotoxins exhibit their effect via different mechanisms. For example, mercury binds to sulfhydryl, phosphoryl, carboxyl, amide, and amine groups of proteins resulting in protein precipitation, enzyme inhibition, and general destructive action. Mercury neurotoxicity is caused by the presence of reactive oxygen species which act through diminution of glutathione, amending Na+/K+ ATPase activity, and reducing mitochondrial function.

Designer Drug as Neurotoxins

Designer Drug-TFMPP affects the major neurotransmitters and thereby alters neurotransmission: Addiction is defined as a state of incontrollable repetitive usage of substance or engagement in activity to seek rewarding stimuli even though it results in harmful consequences. The main reasons that lead to this detrimental behavior include seeking the feeling of pleasure, alleviating stress, and enhancing performance. Addiction usually starts as casual use which then develops to abuse and dependence. Addiction can be accompanied with self-awareness of the harmful outcomes and the inability to stop due to dependence. Interestingly, the abuse of psychoactive substance is the primary cause of avoidable diseases and premature death. Psychoactive substances provoke pleasure and dependence primarily through their action in the dopaminergic neuronal tract (mesolimbic system) and glutamatergic pathway in the prefrontal cortex. Addiction and dependence have been well-known throughout the history of civilizations. In ancient Egypt, blue lotus flowers were ingested for their sedative and euphoric effects. In Central Asia, a mushroom named Amanita muscaria which has a psychoactive substance, has largely been consumed for the past 4000 years. In the 7th century, Islamic literature mentions people who were suffering from alcoholism were tied and isolated in an attempt to overcome their addiction. Moreover, during the middle ages in Rome, debtors who were unable to repay their debt due to addiction were forced to work as slaves. In the 18th century, opium addiction became a global problem which forced the authorities to ban its planting and consumption. Also, in

Europe, alcoholism was a serious issue among the working classes. Similarly, in American continent, substance abuse became a threat to the society in 19th century when morphine, heroin, and cocaine were marketed as unregulated therapeutic agents. In 1864, The New York State Inebriate Asylum was established as the first hospital to consider alcoholism as a mental health condition. With globalization and industrial development, addiction became a worldwide public health problem[64]. Furthermore, clandestine drug manufacturers were continuously searching for new psychoactive substances of abuse leading to the development of "Designer drugs". Designer drugs are synthetic structural analogues that are synthesized to mimic the psychoactive effects of prohibited drug of abuse. Examples of designer drugs include substituted amphetamines, synthetic cathinones, synthetic cannabinoids, and piperazine derivatives. Designer drugs are structural analogues of drug of abuse, they may produce comparable toxicological and pharmacological actions resulting in neurotoxicity and neuronal cell death. They may exert their neurotoxicity by generating oxidative stress, inducing mitochondrial dysfunction, and initiating apoptosis. As mentioned previously, these mechanisms have been associated with neurodegenerative diseases such as AD and PD. Piperazine drug of abuse derivatives can easily cross blood-brain barrier making the areas that are affected by neurodegenerative disease susceptible to their actions. When designer drugs were introduced to the market, they were not classified as controlled substances. However, with research and clinical findings some designer drugs were listed as controlled substance limiting their use and manufacture due to their harmful effects which, in some cases, involved death. Frequently designer drugs are marketed as safe alternative to controlled drug. This claim is based on the lack of data reporting their effects, suggesting the need for more research to provide better understanding of their complete effects. 3-Trifluoromethylphenylpiperazine (3-TFMPP) (Figure 1.10.) is a well-known designer drug that

is being sold and abused on a global scale as party pills. Its combination with the other piperazine designer drug "benzylpiperazine" is currently being falsely marketed as legal and safer alternative to MDMA "Ecstasy" and even called Legal X. The pharmacologic and toxic actions of 3-TFMPP are not fully understood due to the limited research involving these drugs. Further research on 3-TFMPP effects is needed in order to establish suitable treatment approaches to avoid its probable toxic effects and dependence as well.

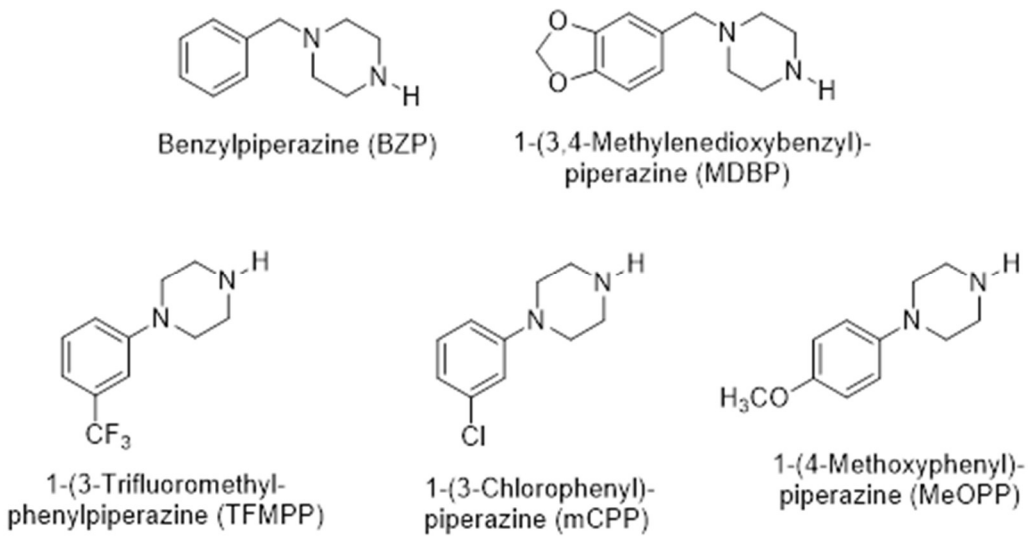

Figure 1.10. Piperazine designer drugs

Piperazine is heterocyclic molecule that is composed of two nitrogen atoms in opposite positions and four carbons dispersed between them (Figure 1.10.). Designer drugs that have piperazine structures can be categorized into one of two groups. First, the benzylpiperazines group which include substances like N-benzylpiperazine (BZP) and 1-(3,4- methylenedioxybenzyl) piperazine (MDBP). Second, the phenylpiperazines group which includes substances like 1-(3-chlorophenyl) piperazine (mCPP), 1-(3-trifluoromethylphenyl) piperazine (TFMPP), and 1-(4-

methoxyphenyl) piperazine[65] (Figure 1.10.). These substances gained their popularity as party pills under different street names such as "natural highs, Legal X, Pep X, Frenzy or Nemesis" [66]. Generally, the combination of BZP and TFMPP are the most common misused piperazines since they are marketed as harmless and risk-free alternates to 3,4- methylenedioxymethamphetamine (MDMA) and amphetamines. BZP acts primarily on dopamine pathway while TFMPP affects the serotonin pathway. Trifluoromethylphenylpiperazine (TFMPP) is a piperazine designer drugs which is classified as scheduled I controlled substance. Piperazine designer drugs gained popularity among people due to their psychoactive properties. TFMPP is a substituted phenyl amine which is synthesized by altering the original piperazine structure (Figure 1.10.). It is usually administered in combination with BZP to gain synergistic psychostimulatory effects which are analogous to the illegal drug, MDMA. TFMPP is marketed as safer replacement to MDMA under several street names such as "X4, PEP, Twisted, Flying Angel and Wicked High". Piperazines were synthesized primarily as anti-helminthic. However, N-benzylpiperazine was found to increase the cholinergic neurotransmitter acetylcholine. Enhancement of cholinergic neurotransmission results in enhanced learning and memory. Several studies reported the abuse of TFMPP globally[66–81]. TFMPP was utilized in several studies as a valid pharmacological tool (table 1.1).

Use	References
Evaluate the role of monoamines in Addiction	82,83
Study of Aggressive actions	84,85
Role of monoamines and hormones in Anorexia	86,87
Establish the effect of monoamines on o Learning abilities	88–90
Establish the effect of monoamines on Memory formation	91
Establish the effect of monoamines on Locomotory ability	92
Establish the effect of monoamines on Psychoactive behavior	75
Influence of monoamine in Convulsion	93,94
Consequences of monoamines and hormones in Depression	95,96
Mechanisms involved in Neuronal firing	97
Nociception mechanisms	98
Release of Neurotransmitters mechanisms	99
Reward pathway	100
Synaptic Neurotransmission	101
Synthesis of Neurotransmitters	102

Table 1.1. TFMPP as a valid pharmacological tool

Applications/ Uses of Neurotoxins

Neurotoxins have been studied to be the major cause of deadly disease, but they have also been used as pharmacologic agents to treat various conditions. If neurotoxins enter the body in the outcome is most often deadly, but through the use of pharmacological agents and intense monitoring they could provide a benefit for patients. This is one example of how scientist can use toxic pathogens and modify them to have a beneficial impact.

Neurotoxins have been used for:

- Cosmetic purpose
- Pesticides
- Food preservative
- As a pharmaceutical agent: psychostimulant, dystonia and pain
- Model for neurodegenerative diseases

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2. Investigate the Pharmacokinetic and Pharmacodynamic Effects of Trifluoromethylphenylpiperazine (TFMPP) Derivatives

2.1. Introduction

The abuse of TFMPP derivatives has increased in the United States of America and throughout the World making it important to fully understand its pharmacological effects. Generally, TFMMP is consumed orally and it is available as powder, tablet or capsule. A single oral dose of 3-TFMPP 60mg resulted in plasma concentration of 24ng/mL (Tmax = 90 minutes)[1]. TFMPP showed an oral clearance (CL/F) of 384 L/hour and had two disposition phases with calculated half-lives of 2 hours and 6 hours[1]. The lipophilic structure of TFMPP allows its passage through the blood brain barrier (BBB). The increased levels of TFMPP can lead to augmented effects on mood[2]. An interesting study of the distribution of TFMPP in the body showed that half an hour after TFMMP consumption[3]. With regard to metabolism, CYP2D6, CYP1A2 and CYP3A4 metabolize TFMPP. CYP2D6 was found to be responsible for 80.9 % of TFMPP metabolism. While CYP1A2 and CYP3A4 controlled 11.5% and 7.6 % of TFMPP metabolism respectively[4,5]. Hydroxylation was found to be the major reaction in TFMPP metabolism[4]. Multiple studies demonstrated that TFMPP undergoes extensive metabolism mostly by hydroxylation of the aromatic ring and by degradation of the piperazine moiety to N-(3-trifluoromethylphenyl)

ethylenediamine, N-(hydroxy-3-trifluoromethylphenyl) ethylenediamine, 3-trifluoromethylaniline, and hydroxy-3-trifluoromethylaniline[4,6,7]. TFMPP can have significant effects in both brain and peripheral nervous system. TFMPP acts mainly on monoaminergic neurotransmitters such as serotonin (5-HT) dopamine (DA) and norepinephrine (NE). However, the main effects of TFMPP are due to its high affinity towards 5-HT receptors (5-HT1A, 5-HT1B, 5-HT1D, 5-HT2A, and 5-HT2C). TFMPP acts as an agonist at all 5-HT receptors excluding the 5-HT2A receptor where it displays weak partial agonist or antagonist action[8,9]. Because of its action on monoaminergic neurotransmitters. TFMPP agonistic activity on 5-HT neurotransmission result in its hallucinogenic effects[10], anti-nociceptive effects[11] and anxiogenic effect[12]. Additionally, Elverfors & Nissbrandt, 1992[13] confirmed that TFMPP induced DA release in the substantia nigra, striatum and limbic forebrain. Furthermore, TFMPP inhibit the K+-evoked release of acetylcholine[14] and increase DA release in dose dependent manner[15]. Correspondingly, TFMPP caused a reduction in epinephrine content in rat hypothalamus[16] and decreases the frequency of pilocarpine-induced epilepsy in rats[17].

Receptor-binding assays are a critical component in identification and characterization processes of most known and unknown drug targets. It is used to evaluate the interactions between a chemical and receptors. It is highly beneficial tool in the studies of receptor-ligand interactions. Receptor binding assays can provide an illumination about pharmacodynamic mechanisms of actions of drugs and substances of abuse including TFMPP derivatives. These assays provide screening of novel psychoactive compounds for pharmacological and functional activity of central nervous system (CNS) receptors, channels, and transporters in mice. Inadequate ADME (absorption, distribution, metabolism, and excretion) properties of a possible new drug is the

main cause behind about 40-50% of drug failure in clinical trials [18,19]. Preclinical ADME properties screening will lead to the early exclusion of non- suitable drug candidates that shows unsatisfactory features leading to a better attention to potential drug candidates. Using software that can evaluate the ADME properties will indeed help in the process of selecting probable drug candidates and suitable dosage forms. Therefore, precise prediction of ADME properties of any drug before starting experimental testing can save time, reduce cost and even help in new drugs optimization in way that make it demonstrate adequate ADME performances [20]. Professor William L. Jorgensen invented QikProp software which provides a fast, precise and convenient ADME prediction. It can predict molecular properties in addition to correlating new drug properties with those of 95% of well-established drugs. QikProp software establishes its pharmaceutical properties prediction based on the full 3D molecular structure which expediates and improve decisions about a molecule's appropriateness for further research. Recently, It has been used in several studies to virtually elucidate and optimize the properties of new drugs[21–24].

According to Pajouhesh 2005[25], a successful CNS drug should have the following descriptors in QikProp software:

- Potent activity: low to subnanomolar
- Highly selective
- Molecular weight (MW) < 450
- Minimal hydrophobicity (clogp < 5)
- Number of H-bond donor < 3

- Number of H-bond acceptor < 7
- Number of rotatable bonds < 8
- H-bonds < 8
- Polar surface area < 60–70 $Å^2$

Additionally, CNS penetration is likely if:

- MW ≤400
- *cLog p* ≤ 5
- Hydrogen bond donor (HBD) ≤3
- Hydrogen bond acceptor (HBA) ≤7

In summary, data on the pharmacokinetics and pharmacodynamics properties of the piperazine designer drugs are limited. Hence, in this study we assessed piperazine designer drugs receptors binding profile and the pharmaceutical properties.

2.2. Methods

2.2.1. Receptor binding assay

Receptor binding assay for TFMPP derivatives was performed as per the protocol of National Institute of Mental Health Psychoactive Drug Screening Program (PDSP) at the University of North Carolina, Chapel Hill. Initially each isomer was tested in a primary assay at a concentration of 10uM for its ability to displace a standard ligand at each receptor and transporter subtype. Compounds which produced greater than 50% binding inhibition in the primary assay were tested further to determine receptor or transporter affinity constants (Ki: affinity of a ligand for the receptor in nM) in a secondary binding assay.

2.2.2. Computational Assessment

In this study, QikProp filter from Schrödinger was used to calculate a number of pharmacokinetic and pharmacodynamic properties of TFMPP derivatives. Drug molecules with favorable ADME properties have been identified as the primary cause of successful candidate molecules in drug discovery and development. The QikProp set of descriptors (CNS activity, MW, HBD, HBA, QPPCaco, QPlogBB, QPPMDCK, Human Oral Absorption, % Human Oral Absorption, Rule of Five, Rule of Three) were selected to describe this aspect of the compounds permeability and activity in CNS.

2.2.3. Software

Schrödinger Release 2019-2: QikProp, Schrödinger, LLC, New York, NY, 2019.

2.2.4. The Standard Receptor Ligands and Radioligand Assay Conditions Used for The Displacement Studies in The Secondary Binding Assays

2.2.4.a. Radioligand Assay Conditions for Serotonergic Receptor Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
5-HT1A	[^{3}H]8-OH-DPAT (0.5 nM)	Methysergide	Standard Binding Buffer
5-HT1B	[^{3}H]GR127543 (0.3 nM)	Ergotamine	Standard Binding Buffer
5-HT1D	[^{3}H]GR127543 (0.3 nM)	Ergotamine	Standard Binding Buffer
5-HT1E	[^{3}H]5-HT (3 nM)	5-HT	Standard Binding Buffer
5-HT2A	[^{3}H]Ketanserin (0.5 nM)	Chlorpromazine	Standard Binding Buffer
5-HT2B	[^{3}H]LSD (1 nM)	Methysergide	Standard Binding Buffer
5-HT2C	[^{3}H]Mesulergine (0.5 nM)	Chlorpromazine	Standard Binding Buffer
5-HT3	[^{3}H]LY278584 (0.3 nM)	LY278584	Standard Binding Buffer
5-HT5a	[^{3}H]LSD (1 nM)	Ergotamine	Standard Binding Buffer
5-HT6	[^{3}H]LSD (1 nM)	Chlorpromazine	Standard Binding Buffer
5-HT7	[^{3}H]LSD (1 nM)	Chlorpromazine	Standard Binding Buffer

Standard Binding Buffer: 50 mM Tris HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4

Table 2.1. Assay conditions for serotonergic receptor affinity

2.2.4.b. Radioligand Assay Conditions for Adrenergic Receptor Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
Alpha1A	[^{3}H]Prazosin (0.7 nM)	Urapidil	Alpha1 Binding Buffer
Alpha1B	[^{3}H]Prazosin (0.7 nM)	Corynanthine	Alpha1 Binding Buffer
Alpha2A	[^{3}H]Clonidine (1 nM)	Oxymetazoline	Alpha2 Binding Buffer
Alpha2B	[^{3}H]Clonidine (1 nM)	Prazosin	Alpha2 Binding Buffer
Alpha2C	[^{3}H]Clonidine (1 nM)	Prazosin	Alpha2 Binding Buffer
Beta1	[^{125}I]Iodopindolol (0.1 nM)	Atenolol	Beta Binding Buffer
Beta2	[^{125}I]Iodopindolol (0.1 nM)	ICI118551	Beta Binding Buffer
Beta3	[^{125}I]Iodopindolol (0.1 nM)	ICI118551	Beta Binding Buffer

Alpha1 Binding Buffer: 20 mM Tris HCl, 145 mM NaCl, pH 7.4
Alpha2 Binding Buffer: 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.4
Beta Binding Buffer: 50 mM Tris HCl, 3 mM $MnCl_2$, pH 7.7

Table 2.2. Assay conditions for adrenergic receptor affinity

2.2.4.c. Radioligand Assay Conditions for Dopamine Receptor Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
D1	[^{3}H]SCH233930 (0.2 nM)	SKF38393	Dopamine Binding Buffer
D2	[^{3}H]N-methylspiperone (0.2 nM)	Haloperidol	Dopamine Binding Buffer
D3	[^{3}H]N-methylspiperone (0.2 nM)	Chlorpromazine	Dopamine Binding Buffer
D4	[^{3}H]N-methylspiperone (0.3 nM)	Chlorpromazine	Dopamine Binding Buffer
D5	[^{3}H]SCH233930 (0.2 nM)	SKF38393	Dopamine Binding Buffer

Table 2.3. Assay conditions for dopamine receptor affinity

2.2.4.d. Radioligand Assay Conditions for Muscarinic Receptor Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
M1	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M2	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M3	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M4	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer
M5	[^{3}H]QNB (0.5 nM)	Atropine	Muscarinic Binding Buffer

Muscarinic Binding Buffer: 50 mM Tris HCl, pH 7.7

Table 2.4. Assay conditions for muscarinic receptor affinity

2.2.4.e. Radioligand Assay Conditions for Histamine Receptor Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
H1	[^{3}H]Pyrilamine (0.9 nM)	Chlorpheniramine	Histamine Binding Buffer
H2	[^{3}H]Tiotidine (3 nM)	Cimetidine	Histamine Binding Buffer
H3	[^{3}H]alpha-methylhistamine (0.4 nM)	Histamine	Histamine Binding Buffer
H4	[^{3}H]Histamine (5 nM)	Clozapine	Histamine Binding Buffer

Histamine Binding Buffer: 50 mM Tris HCl, 0.5 mM EDTA, pH 7.4

Table 2.5. Assay conditions for histamine receptor affinity

2.2.4.f. Radioligand Assay Conditions for GABAA, BZP and PBR Receptor Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
GABAA	[^{3}H]Muscimol (1 nM)	GABA	50 mM Tris Acetate, pH 7.4
BZP	[^{3}H]Flunitrazepam (0.5 nM)	Diazepam	50 mM Tris HCl, 2.5 mM $CaCl_2$, pH 7.4
PBR	[^{3}H]PK11195 (1 nM)	PK11195	50 mM Tris HCl, pH 7.4

Table 2.6. Assay conditions for GABAA, BZP and PBR receptor affinity

2.2.4.g. Radioligand Assay Conditions for Delta, Kappa and Mu Opioid Receptors Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
Delta OR	[^{3}H]DADLE (0.3 nM)	Naltrindole	Standard Binding Buffer
Kappa OR	[^{3}H]U69593 (0.3 nM)	Salvinorin A	Standard Binding Buffer
Mu OR	[^{3}H]DAMGO (0.3 nM)	DAMGO	Standard Binding Buffer

Table 2.7. Assay conditions for Delta, Kappa and Mu opioid receptors affinity

2.2.4.h. Radioligand Assay Conditions for SERT, NET and DAT Transporters Affinity

Receptor	Radioligand (Assay Conc.)	Reference	Assay Buffer
SERT	[^{3}H]Citalopram (0.5 nM)	Amitriptyline	Transporter Binding Buffer
NET	[^{3}H]Nisoxetine (0.5 nM)	Desipramine	Transporter Binding Buffer
DAT	[^{3}H]WIN35428 (0.5 nM)	GBR12909	Transporter Binding Buffer

Table 2.8. Assay conditions for SERT, NET and DAT transporters affinity

2. 3. Results

2.3.1. Receptor Binding Assay

Receptor binding assays exhibits that TFMPP derivatives had significant binding on the serotonergic, adrenergic (alpha prominently and beta less significantly), histaminic, and the reuptake pump. All three derivatives (2-, 3- and 4-TFMPP) were tested for their receptor binding profiles by the University of North Carolina's PDSP. The top number in each row and column represents the mean percent inhibition (N = 4 determinations) of standard ligand binding for each compound tested at receptor and transporter subtypes. Significant inhibition is considered > 50%. In cases where negative inhibition (-) is seen, this represents a stimulation of binding; occasionally, compounds at high concentrations will non-specifically increase binding. Those compounds which produced greater than 50% binding inhibition in the primary assay were tested further to determine receptor or transporter affinity constants (Ki values in nM) in a secondary binding assay. The Ki (nM) values were obtained from non-linear regression of radioligand competition binding isotherms in this assay and calculated from best fit IC_{50} values using the Cheng-Prusoff equation. The results obtained are presented as the bottom number in each row/column.

2.3.1.a. Effect of TFMPP Derivatives on Serotonergic Receptor Affinity

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
Serotonin receptors	5-HT1A	51.5	54.1	9.1
	5-HT1B	778	181	
		25.1	69.5	13
	5-HT1D	41.5	402.3(AVE)	40.3
			86.4	
	5-HT1E	-0.7	429.0(AVE)	34.9
	5-HT2A	42.4	27.5	63.2
			80	
	5-HT2B	90.1	144.0(AVE)	510(AVE)
			88.5	84.3
	5-HT2C	20	10.7(AVE)	1118(AVE)
		87.3	91.4	84.3
	5-HT3	409.3(AVE)	123(AVE)	400.7(AVE)
		74.5		
	5-HT5A	1,236.7(AVE)	42.3	17.3
		53.1	52.8	
	5-HT6	2,053	2.573	17.9
		61	83.9	74
	5-HT7	1632(AVE)	571.7(AVE)	945.3(AVE)
		81	90.1	75.6
		243.5(AVE)	62.3(AVE)	1343.3(AVE)

Table 2.9. Effect of TFMPP derivatives on serotonergic receptor affinity

5-HT1A Receptors

At 5-HT1A receptors only the 2- and 3-TFMPP regioisomers 10uM produced greater than 50% inhibition of receptor binding. Of these two compounds the 3-TFMPP isomer displayed the highest binding affinity with a Ki of 181 nM and this is consistent with the value reported in the literature (288 nM) using a similar assay (figure 2.1.) [26].

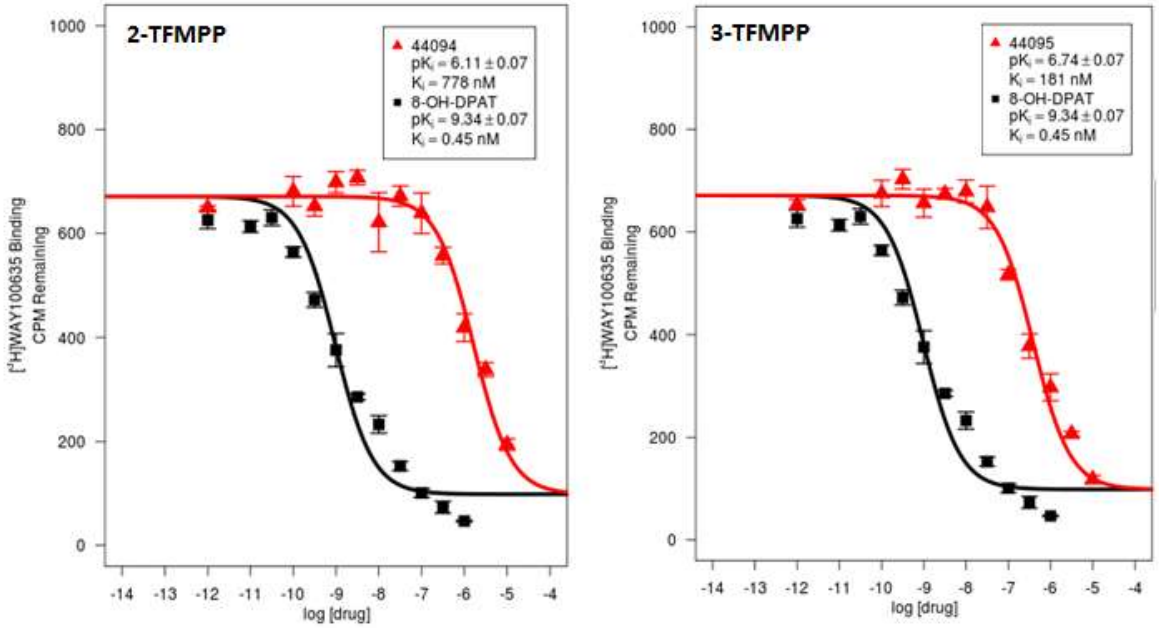

Figure 2.1. Binding of 2-TFMPP and 3-TFMPP at 5-HT1A receptors

5-HT1B Receptors

At 5-HT1B receptors, the 3-isomer produced greater than 50% inhibition of binding and the Ki was determined to be 402 nM (table 2.9.). This is consistent with the value reported in the literature (232 nM) using a similar assay[26].

5-HT2A Receptors

At 5-HT2A receptors, the 3- and 4-TFMPP 10uM regioisomers produced greater than 50% inhibition of receptor binding. Of these two compounds the 3-TFMPP isomer displayed the highest binding affinity with a Ki of 144 nM and this is consistent with the value reported in the literature (269 nM) using a similar assay [26] (table 2.9.).

5-HT2B Receptors

At 5-HT2B receptors only the 2- and 3-TFMPP 10 uM regioisomers produced greater than 50% inhibition of receptor binding. Of these two compounds the 3-TFMPP isomer displayed the highest binding affinity with a Ki of 10.7 nM, nearly twice the affinity of the 2-isomer (figure 2.2.).

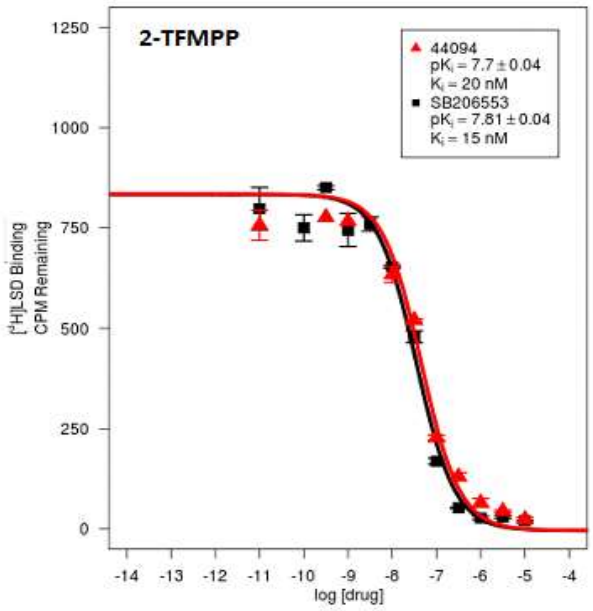

Figure 2.2. 5-HT2B receptor binding profiles for 2-TFMPP

5-HT2C Receptors

At 5-HT2C receptors all three regioisomers produced greater than 50% inhibition of receptor binding at 10 uM. Of these three compounds the 3-TFMPP isomer displayed the highest binding affinity with a Ki of 123 nM, approximately 4 times higher than the affinities of the 2- and 4-isomers (table 2.9.). The affinity of 3-TFMPP for this receptor subtype is consistent with value reported in the literature (62 nM) using a similar assay[26].

2.3.1.b. Effect of TFMPP Derivatives on Adrenergic Receptor Affinity

The results show that none of the TFMPP regioisomers produced greater than 50% inhibition of receptor binding at alpha-1 receptors. While displaying weak affinity for alpha-2 receptor subtypes, all three isomers had Ki values greater than 700 nM for this receptor population. And only the 3-TFMPP isomer had some affinity for beta-1 receptors, but with a Ki of 875 (table 2.10.).

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
Adrenergic receptors	Alpha1A	33.4	28.7	38.4
	Alpha1B	15.8	24.9	37.8
	Alpha1D	37.7	12.1	42.1
	Alpha2A	78.3 893(AVE)	86.6 832(AVE)	81 1697.5(AVE)
	Alpha2B	64.3 2,430.00	78.6 1600	74 2,069

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
	Alpha2C	84.7 764.8(AVE)	85.8 1,174(AVE)	53.4 2814.3(AVE)
	Beta1	31.3	57.3 875	48
	Beta2	21.5	38	47.6
	Beta 3	-1.6	12.7	6.6

Table 2.10. Effect of TFMPP derivatives on adrenergic receptor affinity

2.3.1.c. Effect of TFMPP Derivatives on Dopamine Receptor Affinity

The results show that none of the TFMPP regioisomers produced greater than 50% inhibition of receptor binding at any DA receptor subtype. Even at D4 receptors where weak binding was noted, the Ki values were greater than 1500 nm (table 2.11.). Note that the standard ligands for these receptors have affinities < 1.0 nM.

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
Dopamine receptors	D1	30.2	9.5	23.3
	D2	29.4	7.3	18.3
	D3	18	24.8	-1.3
	D4	26	46.9	50 1,572(AVE)
	D5	13.9	10.5	8.5

Table 2.11. Effect of TFMPP derivatives on dopamine receptor affinity

2.3.1.d. Effect of TFMPP Derivatives on Muscarinic Receptor Affinity

Receptor affinity results show that none of the TFMPP regioisomers produced greater than 50% inhibition of receptor binding at any muscarinic receptor subtype, except for the 2-TFMPP isomer at M5 receptors which shows extremely high affinity (Ki > 6000 nM). The standard muscarinic receptor ligand atropine has an affinity in the subnanomolar range at these receptors (table 2.12.).

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
Muscarinic receptors	M1	24.5	9.2	9
	M2	20.7	18.2	0.9
	M3	49.8	32.9	21.7
	M4	41.7	19.5	25.8
	M5	63.6 6.464.00	44.7	40.3

Table 2.12. Effect of TFMPP derivatives on muscarinic receptor affinity

2.3.1.e. Effect of TFMPP Derivatives on Histamine Receptor Affinity

Receptor affinity results show that none of the TFMPP regioisomers produced greater than 50% inhibition of receptor binding at -2, -3 or -4 receptor subtypes. These compounds did display relatively weak binding at H-1 receptors, with the 2- and 3-TFMPP isomers having Kis in the 400 nM range (table 2.13.).

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
Histamine receptors	HI	66.9 460	60.5 456	80.9 146.5(AVE)
	H2	5.5	19	35.2
	H3	-0.7	6.7	10.2
	H4	-4.7	3.1	18.3

Table 2.13. Effect of TFMPP derivatives on histamine receptor affinity

2.3.1.f. Effect of TFMPP Derivatives on GABA-A, Central and Peripheral Benzodiazepine Receptors Affinity

Receptor affinity results show that none of the TFMPP regioisomers produced greater than 50% inhibition of receptor binding in any of these assays, while the assay standards have Ki values in the 1 nM range (table 2.14.).

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
GABA receptor	GABAA	19.5	-11.3	-8.8
	Sigma2	39.5	75.4	89
BZP rat site		3.2	12.9	22.5
Peripheral benzodiazepine receptors	PBR	-10.8	-4.7	-6.0

Table 2.14. Effect of TFMPP derivatives on GABAA, BZP and peripheral receptor affinity

2.3.1.g. Effect of TFMPP Derivatives on Delta, Kappa and Mu Opioid Receptors Affinity

Receptor affinity results show that none of the TFMPP regioisomers produced greater than 50% inhibition of receptor binding in any of these assays, while the assay standards have Ki values in the <1 nM range (table 2.15.).

Receptors	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
Opioid receptors	MOR	30.8	37.5	33.3 10,000.0(AVE)
	KOR	22	0.8	-4.9
Sigma receptors	Sigma1	79.5 529	79.5	93.5
	Sigma2	39.5	75.4	89

Table 2.15. Effect of TFMPP derivatives on Delta, Kappa and Mu opioid receptors affinity

2.3.1.h. Effect of TFMPP Derivatives on Serotonin (SERT), Norepinephrine (NET) and Dopamine (DAT) Transporters Affinity

Receptor affinity results show that none of the TFMPP regioisomers produced greater than 50% inhibition of binding in the DAT and NET assays. The 3- and 4-TFMPP isomers did display weak inhibition of SERT transporters (figure 2.3.). In this assay the 4-TFMPP isomer with a Ki of 201 nM was about 5-times more potent as an inhibitor of 5-HT reuptake than 3-TFMPP (Ki 1087 nM). Standard inhibitors of SERT transporters such as amitriptyline have Ki values < 1 nM (table 2.16.).

Transporters	Types	Ki Value 2-TFMPP	Ki Value 3-TFMPP	Ki Value 4-TFMPP
	DAT	10.5	5.5	24.9
	NET	48.2	10.4	11.5
	SERT	-2	60.6 1087	80.6 201

Table 2.16. Effect of TFMPP derivatives on SERT, NET and DAT transporters affinity

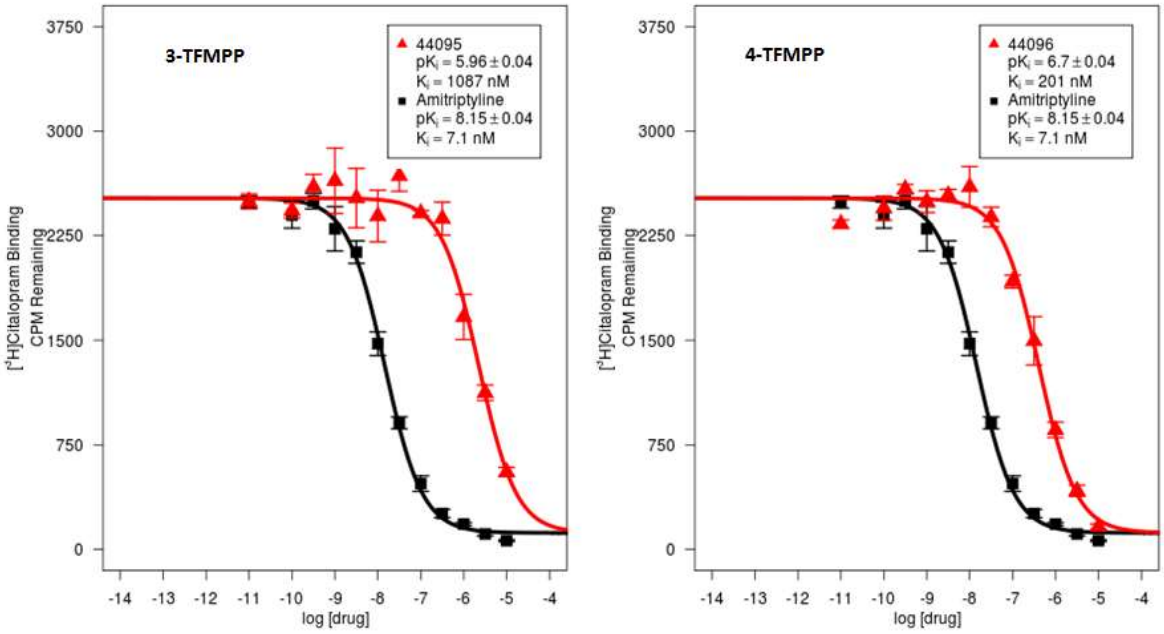

Figure 2.3. SERT binding profile of 3-TFMPP and 4-TFMPP

2.3.2. Evaluation of The Pharmacodynamics and Pharmacokinetic Properties of TFMPP Derivatives Using Computational Design.

The possible pharmacodynamics and pharmacokinetic effects of 2-TFMPP, 3-TFMPP and 4-TFMPP was assessed using QikProp software. All the three TFMPP Derivatives showed no violation of Lipinski's rule of five and Jorgensen's rule of three, which make them likely to be orally active drugs. Moreover, all the derivatives have low MW (below 350), which is required for a drug to penetrate the BBB. Likewise, all compounds displayed optimum cLogP values less than 5 which is comparable to successful CNS drug. With regard to oral absorption, TFMPP Derivatives results showed 100% absorption in Percent Human Oral Absorption parameter, 3 which indicates high absorption according to Human Oral Absorption parameter. Additionally, QPPCaco parameter displayed high GI membrane permeability through Passive diffusion. Marketed CNS drugs should have HBDs number lower than 3 and HBAs lower than 7 which was shown for all TFMPP derivatives. Also, according to QPlogBB and QPPMDCK parameters results, all derivatives were found to cross the BBB through Passive diffusion. Finally, the CNS activity parameter results show that TFMPP derivatives are CNS active (table 2.17).

Descriptor / Molecule	3-TFMPP	2-TFMPP	4-TFMPP
CNS activity	2	2	2
Molecular Weight	230	230	230
Hydrogen Bonds Donor	1	1	1
Hydrogen Bonds Acceptor	2.5	2.5	2.5
cLogP	2.714	2.474	2.658
QPPCaco	1285.308	1286.27	1243.336
QPlogBB	0.991	0.942	0.98
QPPMDCK	3162.021	2264.398	3060.521
Human Oral Absorption	3	3	3
% Human Oral Absorption	100	100	100
Rule of Five	0	0	0
Rule of Three	0	0	0

Table 2.17. QikProp set of descriptors for TFMPP derivatives

2.4. Discussion

The major mechanisms of action attributed towards the addictive and abusive effects of TFMPP derivatives may be due to its effect on serotonergic neurotransmission. 5-HT1A is the most common of all the 5-HT receptors. It is involved in the mediation of several physiological such as peripheral vasodilation, appetite, sleep, aggression in addition to sexual drive and arousal. Our data suggest that moving the trifluoromethyl substituent from 3-position to the 2-position significantly reduces affinity, while moving it to the 4-position abolishes 5-HT1A receptor affinity. 5-HT1B receptors located in the frontal cortex can act as postsynaptic receptor inhibiting the release of DA. While, in the basal ganglia and the striatum it acts as autoreceptor inhibiting the release of 5-HT. Our data displays clearly that moving the position of the trifluoromethyl group from the 3-position eliminates significant affinity for this receptor subtype. Similar results were obtained in binding studies with the 5-HT1D receptor subtype where only 3-TFMPP showed significant affinity with a Ki of 429 nM. 5-HT2A is the main excitatory G protein-coupled receptor for 5-HT. Physiological effects mediated by 5-HT2A include neuronal excitation, behavioral effects (hallucinogenic, addiction, anxiety), platelet aggregation and smooth muscle contraction. Our data suggest that moving the trifluoromethyl substituent to the 4-position significantly reduces affinity, while moving it to the 2-position abolishes 5-HT1A receptor affinity. Interestingly earlier pharmacologic studies have demonstrated that 3-TFMPP is a partial agonist at 5-HT2A receptors[27]. Another 5-HT receptor is 5-HT2B which is involved in

the mediation of several physiological action like anxiety, contraction of smooth muscle and regulation of cardiac structure and functions. Binding data for 3-TFMPP at 5-HT2B receptors is not reported. However, our data demonstrates that moving the trifluoromethyl substituent from the 3-position to the 2-position significantly reduces 5-HT2B receptor affinity, while moving it to the 4-position abolishes 5-HT2B receptors affinity. The excitatory 5-HT2C receptors are found mainly in the choroid plexus where they are involved in cerebrospinal fluid secretion. Our data indicates that moving the trifluoromethyl substituent from the 3-position to the either the 2- or 4-positions significantly reduces 5-HT2C receptors affinity. With regard to adrenergic receptor subtype, our data shows that TFMPP derivatives have no significant affinity for any adrenergic receptor subtype.

TFMPP regioisomers showed weak binding affinity toward DA, histamine and muscarinic receptors. However, the standard histamine receptor ligands have affinities <10 nM, thus it is unlikely the TFMPP compounds would produce significant histaminic effects in animal models. Also, it is unlikely the TFMPP compounds would produce significant GABA or benzodiazepine-mediated effects in animal models. Moreover, TFMPP regioisomers did not exceed 50% inhibition of binding in the DAT and NET assays, suggesting that these compounds would not produce significant reuptake of these neurotransmitters. The 3- and 4-TFMPP isomers did display weak inhibition of SERT transporters. In this assay the 4-TFMPP isomer with a Ki of 201 nM was about 5-times more potent as an inhibitor of 5-HT reuptake than 3-TFMPP (Ki 1087 nM). Standard inhibitors of SERT transporters such as amitriptyline have Ki values < 1 nM, therefore it is not clear if 4-TFMPP would produce significant inhibition of this transporter in animal models.

The structural changes performed on parent molecule in order to synthesize designer drugs can lead to increase in pharmacological potency or toxicity, making them even more stimulatory, addictive and toxic than their parent compound (stimulants / drugs of abuse). These changes can certainty affect the ADME profile of the molecule making ADME profiling an important first step before initiating experimental testing. QikProp software obtained from Schrödinger, LLC provide ADME prediction by analyzing the structure of molecule of interest. ADME prediction is based on several properties and descriptors that are given as numerical averages. In this study, we focused on the parameters that are related to the CNS and oral availability. For example, CNS descriptor which predict CNS activity on a –2 (inactive) to +2 (active) scale. Also, we evaluated the predicted numbers of HBD and HBA of TFMMP derivatives. These parameters reflect the estimated number of hydrogen bonds that would be donated/ accepted by the solute to/from water molecules in an aqueous solution. One more important parameter that was examined is QPPCaco. QPPCaco predict apparent Caco-2 cell (model for the gut blood barrier) permeability in nm/sec where numbers > 500 indicate great permeability. Likewise, QPlogBB parameter predict brain/blood partition coefficient and QPPMDCK predict MDCK cell permeability which imitate the blood-brain barrier in nm/sec. numbers > 500 in MDCK cells are considered to be a great indication of drug ability to cross the BBB. Oral absorption is an important factor in drug discovery and formulation. QikProp can predict Human Oral Absorption on a scale from 1 for low, 2 for medium, or 3 for high human oral absorption. The estimate relies on knowledge-based set of rules, including checking for suitable values of Percent Human Oral Absorption, number of metabolites, number of rotatable bonds, logP, solubility and cell permeability. Another parameter that help to calculate oral absorption is Percent Human Oral Absorption. This parameter calculates human oral absorption on 0 to 100% scale where >80% is considered to

reflect high human oral absorption. Another important descriptor is Rule of Five which represents number of violations of Lipinski's rule of five. The rules include: mol_MW < 500, QPlogPo/w <5, donorHB ≤5, accptHB ≤10. Molecules that satisfy these rules are considered suitable to be used as drugs. Furthermore, Rule of Three displays the number of violations of Jorgensen's rule of three. The three rules include QPlogS > -5.7, QPPCaco > 22 nm/s, # Primary Metabolites < 7. Molecules with fewer violations of these rules are more likely to be orally available [28].

2.5. Conclusion

Piperazine designer drugs have very minimal binding affinity (did not have greater than 50% inhibition of receptor binding) towards muscarinic (M_1, M_2, M_3, M_4, M_5), histaminic (H_1, H_2, H_3, H4), GABA-A, BDZ-site on GABA, sigma-1, sigma-2, dopamine (DA_1, DA_2. DA_3. DA_4, DA_5), Alpha-1A adrenergic, Alpha-1B adrenergic, Alpha-1D adrenergic, Alpha-2A adrenergic, Alpha-2B adrenergic, Alpha-2C adrenergic, Beta-1 adrenergic, Beta-2 adrenergic, Beta-3 adrenergic receptors. Similarly, the designer drugs had minimal binding toward the dopamine (DAT) and norepinephrine (NET) transporters. However, piperazine designer drugs showed significant binding on various serotonin receptors and transporters (SERT). Based on Lipinski's rule of five, Jorgensen's rule of three, Human Oral Absorption, % Human Oral Absorption and the QPPCaco descriptors, the TFMPP derivatives can undergo passive diffusion and can be highly absorbed after oral administration (100% oral absorption). TFMPP derivatives have lower cLogp, HBD and HBA values. Furthermore, all derivatives have an optimum MW and showed a capability to have high CNS activity. Moreover, QPlogBB and QPPMDCK descriptors confirmed that TFMPP derivatives have High brain/blood partition coefficient and great ability to cross the BBB. Therefore, TFMPP derivatives designer drugs can cross the BBB and possibly exhibit significant pharmacodynamic effects in the CNS. These pharmacodynamic and pharmacokinetic studies has demonstrated the piperazine designer drugs when abused orally can readily cross the CNS and affect the monoaminergic neurotransmission to elicit a significant pharmacological or toxicological effect in the humans. Our next step was to study the effect on hippocampal and dopaminergic neurons.

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3. Elucidate the Hippocampal Neurotoxicity of TFMPP Derivatives

3.1. Introduction

Designer drugs are synthetic compounds that have been modified chemically to resemble the structure of controlled substances in order to mimic their effects. Designer drugs are marketed as "legal highs" since they are not controlled appropriately under current drug regulations. The United Nations Office on Drugs and Crime has defined as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat"[1]. The misuse of designer drugs for their psychoactive properties has been increasing globally with limited data on its safety and toxicity[2]. Designer drugs demonstrated an ability to affect several brain regions such as the basal ganglia, hippocampus, cerebellum, parietal lobe, and globus pallidus[3]. The alteration caused by designer drugs on different brain regions was linked to several health issues such as anxiety, seizures, agitation, aggression and acute psychosis[2]. Furthermore, long-lasting depression, confusion, memory and attention impairments were reported in designer drugs users[4,5]. However, the mechanisms associated with memory and attention impairments associated with the use of designer drugs are not elucidated.

Alzheimer's disease (AD), is an irreversible, progressive and chronic neurodegenerative disease that begins diminishing memory and thinking skills slowly and worsen gradually to eventually leading to the patient inability to perform simple tasks independently. AD most distinguished symptoms are memory impairment and inability to create new memories. Symptoms of AD may also include mental decline and difficulty in understanding simple math or common things, mental confusion, delusion, disorientation and difficulty concentrating. Furthermore, behavioral changes have been noticed in AD patients, these changes include agitation, personality changes, getting lost easily and inability to perform self-care independently. Symptoms usually starts in patients over their 60s. AD is the leading cause and form of dementia which is the loss of formerly attained cognitive abilities comprising 60-70% of the cases[6].

Many ancient civilizations have linked aging with dementia. However, AD first modern-day report was by German psychiatrist Dr. Alois Alzheimer in 1906. Dr. Alzheimer identified abnormal changes in the brain tissue of 50-year-old female patient whom he followed her unusual mental illness from 1901 until she died on 1906 [7]. According to Alzheimer's association 2019 report, AD is ranked 6th among the causes of death in the United States with an increase of deaths from AD in the past two decades by 145%. Currently, 5.8 million Americans are suffering from AD with a new patient every 65 seconds. In 2019, AD and dementia will cost around $290 billion and if no treatment was developed to treat AD the cost is expected to rise up to $1.1 trillion by 2050[8]. With respect to the age of onset of AD, it can be classified into early-onset AD (EOAD) (patients less than 60 years of age) and late-onset AD (LOAD) (patient older than 60 years of age). 5% of AD cases are EOAD and it has been linked to 230 gene mutations with nearly 50% of the cases carrying mutations in amyloid precursor protein (APP) or

presenilin-1 (PSEN-1), presenilin-2 (PSEN-2) genes[9]. LOAD is more common, and it is commonly associated with multiple risk factors such as diabetes mellitus, hypertension, hypercholesterolemia, obesity, and metabolic syndrome[10]. Generally, neurodegenerative diseases are accompanied with oxidative stress, mitochondrial and synaptic dysfunction in addition to neuroinflammation[11]. Furthermore, both categories of AD are characterized by the irregular extracellular buildup of amyloid-β peptide (Aβ) leading to amyloid plaques formation and intracellular tau protein accumulation in neuro fibrillary tangles (NFTs)[12]. The accumulation of (Aβ) peptide and tau protein result in alteration of blood-brain barrier permeability and eventually initiate synaptic and neuronal dysfunction[13].

However, since AD is a multifactorial disease, multiple etiological hypotheses have been suggested to explain the pathology of AD, these hypotheses include: the amyloid hypothesis, the tau hypothesis, the cholinergic hypothesis, the mitochondrial cascade hypothesis, the metabolic hypothesis and the vascular hypothesis[14]. The Amyloid Hypothesis relies on the notion of the augmentation of neuronal cell damage and death by the toxic aggregation of Aβ plaques resulting ultimately in cognitive and behavioral abnormalities[15]. Tau proteins act by connecting, stabilizing and polymerizing axonal microtubules by isoforms formation in addition to phosphorylation. Hyperphosphorylation of Tau proteins result in its accumulation to form insoluble NFTs. NFTs results in deficit in the normal function of tau protein leading to its inability to stabilize the microtubule. This leads to the disintegration of the microtubule resulting in dysfunctional axonal transport, synaptic signaling and neuronal cell death[16]. According to the cholinergic hypothesis the loss of the transmission of the electrical signals by decreased acetylcholine level in addition to Aβ and NFTs aggregation caused by acetyl cholinesterase

(AChE) hydrolysis of acetylcholine are one of the etiologies contributing in AD development and progression[17]. The mitochondrial cascade hypothesis suggest that mitochondrial dysfunction caused by mitochondrial DNA mutations, oxidative stress in addition to the precipitation of Aβ in mitochondria can result in the development of AD by intiating neuronal cell apoptosis[18]. According to the metabolic hypothesis, AD can result from metabolic disorders such as obesity, diabetes mellitus and hypercholesterolemia. Insulin signaling plays a key role in the normal functioning of neurons, regulation of tau phosphorylation in addition to its role in the metabolism and clearance of Aβ. Therefore, inconsistent insulin level may have a declining effect on the normal functioning of neurons[19]. In the vascular hypothesis, AD development can be a result of the blood-brain barrier impairment through its effect on oxygen supply, glucose delivery and removal of toxic substances leading to neuronal cells death [20]. There are various *in vitro* and *in vivo* models to study the etiopathology and novel drug therapy for AD. The various models are:

- ✓ Transgenic:
  - ❖ APP transgenic mice (PDAPP, APP23, Tg2576, PS2APP)
  - ❖ Tau transgenic mice (PrP-Tau, Thy-Tau22, GFAP-Tau)
  - ❖ APP/Tau double transgenic mice
  - ❖ Triple transgenic mice
  - ❖ 5X transgenic mice
- ✓ Natural AD models: Rodents (SAMP8 mice, OXIX rats, Dog, Non-human primates)
- ✓ Chemically-induced models
  - ❖ High-fat diet
  - ❖ Streptozotocin

- ❖ Scopolamine

- ✓ Cells / Tissue models:
  - ❖ Cultured slices (Hippocampal, cortex)
  - ❖ Brain Slice
  - ❖ Cells (neuroblastoma, induced pleuripotent stem cells)

HT-22 cells are subcloned immortalized mouse hippocampal cell line obtained from the HT-4 cell line. HT-22 is highly sensitive to glutamate and is thus frequently used as a model system to study glutamate-induced toxicity in neuronal cells. Glutamate exerts its action by binding trough glutamate receptors (NMDA, AMPA, kainate and metabotropic receptors-mGluR). Normal content of glutamate are essential for the physiological actions, nevertheless excess content results in neuronal hyperexcitation resulting in neurodegeneration leading to neuronal death. Excitotoxicity plays a vital role in various neurodegenerative disorders such as AD, Huntington's disease and Parkinson's disease along with other pathologies such as spinal cord injury and multiple sclerosis. HT-22 cells have been used to study the etiopathology of AD[21] and also has been used to explore the neuroprotective effects of synthetic drugs[22] and botanicals[23]. In addtion to the markers of excitotoxicity the HT-22 cells also possees markers of cholinergic neurotransmission such as choline transporter, choline acetyltransferase (ChAT), vesicular acetylcholine transporter, and muscarinic acetylcholine receptors[21]. Decreased cholinergic neurotransmission and excitotoxicity are the major neurotoxic mechanisms assocaited with neurodegernation in AD. Since HT-22 cells contain all the above markers and this makes it a valid *in vitro* model to use in our current study.

In summary, the mechanisms associated with the piperazine designer drugs mediated neurotoxicity leading to AD are poorly understood. Thus, in this study we evaluated the effect of piperazine designer drugs on cholinergic metabolism (choline acetyltransferase, acetylcholine esterase), oxidative stress, mitochondrial dysfunction, apoptosis, inflammation and excitotoxicity.

3.2. Materials and Methods

3.2.1 Chemicals and Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT), Neostigmine and Aldrithiol were bought from Tokyo Chemical Industry America (Portland, OR). Dulbecco's Modification of Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin solution were purchased from Corning® (Corning, NY). Griess reagent, Sodium nitrite Phosphate buffer saline (PBS), Dimethyl sulfoxide (DMSO), Nicotinamide adenine dinucleotide (NADH), 2`, 7-dichlorofluorescindiacetate (DCFH), Pyrogallol, Hydrogen Peroxide (H_2O_2), Phosphoric acid, O-phthalaldehyde (OPT), Glutathione (GSH), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Kynuramine and EDTA were purchased from Sigma Aldrich (St. Louis, MO). AC-YVAD-AMC and AC-DEVD-AMC were purchased from Enzo Life Sciences (Farmingdale, NY). Acetylthiocholine iodide, 5,5'-Dithiobis (2-nitrobenzoic acid) (Ellmans reagent, DTNB) and 4 X reducing Laemmli SDS sample buffer were purchased from Alfa Aesar (Haverhill, MA). Cell lysis buffer was purchased from Cell Signaling Technologies (Danvers, MA). Acetyl Coenzyme A was obtained from Chem Impex Intl Inc (Wood Dale, IL). Highly pure Choline chloride and purity Trypsin (0.25%) EDTA was acquired from VWR (Radnor, PA). Anti-NFKB p65 antibody, anti-IKKB antibody, anti-GLUR1 antibody, anti-PSD95 antibody, anti-NR2A antibody, anti-GADPH antibody and anti-β-actin antibody were purchased from abcam

(Cambridge, MA). N-PER™ Neuronal Protein Extraction Reagent and PVDF membrane were purchased from Thermo Fisher Scientific (Waltham, MA). A Thermo Fisher Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL) for protein quantification.

3.2.2. HT-22 Mouse Hippocampal Neuronal Cell Line

HT-22 mouse hippocampal neuronal cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L Glucose, 4mM L-Glutamine, 10% Fetal Bovine Serum, 100 units/mL Penicillin and 50 µg/mL Streptomycin. Cells were grown into 75 cm^2 flasks and kept in a humidified incubator at 37°C and supplemented with 5% CO_2. HT-22 cells were collected by trypsinization (0.25% (w/v) Trypsin-0.53mM EDTA) after reaching 70% confluency (2-3 days) and centrifuged at 300 x g for 2-3 minutes to precipitate the cells. For the MTT assay, cells were seeded into 96 well plates at a density of 1 x 10^5 cells/well. Cells were used within 3-10 passages after they were received [24,25].

3.2.3. Treatment Design

Prior to each experiment, 2-TFMPP, 3-TFMPP and 4-TFMPP were freshly prepared and diluted in PBS to a 10mM stock solution. To evaluate the cytotoxicity, different concentrations of 2-TFMPP, 3-TFMPP and 4-TFMPP were prepared by serial dilution with PBS followed by additional dilution in serum enriched fresh culture medium. With regard to the control, HT-22

mouse hippocampal neuronal cells were treated with PBS. Cells were exposed to different concentrations of 2-TFMPP, 3-TFMPP and 4-TFMPP for 24 and 48 hours. However, to establish the effect of 2-TFMPP, 3-TFMPP and 4-TFMPP on oxidative stress, mitochondrial dysfunction, apoptosis, inflammation and excitotoxicity, the HT-22 mouse hippocampal neuronal cells were exposed TFMPP derivatives for 18 hours. All stock solutions were stored at -20 ºC and freshly diluted on the day of the experiment.

3.2.4. Cytotoxicity Assay

For the evaluation of cytotoxicity, MTT cell viability assay was performed. The concept of MTT assay is that the mitochondria of viable cells through succinate dehydrogenases reduce the yellow colored water soluble tetrazole reagent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble blue crystal formazan that can be measured colorimetrically[26,27]. After 24 hours and 48 hours incubation with 3-TFMPP in serum-fed and serum-free medium, 12 mM MTT stock solutions was prepared and then added on each well along with fresh culture medium. Following a 2 hours incubation at 37 ºC the medium was aspirated and 200 µl of DMSO was added to solubilize the formazan crystal. Afterward 10 minutes incubation at 37 ºC the absorbance was measured using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) at 570 nm.

3.2.5. Protein quantification

Protein was quantified using Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

3.2.6. Quantifying Reactive Oxygen Species

The generation of reactive oxygen species (ROS) in the cells treated with 3-TFMMP, 2-TFMPP and 4-TFMPP was estimated spectrofluorometrically by measuring the conversion of non-fluorescent chloromethyl-DCF-DA (2′,7-dichlorofluorescindiacetate, DCF-DA) to fluorescent DCF using excitation wavelength of 492 nm and emission wavelength of 527 nm. A mixture of 0.05% w/v solution of DCFDA in ethanol (10 µl), phosphate buffer (150 µl) and cell homogenate (40 µl) were incubated for 1 h at 37 °C. DCFH reacted with ROS to form the fluorescent product DCF. Readings were measured by BioTek Synergy HT plate reader (BioTek, VT, USA). Results were expressed as percentage change from the control[28].

3.2.7. Nitrite Content

Nitric oxide (NO) oxidation pathways produce nitrite and nitrate as final products which allow to use their concentrations as an expression of NO production. Nitrite was measured spectrophotometrically using Griess reagent (St. Louis, MO) which was developed by Griess in 1879. This method relies on Reaction of NO2 with sulfanilamide under acidic condition resulting in the production of diazonium ion which then combine with N-(1-naphthyl) ethylenediamine to

form chromophoric azo product which can be measured spectrophotometrically at 546 nm. Griess reagent was added to Cell homogenate after 3 minutes shaking the mixture was incubated for 15 minutes. A sodium nitrite standard curve was prepared from commercially acquired sodium nitrite. Results were expressed as percentage change from control[29,30].

3.2.8. Lipid Peroxide Content

Lipid peroxidation is a sequence reaction process in which ROS attack polyunsaturated fatty acids causing the oxidative breakdown of lipids. Lipid peroxidation content was measured by calculating the quantity of malondialdehyde (MDA) content in the form of Thiobarbituric acid-reactive substances (TBARS)[31]. Ice cold TCA (20 % w/v) was added to Cell homogenate then it was mixed with Thiobarbuturic acid (TBA) (0.5 % w/v) and deionized water. Additionally, the mixture was incubated in water bath for 15 minutes (80º C) then cooled at ice for 5 minutes. Afterwards the mixture was centrifuged at 4°C for 5 minutes at 10,000 RPM. Following the centrifuging, samples supernatant was placed at 96-well plate and the absorbance was measured at 532 with a plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) using duplicate reading and MDA levels were calculated as TBARS reactive substances per mg protein. Results were expressed as percentage change from control[32,33].

3.2.9. Superoxide Dismutase Activity

The autoxidation of pyrogallol in an alkaline environment results in the generation of superoxide anion radicals. Superoxide dismutase (SOD) is an antioxidant enzyme that rapidly dismutase superoxide anion radicals into H_2O_2 and water. Spectrophotometric measurement of the inhibition of pyrogallol autoxidation induced by SOD can be performed rapidly and conveniently by reading the absorbance of a mixture of 2 mM pyrogallol solution, 50 mM Tris buffer pH 8.2 and cell homogenate using visible light at 420 nm for 3 minutes. SOD activity was measured as the change in absorbance at 420 nm and expressed as percentage change from control [34].

3.2.10. Catalase Activity

Catalase is an antioxidant enzyme that stimulate the transformation of H_2O_2 into water and oxygen. An assay mixture of 50 mM PBS at pH 7.0 and cell homogenate was prepared. Following the addition of 30 mM H_2O_2, which yielded approximately 0.5 absorbance, the decomposition of H_2O_2 was monitored spectrophotometrically using ultraviolet light at 240 nm for 1 minute[35]. A standard curve was created from commercially procured H_2O_2. The change in absorbance was observed and the enzyme activity was calculated as percentage change from control[36].

3.2.11. Glutathione Content

In the presence of GSH, Glutathione peroxidase (GSH-Px) stimulate the conversion of H_2O_2 to water. The condensation reaction between GSH and o-phthalaldehyde (OPT) produce a fluorescence at pH 8.0 that can be measured spectrofluorometrically[37]. The assay mixture was made of cell homogenate, 0.1 M phosphoric acid, 0.1% OPT solution in methanol and 0.01 M phosphate buffer. In the beginning of the experiment, cell homogenate was mixed with the 0.1 M phosphoric acid in order to precipitate the protein. Then, the mixture was centrifuged at 12000 RPM for 10 minutes. Following the addition of OPT to the supernatant, the mixture was incubated in dark for 20 minutes at room temperature. Fluorometric readings were taken at an excitation wavelength of 340 nm and an emission wavelength of 420 nm. A GSH standard curve was prepared from commercially acquired GSH. The GSH content was calculated as mmol of GSH/μg protein and expressed as percentage control[32,36].

3.2.12. Monoamine oxidase activity

Total monoamine oxidase (MAO) activity was measured fluorometrically by determining the amount of 4-hydroxyquinoline formed as a result of kynuramine oxidation[38]. MAO activity was reported as 4-hyroxyquinoline formed/hour/mg protein[32,36,39].

3.2.13. Mitochondrial Complex-I Activity

NADH oxidation to NAD^+ is catalyzed by Mitochondrial Complex-I (NADH dehydrogenase). Cell homogenate was added to phosphate buffered saline and NADH in order to measure NADH dehydrogenase activity spectrophotometrically at 340 nm using visible light. A standard curve was composed from commercially obtained NADH. The extent of NADH oxidation was quantified by determining the decrease in absorbance at 340 nm for 3 minutes. Results were reported as percentage change from the control[40].

3.2.14. Mitochondrial Complex-IV activity

Cytochrome C oxidation is catalyzed by Mitochondrial complex IV (Cytochrome C oxidase). Cell homogenate was added to phosphate buffered saline and Cytochrome C in order to determine the activity of the Cytochrome C oxidase activity spectrophotometrically at 550 nm using visible light. A standard curve was created from commercially obtained Cytochrome C. The magnitude of Cytochrome C oxidation was measured by following the oxidation of reduced Cytochrome C as an absorbance decrease at 550 nm for 3 minutes. Results were reported as percentage change from the control[40,41].

3.2.15. Caspases-1 activity

The activity of Caspase-1 was measured fluorometrically using the fluorogenic caspase-1 substrate AC-YVAD-AMC at excitation wavelength: 340-360 nm and emission wavelength:440-460nm. Caspase -1 recognize the amino acid sequence YVAD and form a cleavage with it on precursor interleukin-1beta Asp116 cleavage site resulting in the liberation of fluorescent AMC which can be quantified[42].

3.2.16. Caspases-3 Activity

The activity of Caspase-3 was measured fluorometrically using the fluorogenic caspase-3 substrate AC-DEVD-AMC at excitation wavelength: 340-360 nm and emission wavelength:440-460nm. Caspase -1 recognize the amino acid sequence YVAD and form a cleavage with it on the PARP cleavage site Asp216 resulting in the liberation of fluorescent AMC which can be quantified[43].

3.2.17. Acetylcholinesterase Activity

AChE is the principal cholinesterase in the body which is responsible for the metabolism of acetylcholine. AChE activity was measured spectrophotometrically using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB). The AChE activity refers to the amount of 5-thio-2-nitrobenzoate formed/mg protein. 5-thio-2-nitrobenzoate is the product formed when thiocholine—the product of the breakdown of acetylcholine—reacts with DTNB[44,45].

3.2.18. Choline acetyltransferase Activity

ChAT catalyzes the transfer of an acetyl group from the coenzyme acetyl-CoA to choline resulting in the synthesis of acetylcholine. ChAT activity was measured spectrophotometrically at 324 nm using choline chloride as a substrate. The ChAT activity refers to amount of 4-thiopyridone formed/mg protein. 4-thiopyridone is the product formed when reduced CoA reacts with 4,4'-dithiopyrdine[46].

3.2.19. Western Blot

After the incubation with TFMPP derivatives, cells were washed with ice cold PBS and the total protein was isolated using a N-PER™ cell lysis buffer (Thermo Fisher Scientific, Waltham, MA) containing a protease inhibitor cocktail (Sigma, St. Louis, MO) and phosphatase inhibitors (Sigma, St. Louis, MO). Protein concentration of each sample was measured using the Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Samples were divided into 50 ug portions and were stored in -80 ºC freezer. Before the experiment, 4 X Laemmli buffer (Alfa Aesar, Haverhill, MA) was added to the samples and mixture was boiled for 5 minutes then loaded into 4-12% SDS-page gel. Samples were electrophoretically transferred onto PVDF membrane (Thermo Fisher Scientific, Waltham, MA) via wet transfer. Non-specific binding sites on the membranes were blocked with 5% non-fat milk in Tris-buffered saline along with 0.1% v/vTween-20 at pH 7.4 (TBST) for two hours. The membranes were washed with TBST (3 X,

each for 5 minutes) and incubated with anti-NFKB p65, anti-IKKB, anti-GLUR1, anti-PSD95 and anti-NR2A overnight at 4 ºC as well as anti-GADPH and anti-β-actin antibody which were used as a loading control. All primary antibodies were constituted in 5% BSA in TBST and made as a 1:1000 dilution. Membranes were incubated at room temperature for 1 hour with Goat anti-Rabbit IgG (H+L) secondary antibody that was constituted with fluorophore DyLight 550 (Thermo Fisher Scientific, Waltham, MA) and prepared in 1:10000 dilution. Then, membranes were imaged with the FluorChem Q System imager (Proteinsimple, San Jose, CA) at excitation wavelength λ535nm and emission wavelength λ606 nm. Band densities for each sample were normalized to their respective β-actin or GAPDH signal and reported as percentage control.

3.2.20. Statistical Analysis

Data was reported as mean ± SEM. Statistical analysis were accomplished using one-way analysis of variance (ANOVA) followed by Turkey 's multiple comparisons test ($p< 0.05$ was considered to be statistically significant). Statistical analysis was performed using Prism-V software (La Jolla, CA, USA).

3.3. Results

3.3.1. Dose-dependent and time dependent effect of TFMPP derivatives on HT-22 Cell Viability:

HT-22 cells were incubated with a range of doses (10μM, 50μM, 100μM, 250μM, 500μM, 1mM, 2.5mM, 5mM, 10mM) of 3-TFMPP, 2-TFMPP, and 4-TFMPP for two different time points (24 and 48 hours). Control cells were incubated under the same conditions as treatment groups without exposure to TFMPP derivatives. After 24 hours incubation, the parent drug 3-TFMPP significantly reduced HT-22 cell viability by (25%) at the dose of 100μM and by 62% at the dose of 250μM ($n=12$, $p<0.01$; Figure 3.1.). Additionally, 2-TFMPP significantly decreased HT-22 cell viability by 31% and 65% at the dose of 100μM and 250μM respectively ($n=12$, $p<0.01$; Figure 3.2.). Moreover, 4-TFMPP reduced HT-22 cell viability 12% at the dose of 100μM and by 69% at the dose of 250μM ($n=12$, $p<0.01$; Figure 4.1). After 48 hours incubation, 3-TFMPP significantly reduced HT-22 cell viability 39% at the dose of 100μM and by 85% at the dose of 250μM ($n=12$, $p<0.01$; Figure 3.1.). Furthermore, 2-TFMPP significantly reduced HT-22 cell viability by 25% and 65% at the dose of 100μM and 250μM respectively ($n=12$, $p<0.01$; Figure 3.2.). Moreover, 4-TFMPP reduced HT-22 cell viability 12% at the dose of 100μM and by 94% at the dose of 250μM ($n=12$, $p<0.01$; Figure 3.3.). Interestingly, the parent drug was the only derivative to reduce cell viability at dose of 10μM after 24 hours incubation by nearly 19%. All three TFMPP derivatives caused 100% cell death at dose of 500μM.

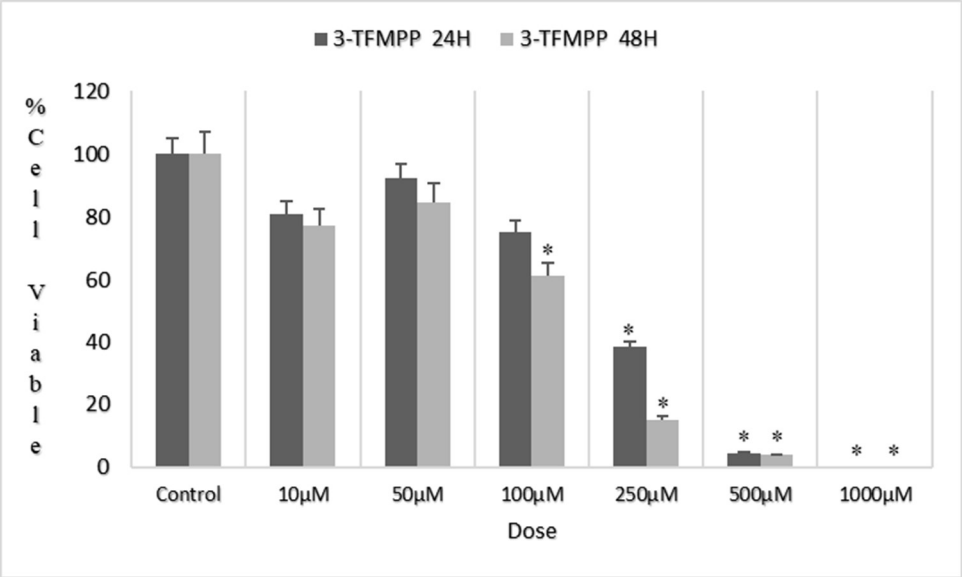

Figure 3.1. Effect of 3-TFMPP on HT-22 mouse hippocampal neuronal viability: Cells were treated with different doses of 3-TFMPP for 24 hours and 48 hours as well at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls, n=12, $p<0.01$.

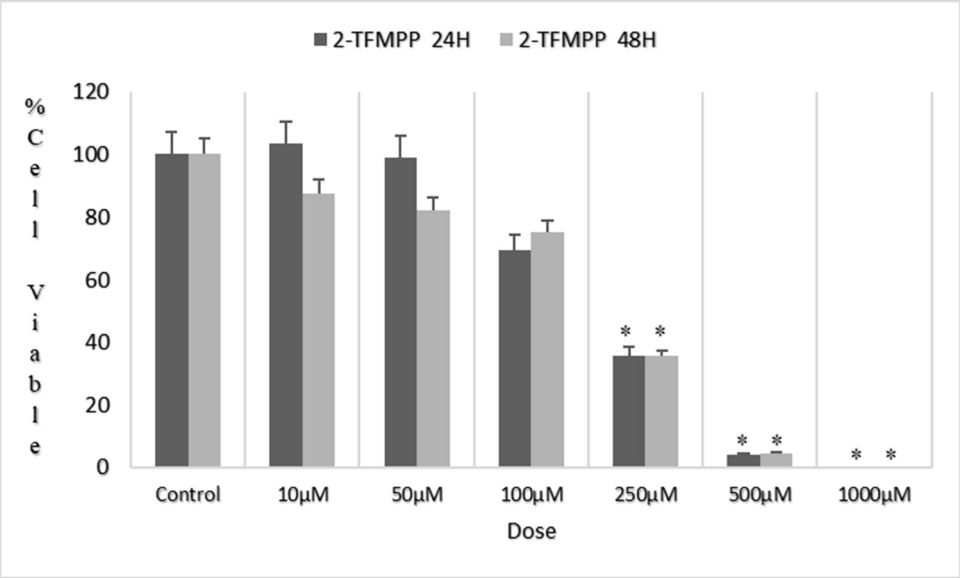

Figure 3.2. Effect of 2-TFMPP on HT-22 mouse hippocampal neuronal viability: Cells were treated with different doses of 2-TFMPP for 24 hours and 48 hours as well at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls, n=12, $p<0.01$

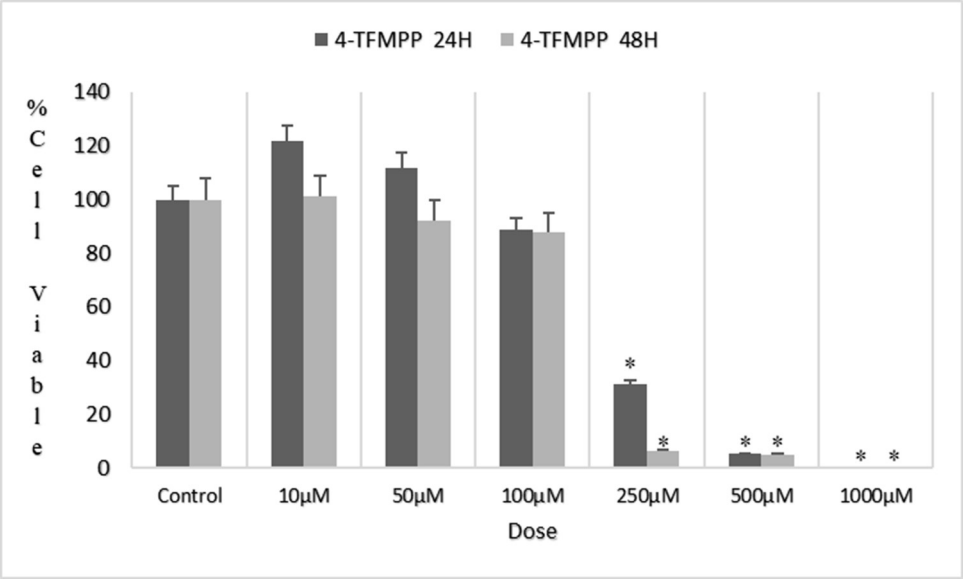

Figure 3.3. Effect of 3-TFMPP on HT-22 mouse hippocampal neuronal viability: Cells were treated with different doses of 4-TFMPP for 24 hours and 48 hours as well at 37°C. Cell viability was evaluated through the MTT reduction assay (n=12). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls, n=12, $p<0.01$

3.3.2. TFMPP Derivatives Generate Reactive Oxygen Species

3-TFMPP, 2-TFMPP and 4-TFMPP significantly and dose-dependently increased ROS generation in HT-22 cells as compared to the control (n=6, p< 0.0001; Figure 3.4.). Interestingly, 3-TFMPP caused higher ROS generation (217% at 100µM, 388% at 250µM) than 2-TFMPP (203% at 100µM, 318% at 250µM) and 4-TFMPP (201% at 100µM, 269% at 250µM).

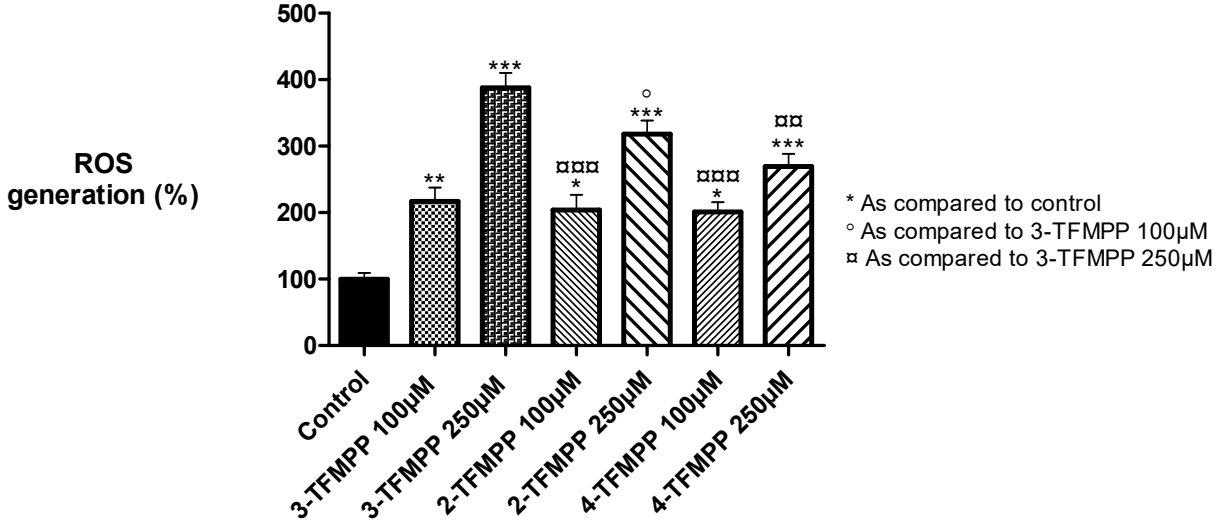

Figure 3.4. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal reactive oxygen species generation: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently generated ROS generation in HT22 cells as compared to the control (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls

3.3.3. TFMPP Derivatives Increase Nitrite Content

TFMPP caused a rise in nitrite content which was significant at the higher dose (250µM) where 3-TFMPP, 2-TFMPP and 4-TFMPP increased the nitrite production by 352%, 266% and 211% respectively (n=6, p< 0.0001; Figure 3.5.). At the same time, 3-TFMPP, 2-TFMPP and 4-TFMPP also increased nitrite formation by 156%, 128% and 145% respectively at the lower dose (100µM). Furthermore, 3-TFMPP had formed greater nitrite content (156% at 100µM, 352 % at 250µM) as compared to 2-TFMMP (127% at 100µM, 266% at 250µM) and 4-TFMPP (145% at 100µM, 211% at 250µM).

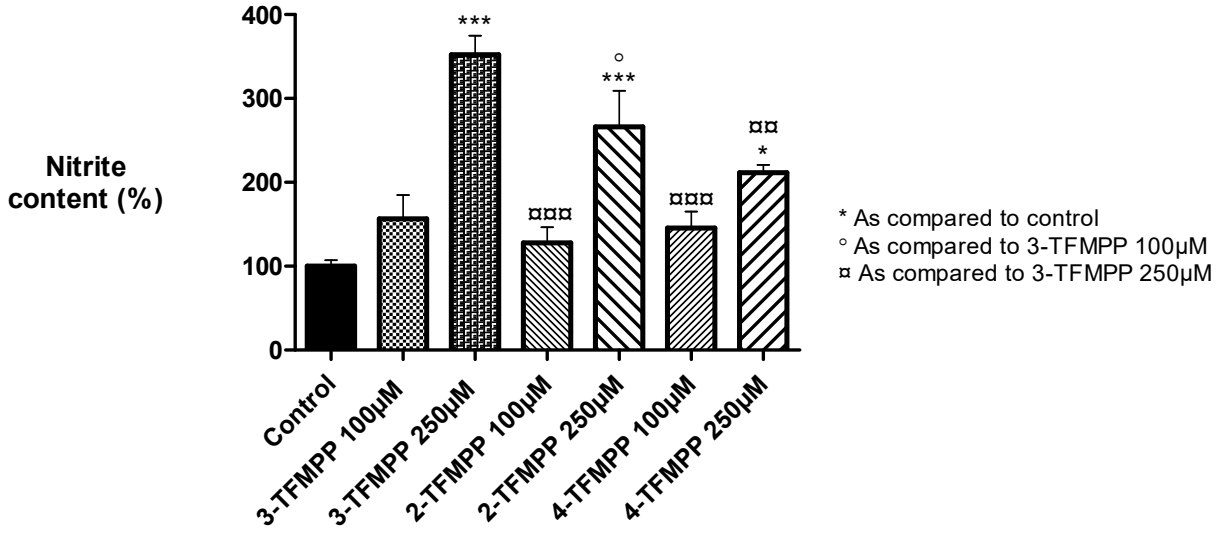

Figure 3.5. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal nitrite content: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently increased nitrite content in HT22 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.4. TFMPP Derivatives Induce Lipid Peroxidation

At dose of 100μM, 3-TFMPP and 2-TFMPP significantly increased lipid peroxidation by 149% and 158% respectively (n=6, p< 0.0001; Figure 3.6.). However, 4-TFMPP increased lipid peroxidation by 145% at dose of 100μM but this increase was not statistically significant. Furthermore, 3-TFMPP, 2-TFMPP and 4-TFMPP increased lipid peroxidation at the dose of (250μM) by 223%, 193% and 177% respectively (n=6, p< 0.0001; Figure)

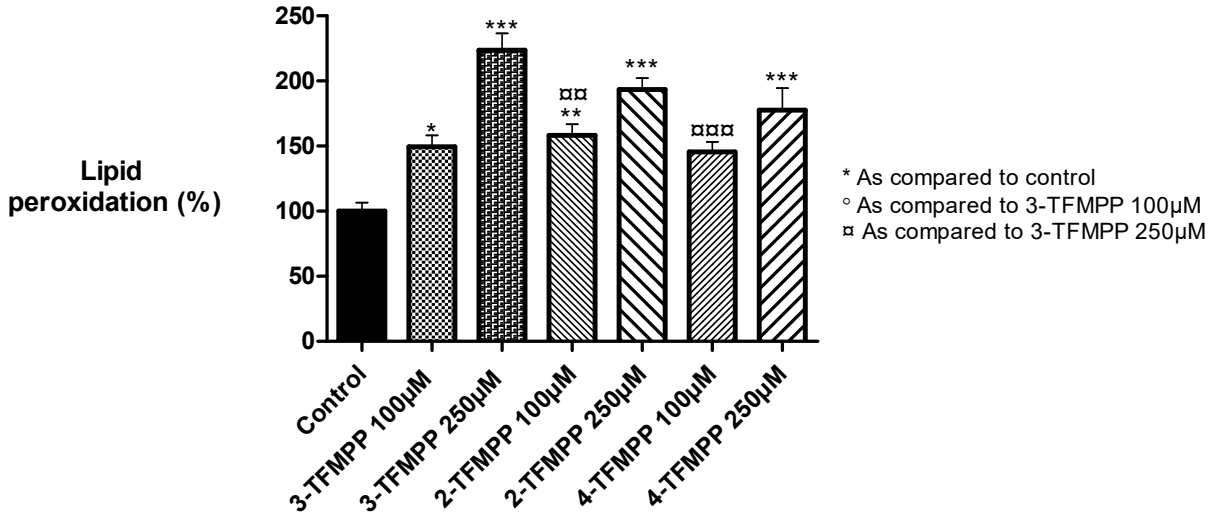

Figure 3.6. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal lipid peroxide content: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently increased lipid peroxide content in HT22 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.5. TFMPP Derivatives Decrease Superoxide Dismutase Activity

SOD is an antioxidant enzyme that protects the neuron by dismutation of superoxide anion to H_2O_2 and water. 3-TFMPP, 2-TFMPP and 4-TFMPP at dose of (250µM) decreased SOD activity significantly by 57%, 68% and 64% respectively (n=6, p <0.0001; Figure 3.7.). Also, at the lower dose (100µM) 4-TFMPP significantly decreased SOD activity by 51% (n=6, p <0.0001; Figure). However, 3-TFMPP and 4-TFMPP had no effect on SOD activity at dose of (100µM).

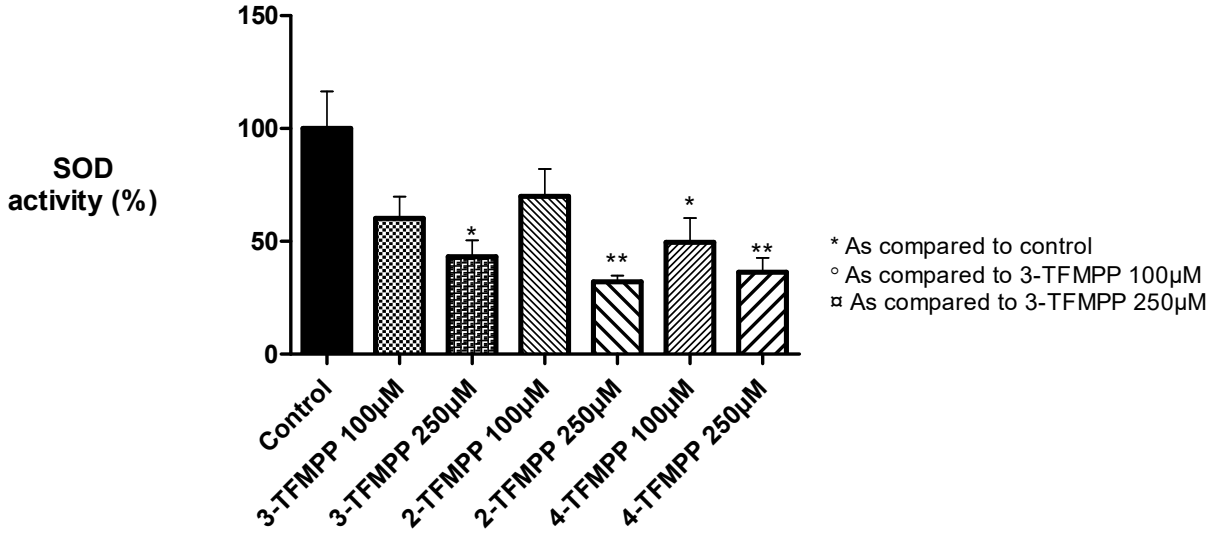

Figure 3.7. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal superoxide dismutase activity: 3-TFMPP, 2-TFMPP and 4-TFMPP significantly decreased SOD activity in HT22 cells at dose of (250µM) as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.6. TFMPP Derivatives Decrease Catalase Activity

Catalase is an antioxidant enzyme that breaks H_2O_2 to water and molecular oxygen. 3-TFMPP and 4-TFMPP reduced catalase activity significantly at the low and the high doses (100µM, 250µM) (n=6, p <0.0001; Figure 3.8.). On the other hand, 2-TFMPP had no effect on catalase activity.

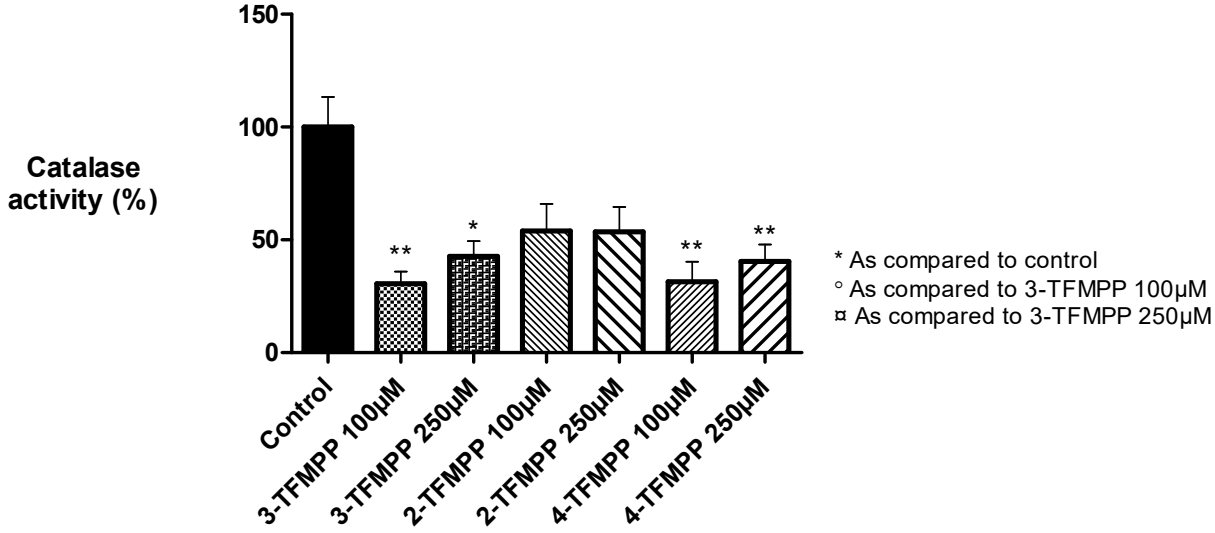

Figure 3.8. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal catalase activity: 3-TFMPP and 4-TFMPP significantly decreased catalase activity in HT22 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.7. TFMPP Derivatives had no Effect on Glutathione Content

3-TFMPP, 2-TFMPP and 4-TFMPP had no effect on GSH content in HT-22 cells (Figure 3.9.).

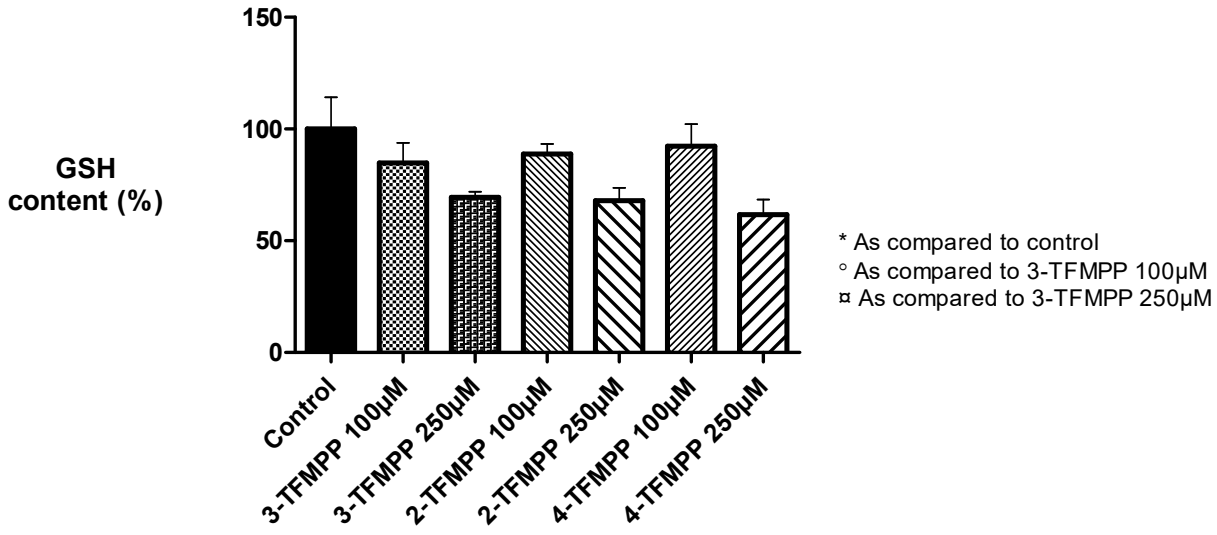

Figure 3.9. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal glutathione content: 3-TFMPP, 2-TFMPP and 4-TFMPP had no effect on GSH content in HT22 cells as compared to the control. (n=6). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.8. 3-TFMPP increase Monoamine oxidase activity

MAO participates in neurodegeneration through several mechanisms such as oxidative stress [47], neuroinflammation [48], apoptosis [49], glial cells activation [50] and decreasing aggregated-protein clearance [51]. 3-TFMPP increased MAO activity in HT-22 cells at dose of (250µM) (n=6, p< 0.0001; Figure 3.10.).

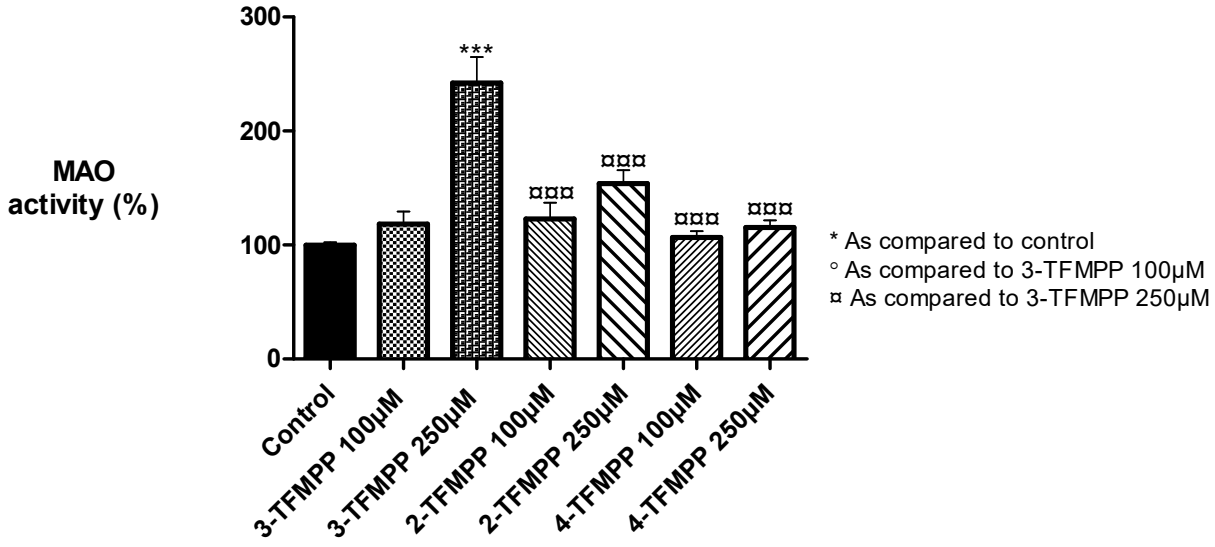

Figure 3.10. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal monoamine oxidase activity: 3-TFMPP at dose of 250µM increased MAO activity in HT22 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.9. TFMPP Derivatives Inhibit Mitochondrial Complex-I Activity

TFMPP derivatives showed significant inhibition of Complex-I activity in a dose-dependent manner (n=, p< 0.0001; Figure 3.11.). At the dose of 250µM, 3-TFMPP, 2-TFMPP and 4-TFMPP decreased Complex-I activity by 90%, 72% and 68% respectively (n=6, p< 0.0001; Figure). While at the lower dose (100µM), 3-TFMPP, 2-TFMPP and 4-TFMPP decreased Complex-I activity by 62%, 57% and 61% respectively (n=6, p< 0.0001; Figure).

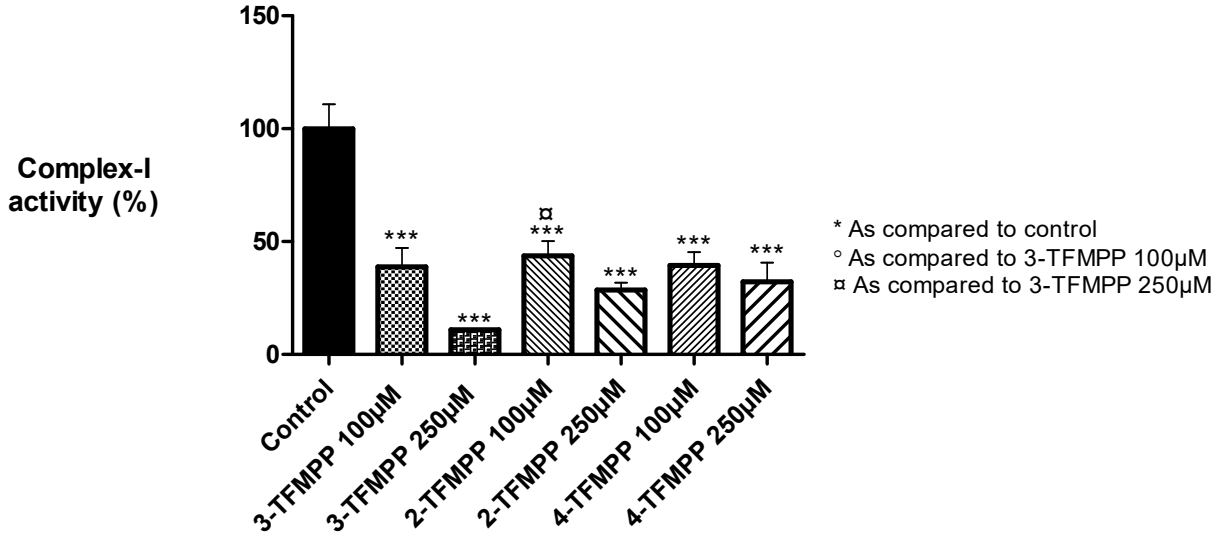

Figure 3.11. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal Complex-I activity: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently decreased Complex-I activity in HT-22 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.10. TFMPP Derivatives Inhibit Mitochondrial Complex-IV Activity

TFMPP derivatives showed significant dose-dependent inhibition of Complex-IV activity (n=, p< 0.0001; Figure 3.12.). At the dose of 250µM, 3-TFMPP, 2-TFMPP and 4-TFMPP decreased Complex-I activity by 90% (n=6, p< 0.0001; Figure 3.12.). Although at the lower dose (100µM), 3-TFMPP, 2-TFMPP and 4-TFMPP decreased Complex-I activity by 82%, 88% and 78 % respectively (n=6, p< 0.0001; Figure).

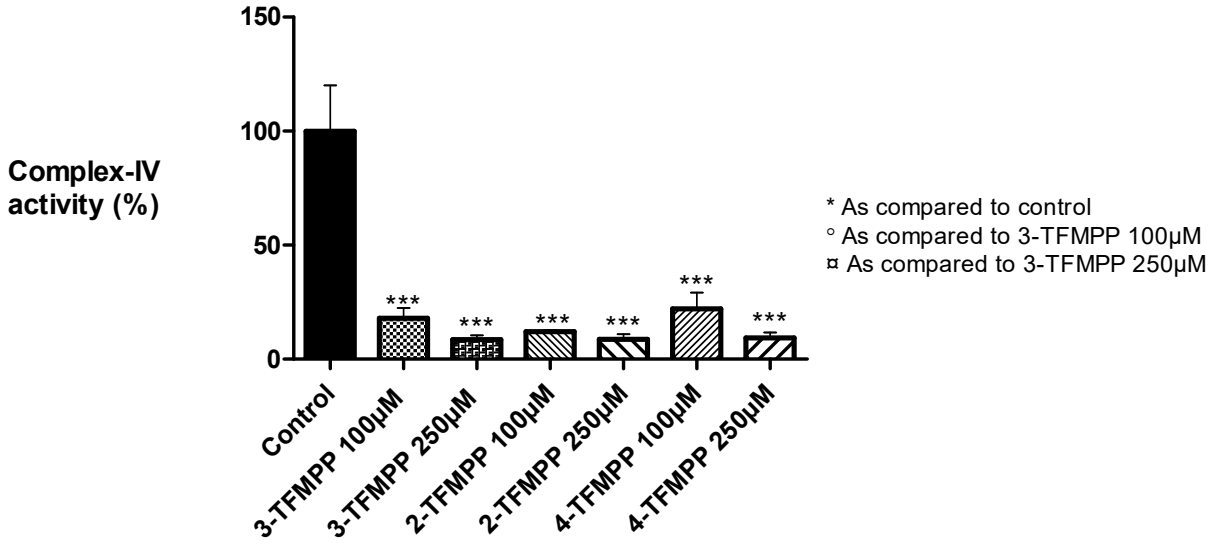

Figure 3.12. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal Complex-IV activity: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently decreased Complex-IV activity in HT2-2 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.11. 3-TFMPP Increase Acetylcholinesterase Activity

AChE is the primary cholinesterase enzyme in the body that catalyzes the breakdown of the neurotransmitter acetylcholine. The parent drug 3-TFMPP significantly increased AchE activity in HT-22 cells at dose of 250µM as compared to the control (n=6, p< 0.0001; Figure 3.13.). 2-TFMPP and 4-TFMPP had no effect on AchE activity in HT-22 cells.

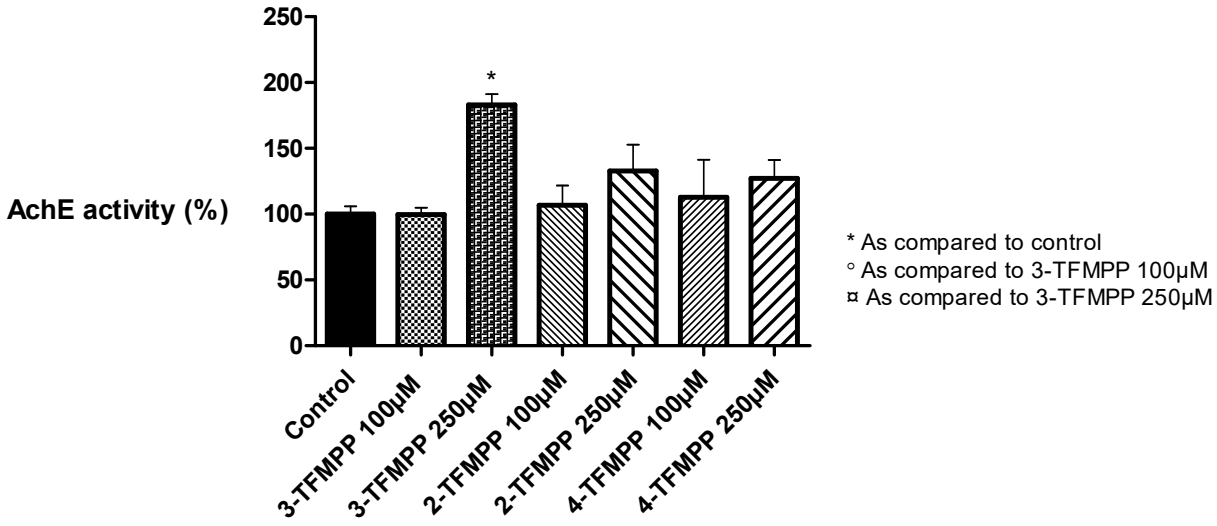

Figure 3.13. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal acetylcholinesterase activity: 3-TFMPP significantly increased AchE activity in HT-22 cells at dose of 250µM as compared to the control (n=6, p< 0.0001) Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.12. TFMPP Derivatives Decrease Choline Acetyltransferase Activity

ChAT is the enzyme responsible for acetylcholine synthesis by catalyzing the transfer of an acetyl group from the coenzyme acetyl-CoA to choline, producing acetylcholine. ChAT activity was significantly decreased in HT-22 cells as compared to the control by 3-TFMPP (33% at 100µM, 37% at 250µM), 2-TFMPP (65% at 100µM, 52% at 250µM) and 4-TFMPP (63% at 100µM, 52% at 250µM) (n=6, p< 0.0001, Figure 3.14.).

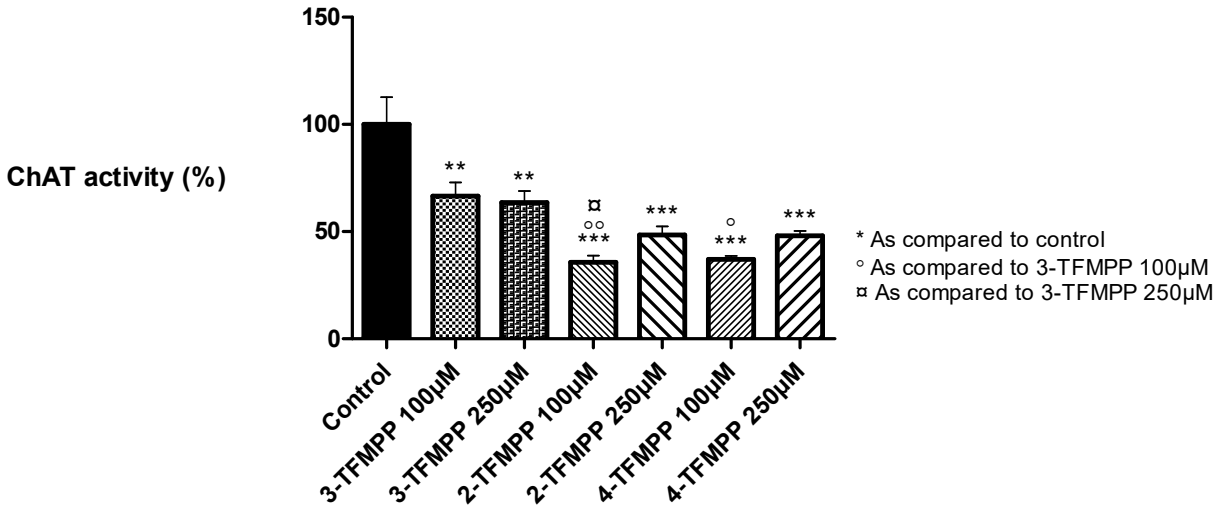

Figure 3.14. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal choline acetyltransferase activity: 3-TFMPP, 2-TFMPP and 4-TFMPP significantly decreased ChAT activity in HT-22 cells as compared to the control (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.13. TFMPP Derivatives had no Effect on Caspase-1 Activity

Caspase-1 is a **c**ysteine protease enzyme that activate inflammatory cytokines interleukin-1β and interleukin-18 to initiate pyroptosis which is a programmed cell death pathway. TFMPP derivatives 3 had no effect on the Caspase-1 activity in HT-22 cells as compared to the control (Figure 3.15.).

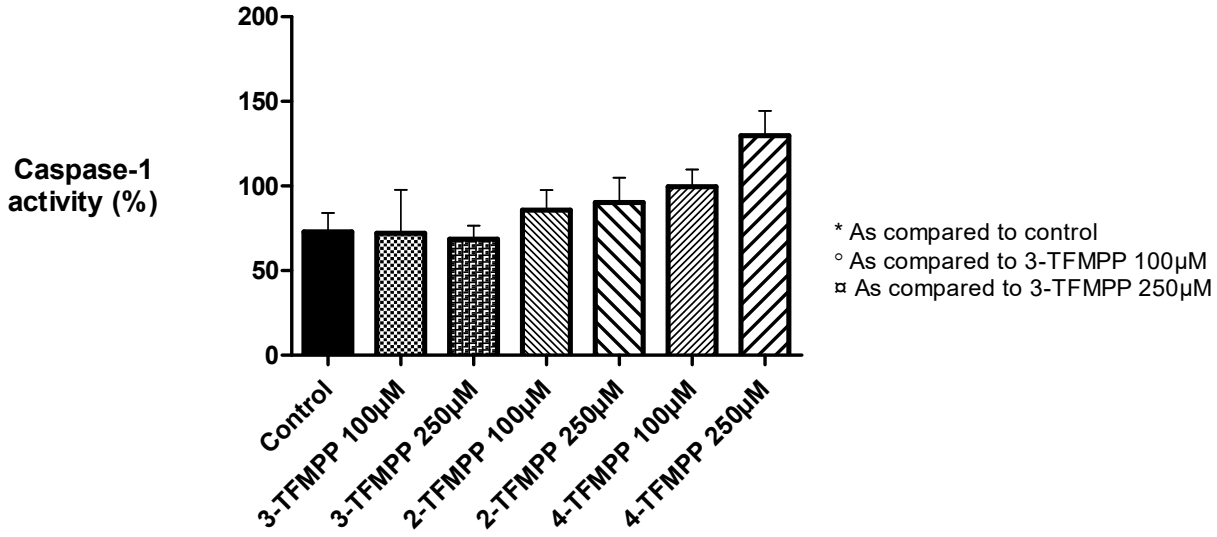

Figure 3.15. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal Caspase-1 activity: 3-TFMPP, 2-TFMPP and 4-TFMPP had no effect on the Caspase-1 activity in HT-22 cells as compared to the control (n=6). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.14. TFMPP Derivatives increase Caspase-3 Activity

Caspase-3 is a **c**ysteine protease enzyme that interacts with Caspase-8 and Caspase-9 as part of sequential activation of cell apoptosis. 3-TFMPP at dose of 250µM increased Caspase-3 activity by (315%) in H-T22 cells as compared to the control (n=6, p< 0.0001; Figure 3.16.). 2-TFMPP and 4-TFMPP had no effect on Caspase-3 activity.

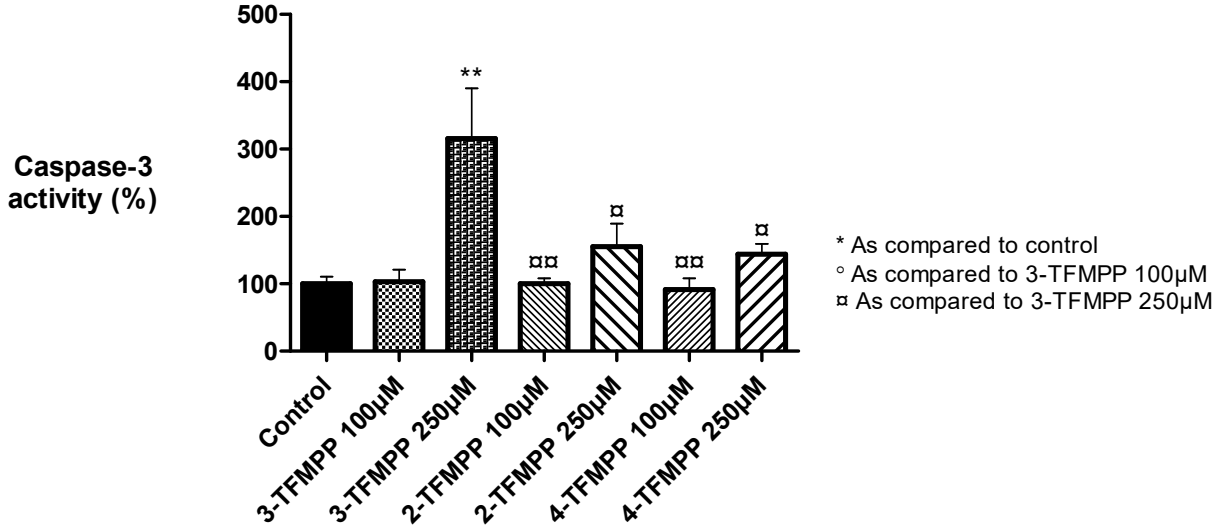

Figure 3.16. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal Caspase-3 activity: 3-TFMPP at dose of 250µM increased Caspase-3 activity in H-T22 cells as compared to the control (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.15. TFMPP Derivatives increase Caspase-9 Expression

TFMPP Derivatives significantly increased Caspase-9 expression in HT-22 cells (n=5, $^{*}p < 0.05$; Figure 3.17.).

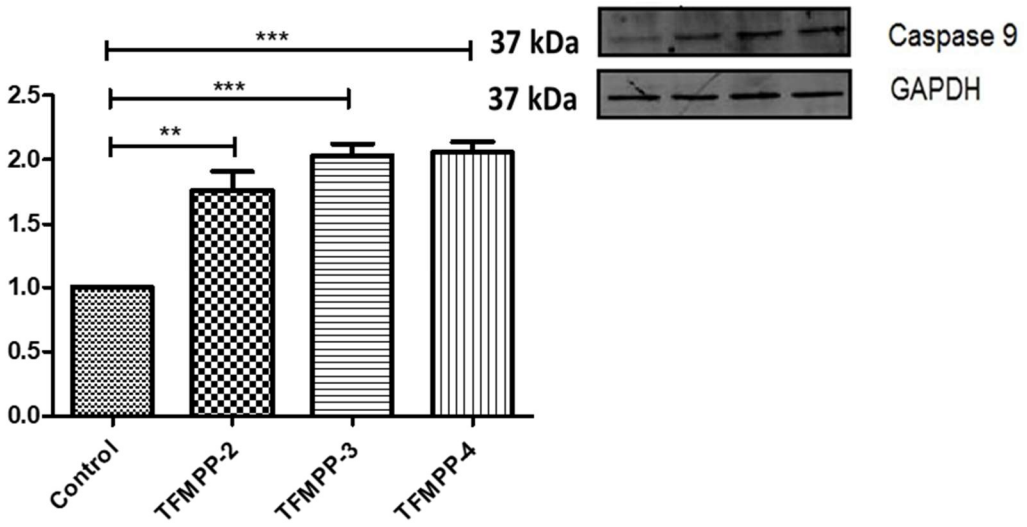

Figure 3.17. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal Caspase-9 expression: 2-TFMPP, 3-TFMPP and 4-TFMPP significantly increased Caspase-9 protein expression in HT-22 cells at dose of 250μM (n=5, $^{*}p < 0.05$). Blots were developed using 1:1000 dilution with primary antibody. GADPH (1:1000) was used as a loading control. Densitometric Analysis was performed with AlphaView software. Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.16. TFMPP Derivatives had no Effect on GLUR1 Expression

TFMPP derivatives had no effect on GLUR1 expression in HT-22 cells (Figure 3.18.).

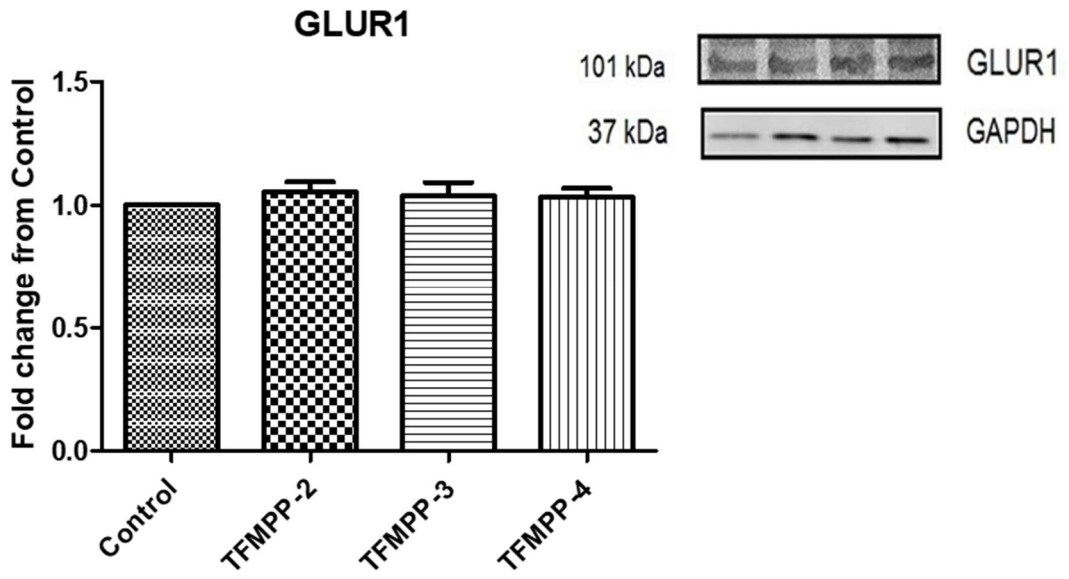

Figure 3.18. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal GLUR1 expression: 2-TFMPP, 3-TFMPP and 4-TFMPP had no effect on GLUR1 protein expression in HT-22 cells at dose of 250μM (n=5). Blots were developed using 1:1000 dilution with primary antibody. GADPH (1:1000) was used as a loading control. Densitometric Analysis was performed with AlphaView software. Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.17. TFMPP Derivatives had no Effect on NR2A Expression

TFMPP derivatives had no effect on NR2A expression in HT-22 cells (Figure 3.19.).

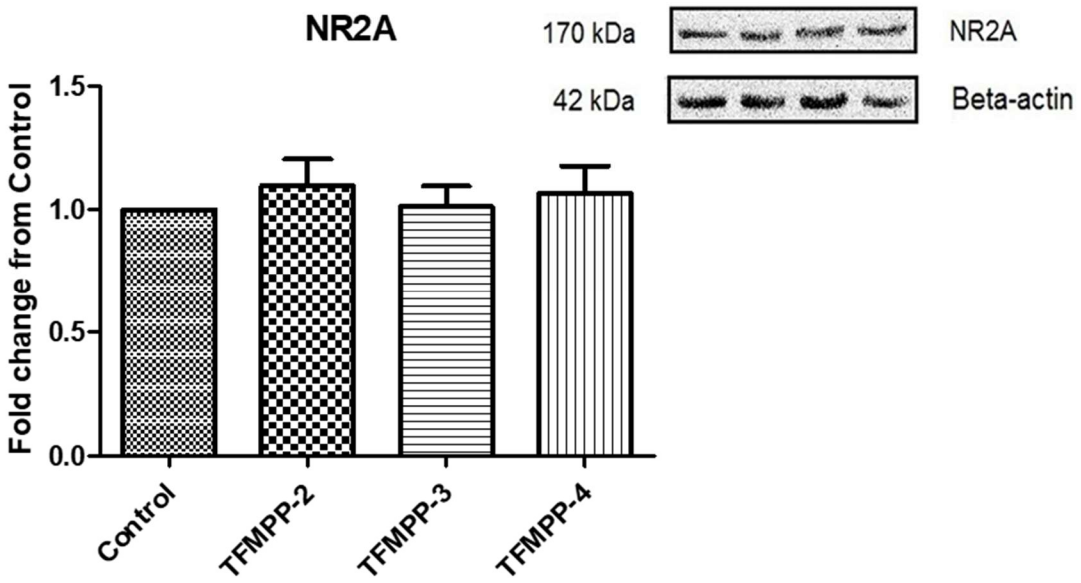

Figure 3.19. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal NR2A expression: 2-TFMPP, 3-TFMPP and 4-TFMPP had no effect on NR2A protein expression in HT-22 cells at dose of 250μM (n=5). Blots were developed using 1:1000 dilution with primary antibody. β-actin (1:1000) was used as a loading control. Densitometric Analysis was performed with AlphaView software. Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.18. TFMPP Derivatives had no Effect on PSD95 Expression

TFMPP derivatives had no effect on PSD95 expression in HT-22 cells (Figure 3.20.).

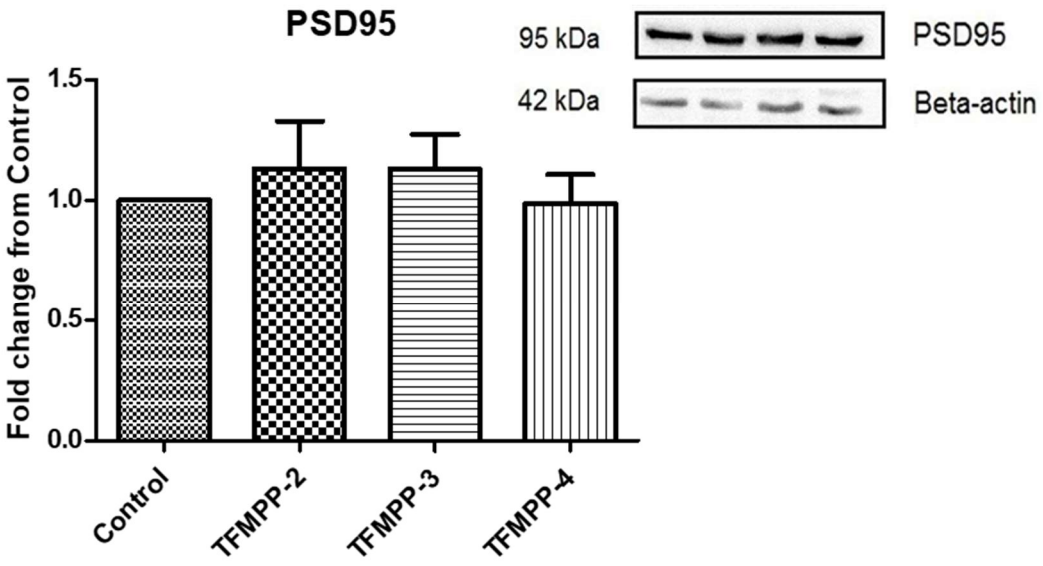

Figure 3.20. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal PSD95 expression: 2-TFMPP, 3-TFMPP and 4-TFMPP had no effect on PSD95 protein expression in HT-22 cells at dose of 250μM (n=5). Blots were developed using 1:1000 dilution with primary antibody. β-actin (1:1000) was used as a loading control. Densitometric Analysis was performed with AlphaView software. Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.19. TFMPP Derivatives increase NFKB Expression

3-TFMPP and 4-TFMPP significantly increased NFKB expression in HT-22 cells (n=5, $^{*}p < 0.05$; Figure 3.21.).

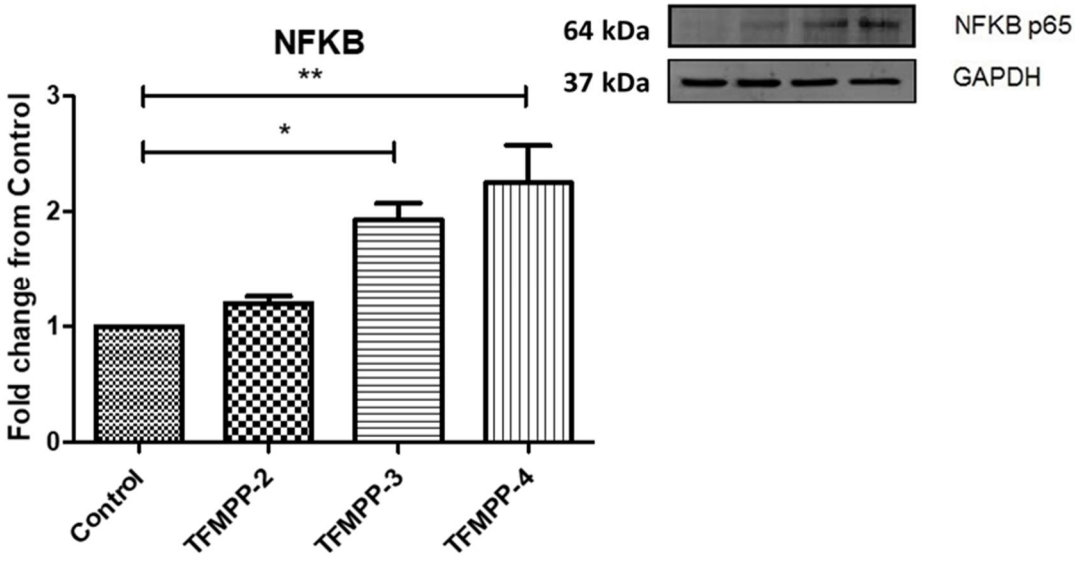

Figure 3.21. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal NFKB expression: 3-TFMPP and 4-TFMPP significantly increased NFKB protein expression in HT-22 cells at dose of 250μM (n=5, $^{*}p < 0.05$). Blots were developed using 1:1000 dilution with primary antibody. GADPH (1:1000) was used as a loading control. Densitometric Analysis was performed with AlphaView software. Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.3.20. TFMPP Derivatives Effect on p-P38 Expression

TFMPP Derivatives significantly increased p-P38 expression in HT-22 cells (n=5, $^{*}p < 0.05$; Figure; 3.22.).

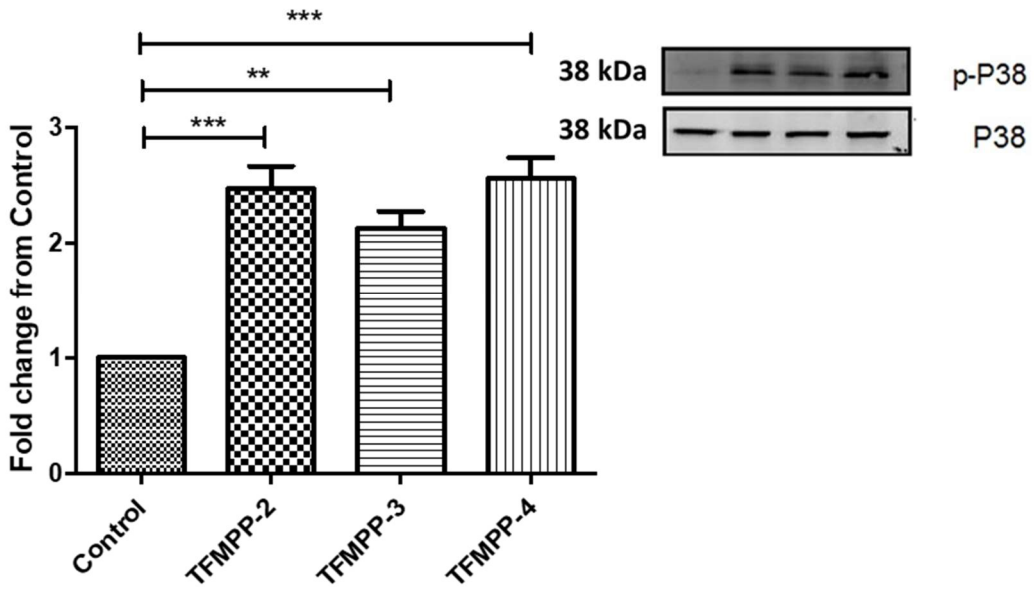

Figure 3.22. Effect of TFMPP derivatives on HT-22 mouse hippocampal neuronal p-P38 expression: 2-TFMPP, 3-TFMPP and 4-TFMPP significantly increased p-P38 protein expression in HT-22 cells at dose of 250μM (n=5, $^{*}p < 0.05$). Blots were developed using 1:1000 dilution with primary antibody. GADPH (1:1000) was used as a loading control. Densitometric Analysis was performed with AlphaView software. Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

3.4. Discussion

Aβ accumulation and plaque buildup produces its neurotoxicity through several mechanisms such as oxidative stress[52], mitochondrial dysfunction[53], energy imbalance[54], neuroinflammation[55], Ca2+ homeostasis disruption[56], interruption of axonal signal transport[57] and initiation of apoptosis[58,59]. All these mechanisms can lead to neuronal cell death resulting in the development and progression of AD. Oxidative stress occurs when the body have more free radicals than antioxidant enzymes. Free radicals cause oxidative damage which results in destruction of biological molecules such as proteins, DNA and lipids. Free radicals are quenched or scavenged in the body by the endogenous antioxidant enzymes or antioxidant molecules. Antioxidants such as catalase, SOD and GSH neutralize the harmful effects of free radicals. Numerous in-vitro and in-vivo studies has concluded that free radicals accelerate neuronal cell damage and death[60,61]. Nunomura, 2001[62] has reported that autopsy from AD patients has shown a significant increase in ROS generation in the brains of the patients. Leskovjan, 2011[63] also reported that in AD pateints there is an increase AL^{3+} and Mn^{2+} content where these metals can induce the production of free radical. Similarly, Siegel, 2007[64] reported the augmented lipid peroxidation in the cerebrospinal fluid of AD patients. Likewise, Markesbery, 1997 and Moreira, 2008[65,66] indicated that the quantities of damaged proteins and DNA are raised in the brain of AD patients. Similarly, Siegel, 2007, Abdul, 2008, Chami, 2012 and Verri, 2012[55,64,67,68] demonstrated that oxidative stress exaggerated mitochondrial dysfunction and Aβ plaque

formation. It is also believed that oxidative stress can be both a primary and secondary cause in the pathogenesis of AD[52,65]. Oxidative stress is one of the major pathogenic causes in mainly all the etiological hypothesis of AD. For example, in the amyloid theory it has been hypothesized that oxidative stress is produced from Aβ aggregation leading to neuronal cell death. With regard to the mitochondrial cascade hypothesis, recent studies showed that oxidative stress can cause mitochondrial dysfunction which result in inadequate ATP production and neuronal cell death. Additionally, The Metabolic Hypothesis suggests that inadequate insulin level is linked to increased generation of oxidative stress. Moreover, according to the vascular hypothesis due to the low blood flow to the brain there is a lower rate of removal of toxic substances from the CNS leading to increased oxidative stress and neuronal cells death. Hence, by inhibiting free radicals, antioxidative therapy has shown to be a valuable option in the treatment of AD[66,68–70]. AD has been found to be associated with abnormalities in the amounts of several neurotransmitters suggesting that these deviations are linked to AD symptoms[71]. Multiple studies have verified the extensive lack in the quantities of cholinergic neurotransmitter not only in the hippocampus but also in the neocortex of AD patients[72–74]. Treatments targeting acetylcholine deficiency showed enhancement in the cognitive function of AD patients which further proved the involvement of cholinergic neurotransmitter in the pathogenesis of AD[74,75]. The low Acetylcholine level can be as a result from increased breakdown of Acetylcholine by AChE or decreased synthesis of acetylcholine resulted from lower ChAT activity. Inflammation plays a crucial dual part in AD. It could be advantageous in the early stages of AD because it helps in the removal of misfolded proteins and toxic substance by phagocytosis. Nevertheless, persistent exposure to these toxicants lead to continuing stimulation of the inflammatory process. As a result, there will be over stimulation of microglia cells and cytokine release causing oxidative stress which will

initiate apoptosis and neuronal cell death. Aβ can trigger inflammation in the brain through Increasing the release of inflammatory cytokines[76] . Furthermore, Aβ provokes ROS generation and triggers oxidative stress which has been suggested as one of the etiologies behind the pathogenesis of AD[77]. Interestingly, activated microglia, astroglia and monocytes are usually found in the parts of the brain that are known to be involved in the pathogenesis of AD such as frontal neocortex, limbic cortex and in the area of Aβ plaques. On the other hand, in the cerebellum there were no indications to the presence of inflammation[78,79]. Correspondingly, anti-inflammatory treatments have shown to be beneficial in reducing the progression as well as delaying the onset of AD[80]. TFMPP derivatives induced an increase in the generation of ROS and nitrite content causing increased interaction of these free radicals with polyunsaturated fatty acids. Consequently, the interaction results in oxidative breakdown of lipids which lead to boosted lipid peroxidation. SOD is an antioxidant enzyme that rapidly dismutase superoxide anion radicals into H_2O_2 and water. Furthermore, MAO stimulate oxidation of monoamine which produces H_2O_2[81,82]. Subsequently the antioxidant enzymes catalase, GSH and GSH-Px converts H_2O_2 into water. TFMPP derivatives caused a decrease in the activity of SOD, catalase and GSH content. This can be explained as a result of the huge increase in the free radical and H_2O_2 content leading to the antioxidants exhaustion and consumption. Deficits in mitochondrial Complex-I and Complex-IV have been linked to several neurodegenerative diseases such as Parkinson's disease, AD, Huntington's disease and Amyotrophic lateral sclerosis[83]. Furthermore, TFMPP derivatives showed an ability to significantly decrease Complex-I and Complex-IV activities. The decline in electron chain enzymes activities demonstrated a trend of escalating with the dose increase. These findings confirm the initiation of mitochondrial dysfunction by TFMPP derivatives leading to diminished ATP production and neuronal cell death. Moreover,

TFMPP derivatives reduced the activity of ChAT suggesting a reduction in the synthesis of acetylcholine. The parent drug (3-TFMPP) exhibited an ability to increase AchE which will lead to an increase in acetylcholine breakdown. With the decrease of acetylcholine synthesis and the increase of its breakdown, TFMPP derivatives abuse can cause a diminished acetylcholine level in the body. With regard to apoptosis, the parent drug (3-TFMPP) caused an increase in the activity of Caspase-3 enzyme implying the ability of the drug to initiate programmed cell death.

From the above literature study on AD, in additon to oxidative stress and mitochondrial dysfunction, it is clear that inflammation, apoptosis and excitotoxicity play a crucial role in cholinergic neurodegeneration. Neuroinflammation plays a central role in the progression of the neuropathological changes in AD[84]. Chronically activated microglia release a variety of proinflammatory mediaotrs and and neurotoxic substances (ROS, NO, and cytokines)[85,86]. Nuclear factor-κB (NF-κB) act as dimeric transcription factors that control the expression of genes affecting several biological processes involving innate and adaptive immunity, inflammation, stress responses, B-cell development, and lymphoid organogenesis[84]. NF-κB proteins are bound and inhibited by IκB proteins. Various pro-inflammatory mediators and antigen receptors activate an IKK complex (IKKβ, IKKα, and NEMO), which phosphorylates IκB proteins. Phosphorylation of IκB leads to its ubiquitination and proteasomal degradation. Active NF-κB complexes are further activated by post-translational modifications (phosphorylation, acetylation, glycosylation) and translocate to the nucleus where, either alone or in combination with other transcription factors including AP-1, Ets, and Stat, they induce target gene expression. NF-κB activators increase the genration of ROS, interleukin 1-beta (IL-1β), tumor necrosis factor alpha (TNF-α)[87].

Endogenous and exogenous neurotoxins can activate stress-activated c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein (MAP) kinase (p38) to alter apoptosis[88]. The possible targets for these kinases include members of the Bcl-2 family proteins, which mediate apoptosis generated through the mitochondria-initiated, intrinsic cell death pathway. The activities of several Bcl-2 family proteins, both pro- and anti-apoptotic, are controlled by JNK phosphorylation[89]. Activated MAP kinase can transmit extracellular signals to regulate cell growth, proliferation, differentiation, migration, and apoptosis. Apoptosis as well as macroautophagy can be induced by extracellular stimuli. Several studies show that p38 and JNK MAP kinase pathways function in the control of the balance of autophagy and apoptosis in response to genotoxic stress[90]. Induction of apoptosis is accompanied by immediate and sustained activation of p38 MAP kinase and various caspases[91]. In our study, the parent drug (3-TFMPP) exhibited significant increase in Caspase-3 activity. Furthemomer, all TFMPP derivatives showed capability to significantly increase Caspase-9 expression in HT-22 cells. Likwise, TFMPP derivatives demonstrated an ability to activate P38 by increasing its phophorylation. These findings suggest the ability of TFMPP derivatives to intiatie apoptosis.

3.5. Conclusion

Hippocampal neurodegeneration is a complex process and therefore continuous researches are being focused to elucidate the toxic mechanisms associated with the decreased functioning of the hippocampal neurons leading to the neuronal death. Novel neurotoxin-induced models have been used to elucidate the neurotoxic mechanisms associated with neuronal death. Piperazine derivatives have shown to exhibit dose-dependent hippocampal neurotoxicity. In the future, piperazine designer drugs can be used as a novel chemically-induced model to study the Hippocampal neurodegeneration. With regard to the neurotoxic mechanisms of Alzheimer's disease, decreased cholinergic neurotransmission coupled with oxidative stress, apoptosis, excitotoxicity, mitochondrial dysfunction and inflammation are the hallmark of the disease. Piperazine derivatives can increase the risk for hippocampal neurotoxicity by decreasing the cholinergic neurotransmission (increase metabolism/breakdown of acetylcholine by increasing AchE activity and decrease synthesis by inhibiting ChAT), induce oxidative stress (increase ROS, NO, LP, MAO activity, decrease SOD, Catalase), decreasing the production of ATP by inhibiting Complex-I and Complex-IV thus leading to mitochondrial toxicity, induce apoptosis (increasing Caspase-3 and caspase-9, pP38) and escalate inflammation by increasing NFKB. Consequently, if proper measures and care is not taken immediately to control the abuse of designer drugs, it can significantly increase the risk for learning and memory impairment in the future generation as well as various types dementia.

3.6. References

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4. Investigate the effect of TFMPP Derivatives on Dopaminergic Neurotransmission

4.1. Introduction

Neurodegeneration symbolizes the progressive loss of structure &/or function of the neurons ("neuro" refers to the neuron / nerves and "degeneration," indicates the progression of losing the structure &/or function). Neurodegenerative diseases characterize a large collection of neurological disorders with diverse clinical and pathological symptoms affecting specific neurons in a specific region of the brain. The major neurodegenerative diseases are Alzheimer disease, Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). The major etiological factors associated with the initiation of neurodegenerative disorders are the genetic, head trauma, drug-induced and environmental factors[1]. Current literature and our earlier studies have shown that any chronic use of stimulants or substance of abuse can increase the risk for dopaminergic neurodegeneration[2,3]. However, the relationship between the use of piperazine designer drugs and the risk for dopaminergic neurodegeneration are not well understood.

PD is a progressive dopaminergic neurodegenerative disease characterized by resting tremor, movement difficulty, low dopamine levels and deterioration of the basal ganglia in the brain[1]. Since the ancient times, PD has been observed by humanity and remarkable symptoms of PD

were reported in various civilizations. For example, in Indian Ayurveda this disease was narrated in 10th century B.C scriptures as disorder which has symptoms that include tremor, salivation and lack of movement. In Ayurveda, this disease was indicated as Kampavata and interestingly was treated with remedies rich in L-DOPA which were extracted from botanicals belonging to Mucuna genus. Later in the 12th century B.C., where an Egyptian papyrus articulates that a king has developed tremor and salvation with ageing. The Greek physician Galen who lived in 2nd century AD has described a disease characterized by tremors, postural changes and paralysis. In 1817, an English apothecary named James Parkinson published a paper titled as "An Essay on the Shaking Palsy" describing six cases that shared similar symptoms and urging more research on this disorder[4]. Later in 1865, a French neurologist called Jean-Martin Charcot and William Rutherford Sanders of Edinburgh acknowledged James Parkinson and named the disease after him. In the 1960s, the low levels of dopamine in the substantia nigra were noticed leading to the introduction of Levodopa, the" golden standard" in the treatment of PD. Until now, there is no cure to PD and the treatment strategy against PD relies on alleviating and containing the symptoms[5]. The symptoms of PD can be classified into motor and non-motor symptoms. They worsen gradually along with aging with the motor symptoms being more abundant in the early stages. Motor symptoms include tremor, rigidity, bradykinesia, akinesia, drooling, hypophonia, dysphagia and postural instability, all these symptoms are often referred communally as "parkinsonism"[6]. Tremor is the most distinguishing and the most common symptom of PD. It occurs in around 70% of the patients and it's mainly appear when the patient is not moving his limb/(s)[7]. Moreover, muscle rigidity is caused by an excessive contraction of muscles leading to joints stiffness. Also, bradykinesia and akinesia are considered as distinctive indicators of PD since both result in movement hindrance and diminishing and can be easily

identified. Drooling and dysphagia are caused by incapability to swallow while hypophonia occur frequently in PD due to the failure in vocal muscle coordination[8].

The exact reason behind the development of PD is still unknown but the loss of dopaminergic neurons leading to insufficiency in dopamine levels is believed to be the main reason[9]. Similar to the general etiological factors associated with the initiation of neurodegenerative disorders, PD also can occur due to ageing, genetic factors, head trauma, drug-induced and exposure to environmental factors. Several mechanisms are suggested to explain the etiology of dopaminergic neuronal death which is abundant in the pars compacta at the substantia nigra in PD patients[10]. These mechanisms include Lewy bodies formation, interruption of autophagy, oxidative stress, mitochondrial dysfunction, neuroinflammation, blood-brain barrier (BBB) disruption and excitotoxicity[11]. Alpha-synuclein (α-synuclein) is insoluble protein that has been found to be increased in the brains of PD patients. Its accumulation results in the formation of the abnormal aggregate called Lewy bodies, an important pathological indicator of PD and dementia[12]. Autophagy is a natural mechanism that plays an important role by maintaining homeostasis of cells and regulating the cells function by eradicating inoperative and defective proteins as well as allowing protein recycling. Interruption of autophagy can result in the accumulation and the formation of protein oligomers and toxic substances. This leads to the Interference with normal cell function and eventually neuronal cell death in Parkinson's disease[13,14]. Several studies demonstrated that agents that boost autophagy exhibited neuroprotective effects that may be due increase clearance of α-synuclein[15–17]. Oxidative stress can result in neuronal cell death in the substantia nigra as a result of several conditions including increased metabolism of dopamine, decreased glutathione content and increased Iron content.

Therefore, enhanced metabolism of dopamine result in increased peroxide production leading to increased lipid peroxidation[18]. Jenner & Olanow 1996 reported that substantia nigra of brains PD patients undergo oxidative stress since it showed augmented iron content, depleted GSH content, and increased oxidative damage to lipids, proteins and DNA[19]. Moreover, enhanced levels of lipid peroxidation have been identified in the substantia nigra but not the cerebellum of PD patients[20,21]. Likewise, the toxic product of lipid peroxidation, 4-hydroxynonenal has been spotted in remaining dopaminergic neurons[22]. Besides, increased levels of protein carbonyls have been revealed in the substantia nigra of PD patients[23–25]. Physiologically, apoptosis (programmed cell death) regulates embryogenesis, tissue renewal, turnover of cells (neurons), development and function of immune system, hormone-dependent atrophy, embryonic formation, and cell (neuronal) death. Change in apoptosis (increase or decrease) leads to several neurodegenerative diseases (Alzheimer's, Parkinson's and Huntington's disease), cardiovascular disorders, autoimmune disorders and several cancers. Patient's with PD show significant apoptosis and autophagic relapse in nigral dopaminergic neurons[26,27]. Likewise, in vitro[28,29] and in vivo[30,31] studies with dopaminergic neurotoxins implicated apoptosis, resulting in dopaminergic neuronal impairment.

The main role of mitochondria in the cell is energy production (ATP) through respiration. Thus, the mitochondria play vital role in regulating cell survival and death. Mitochondria use electron transport chain reaction to transport electrons from a donor to an acceptor through reduction reactions. The transported electron with its coupled H^+ ions is then used by ATP synthase to produce ATP. The members of this chain reaction include Complex-I, Complex-II, Complex-III and Complex-IV. Several studies reported that deficits in Complex enzymes activities are

involved in various neurodegenerative diseases such as PD[32]. Mitochondrial dysfunction in PD was linked to a mutation in PINK1 and Parkin genes. PINK1 and Parkin are responsible for autophagy in mitochondria[33,34]. PINK1 activates Parkin to hydrolyze dysfunctional protein in the mitochondria. Consequently, the mutation in these genes can lead to accumulation of dysfunctional protein in the mitochondria resulting in neuronal cell death[33,34] . Schapira et al 1990 reported a region-specific reduction in Complex-I activity in substantia nigra but not at other regions of PD patients' brain. Additionally, reduction in Complex-I activity was observed in the platelets of PD patients[35]. The loss of dopaminergic neurons in the substantia nigra of PD patients has been connected to neuroinflammation which may be related to the activation of microglial cells. Hence, accumulation of α-synuclein in PD patients result in continuous activation of microglial and chronic release of pro-inflammatory cytokines cells leading to the development of chronic inflammation. Which in turn cause an increase in the generation of ROS, the breakdown of tissue, apoptosis and of the impairment of BBB. BBB sustains the central nervous system homeostasis through permitting the entry of essential nutrient and prevention of the entry of toxins and pathogens[36]. The disruption in BBB ability to control substance passage to substantia nigra of PD patients has been detected[37]. Studies showed that in PD, the development of more capillary is hindered by the inhibition of vascular endothelial growth factor (VEGF) and its receptors[38,39]. This inhibition leads to collapse of present capillaries and loss of connection between cells resulting in the loss of regulation of substance movement throughout BBB.

Excitotoxicity occur when NMDA receptors are excessively stimulated by glutamate leading to the opening of the voltage-gated calcium channels resulting in increased calcium ions influx into

the neurons. The excessive intracellular calcium concentrations tremendously increase the activity of enzymes such as phospholipases and proteases which destroy mitochondria and other cell structures and increase the generation of oxidative stress[40]. Dopaminergic neurons in substantia nigra contain large numbers of different types (NMDA, AMPA, and Kainate) glutamate receptors. Therefore, elevated level of glutamate can lead to excessive neuronal firing and excitotoxicity[41,42] . Increased glutamate level results in excess calcium influx leading to the stimulation of nitric oxide synthase and the formation of nitric oxide an important mediator of excitotoxicity[43] . Studies showed that in nitric oxide synthase knock-out mice the toxicity of MPTP was reduced[44]. Also, the inhibition of nitric oxide synthase demonstrated dopaminergic neurons resistance against MPTP toxicity[45,46]. Nitric oxide exhibited inhibitory effect on mitochondrial respiratory chain specifically Complex-IV[47]. Finally to further confirm the involvement of excitotoxicity in PD[48] showed that NMDA antagonist administration provided a protection to substantia nigra dopaminergic neurons in rat treated with $MPP^{+\cdot}$

In conclusion, the mechanisms associated with the piperazine designer drugs mediated dopaminergic neurotoxicity leading to PD are poorly understood. Hence, in this study we evaluated the effect of piperazine designer drugs on dopamine metabolism.

4.2. Methods

4.2.1. Chemicals and Reagents

Thiazolyl Blue Tetrazolium Bromide (MTT), Dopamine and Homovanillic acid (HVA) were bought from Tokyo Chemical Industry America (Portland, OR). Dulbecco's Modification of Eagle's Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin solution were purchased from Corning® (Corning, NY). Phosphate buffer saline (PBS), Dimethylsulfoxide (DMSO), Hydrogen Peroxide (H_2O_2), Phosphoric acid, Trichloroacetic acid, Heptane sulphonic acid and EDTA were purchased from Sigma Aldrich (St. Louis, MO). Tetrahydrobiopterin (BH_4) was purchased from Enzo Life Sciences (Farmingdale, NY). 3,4-Dihydroxyphenyl acetic acid (DOPAC), 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA), Sodium periodate, Tyrosine, 5,5'-Dithiobis (2-nitrobenzoic acid) (Ellmans reagent, DTNB) were purchased from Alfa Aesar (Haverhill, MA). Cell lysis buffer was purchased from Cell Signaling Technologies (Danvers, MA). Trypsin (0.25%) EDTA was acquired from VWR (Radnor, PA). Phosphoric Acid, Triethyl amine and Acetonitrile were obtained from Fisher Scientific (Hampton, NH). A Thermo Fisher Scientific Pierce 660 nm Protein Assay reagent kit was purchased (Pierce, Rockford, IL) for protein quantification. ZORBAX Extend 300 C18, 4.6 x 250 mm, 5 µm HPLC column was purchased from Agilent Technologies (Santa Clara, CA).

4.2.2. N27 Rat dopaminergic neuronal cells

N27 cell line was harvested from E12 rat mesencephalic tissue and was transfected with SV40 to immortalize the cell line. The N27 cell line, when injected into the striata of 6-hydroxydopamine-lesioned rats (an animal model of PD) caused a time-dependent improvement in neurological deficits. This immortalized cell line has been carefully characterized in studies of dopamine biosynthesis, neurotoxicity and used as a dopaminergic neuron model for *in vitro* and *in vivo* studies. N27 rat dopaminergic neuronal cells were cultured in RPMI 1640 Medium supplemented with 4.5 g/L Glucose, 25 mM HEPES, 4mM L-Glutamine, 10% Fetal Bovine Serum, 100 units/mL Penicillin and 50 µg/mL Streptomycin. Cells were cultivated in 75 cm^2 flasks and kept in a humidified incubator at 37°C and supplemented with 5% CO_2. N27 cells were collected by trypsinization (0.25% (w/v) Trypsin-0.53mM EDTA) after reaching 80% confluency (4-5 days) and centrifuged at 1000 RPM for 5 minutes to precipitate the cells. For the MTT assay, cells were seeded into 96 well plates at a density of 1 x 10^5 cells/well. Cells were used within 6-12 passages after they were received[49].

4.2.3. Treatment Design

Prior to each experiment, 2-TFMPP, 3-TFMPP and 4-TFMPP were freshly prepared and diluted in PBS to a 10mM stock solution. To evaluate the dopaminergic neurotoxicity, different concentrations of 2-TFMPP, 3-TFMPP and 4-TFMPP were prepared by serial dilution with PBS followed by additional dilution in serum enriched fresh culture medium. With regard to the control, N27 dopaminergic cells were treated with PBS. Cells were exposed to different

concentrations of 2-TFMPP, 3-TFMPP and 4-TFMPP for 24 and 48 hours. However, to establish the effect of 2-TFMPP, 3-TFMPP and 4-TFMPP on dopamine synthesis and metabolism, the N27 dopaminergic cells were exposed TFMPP derivatives for 18 h. All stock solutions were stored at -20 ºC and freshly diluted on the day of the experiment.

4.2.4. Cytotoxicity Assay

For the evaluation of cytotoxicity, MTT cell viability assay was performed. The concept of MTT assay is that the mitochondria of viable cells through succinate dehydrogenases reduce the yellow colored water soluble tetrazole reagent MTT to an insoluble blue crystal formazan that can be measured colorimetrically[50,51]. After 24 hours and 48 hours incubation with 3-TFMPP in serum-fed and serum-free medium, 12 mM MTT stock solutions was prepared and then added on each well along with fresh culture medium. Following 2 hours incubation at 37 ºC the medium was aspirated and 200µl of DMSO was added to solubilize the formazan crystal. Afterward 10 minutes incubation at 37 ºC the absorbance was measured using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA) at 570 nm.

4.2.5. Protein quantification

Protein was quantified using Thermo Scientific Pierce 660 nm Protein Assay reagent kit (Pierce, Rockford, IL). Bovine serum albumin (BSA) was used as a standard for protein measurement.

4.2.6. Mitochondrial monoamine oxidase activity

Total monoamine oxidase (MAO) activity was measured fluorometrically by determining the amount of 4-hydroxyquinoline formed as a result of kynuramine oxidation[52]. MAO activity was reported as 4-hyroxyquinoline formed/hour/mg protein[53–55].

4.2.7. Tyrosine Hydroxylase Activity

Tyrosine hydroxylase is the rate-limiting step in dopamine synthesis. It is responsible of oxidizing tyrosine to form L-Dopa, which is further metabolized to dopamine by dopa decarboxylase. The formation of L-Dopa can be estimated by using sodium periodate to oxidize L-Dopa to form the spectrophotometrically detectable chromophore dopachrome using a plate reader at 475nm. A L-Dopa standard curve was prepared from commercially acquired L-Dopa[56].

4.2.8. High-Performance Liquid Chromatography (HPLC)

HPLC was used to measure dopamine content and dopamine turnover in N27 rat dopaminergic cells after incubation with TFMPP derivatives. For analysis of dopamine and its metabolites, cells were collected by trypsinization and washed with ice cold PBS. Chilled 0.4N Perchloric acid + 0.01% EDTA (filtered and degassed) was added on the cells pellet followed by sonication for 6-7 sec until the tissue is completely homogenized. 30µL of the homogenate were drawn out for protein quantification and the remaining homogenate was centrifuged at 12000 rpm for 15 min at 4 °C temperature. 20µL from the supernatant of each sample was injected directly into the

HPLC system (Shimadzu Corporation, Kyoto, Japan) to determine DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-Methoxytyramine (3-MT), and HVA. The system was equipped with Decade Elite electrochemical detector and VT03 cell (Antec Scientific, NV, Zoeterwoude, The Netherlands). Also, the system was equipped with an ion-pair, ultrasphere reverse-phase chromatography column (ZORBAX Extend 300) with 4.6 x 250 mm and 5µm particle size (Agilent Technologies, Santa Clara, CA). The mobile phase was composed of 8.65 mM heptane sulfonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.4–0.45% triethylamine, and 0.20–0.25% phosphoric acid (vol/vol). The flow rate was 0.7 ml/min, and electrodetection was performed at 0.74 V. Results are presented as pmol/mg.

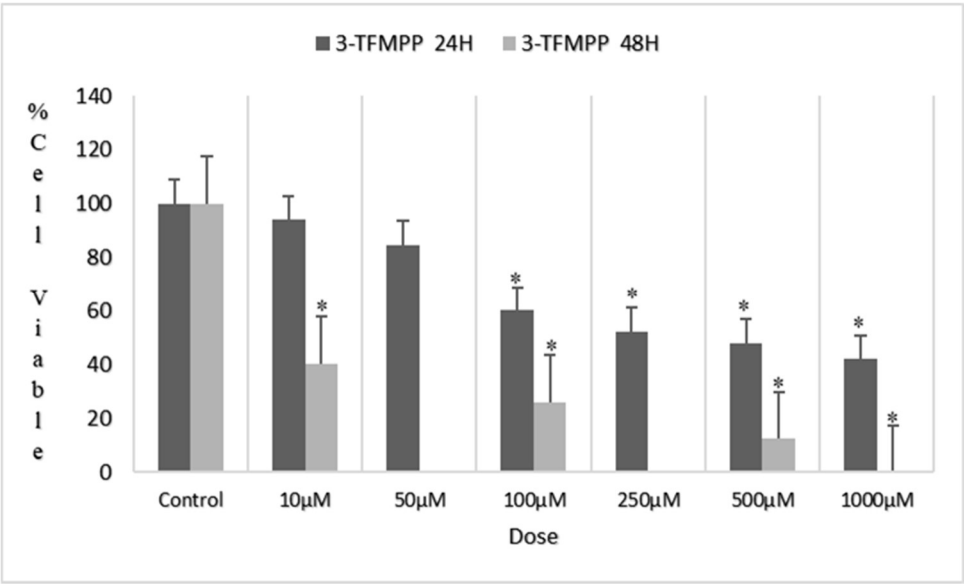
4.2.9. Statistical Analysis

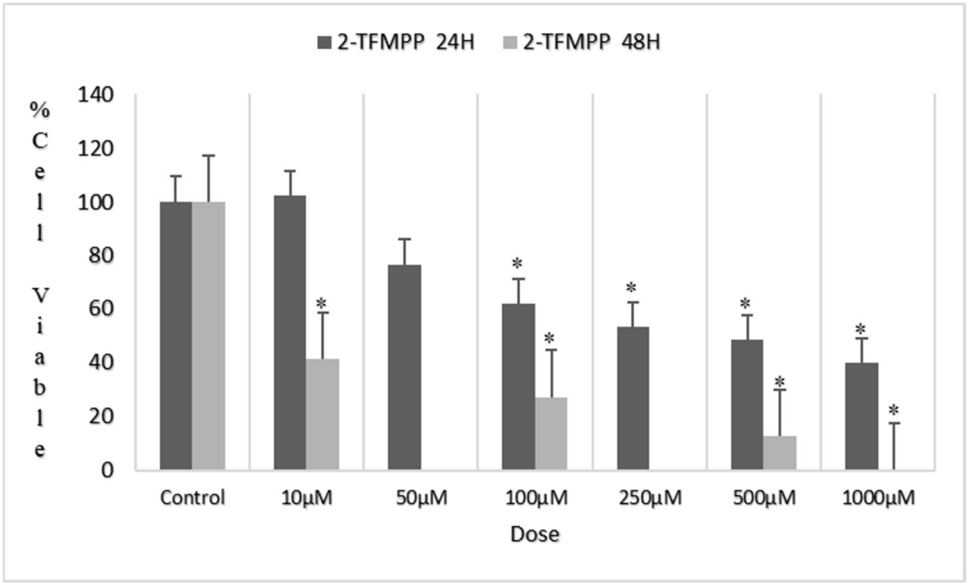
Data was reported as mean ± SEM. Statistical analysis were accomplished using one-way analysis of variance (ANOVA) followed by Turkey 's multiple comparisons test ($p < 0.05$ was considered to be statistically significant). Statistical analysis was performed using Prism-V software (La Jolla, CA, USA).

4.3. Results

4.3.1. Dose-dependent and time dependent effect of TFMPP derivatives on N27 dopaminergic neurons

2-TFMPP, 3-TFMPP and 4-TFMPP induced a dose-dependent and time dependent decrease in dopaminergic neuronal cell viability as compared to the control (n=12, $p < 0.0001$; Figure 4.1.). At the dose of 10 μM, TFMPP derivatives did not exhibit any neurotoxicity. As for the morphological changes in the N27 dopaminergic neuronal cells, TFMPP derivatives-induced well defined neuronal structural deformation compared to the control neurons. There was neuronal shrinkage, changes in the shape of the neurons (rounded and disfigured), decrease in synaptic connections which led to the decreased neuronal viability (figure 4.1.).





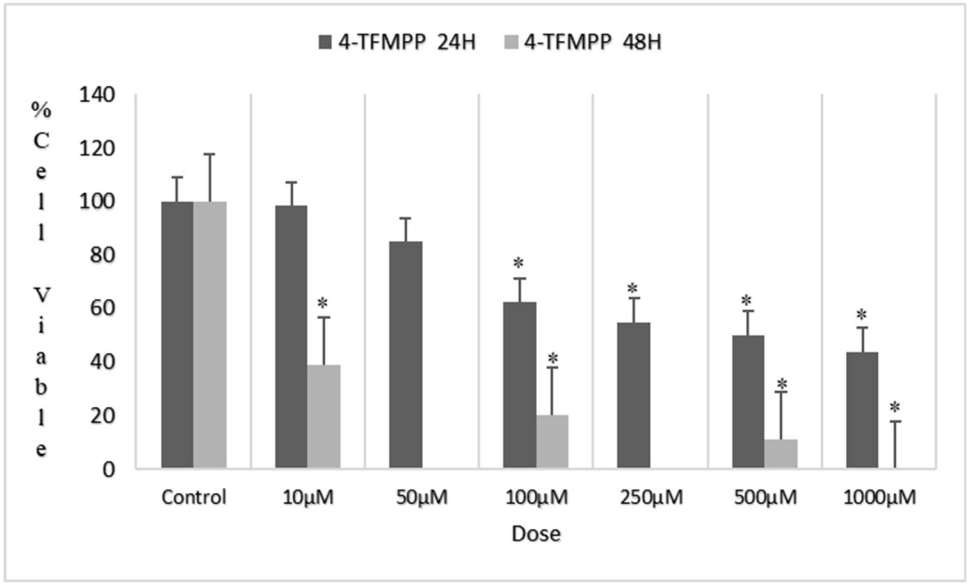


Figure 4.1. Effect of TFMPP derivatives on N27 rat dopaminergic neuronal viability: N27 rat dopaminergic neurons were treated with different concentrations of TFMPP derivatives and incubated for 24 h and 48 h. MTT assay was used to evaluate the neuronal viability. Results are expressed as (%) change as compared to the control, mean ± SEM. 2-TFMPP, 3-TFMPP and 4-TFMPP dose-dependently and time-dependently decreased the neuronal viability significantly as compared to the control ($n=12$, $p < 0.05$).

4.3.2. TFMPP Derivatives Increase Monoamine Oxidase Activity

MAO catalyze the oxidative deamination of dopamine to yield DOPAC which undergoes O-methylation by catechol-O-methyltransferase (COMT) to result in the formation of HVA[57,58]. Total MAO in the control, 3-TFMPP, 2-TFMPP and 4-TFMPP treated N27 rat dopaminergic neuronal cells was measured fluorometrically by determining the amount of 4-hydroxyquinoline formed as a result of kynuramine oxidation. TFMPP derivatives dose-dependently increased MAO activity in N27 cells after 24 hours incubation. as compared to the control (n=5, p< 0.0001; Figure 4.2.).

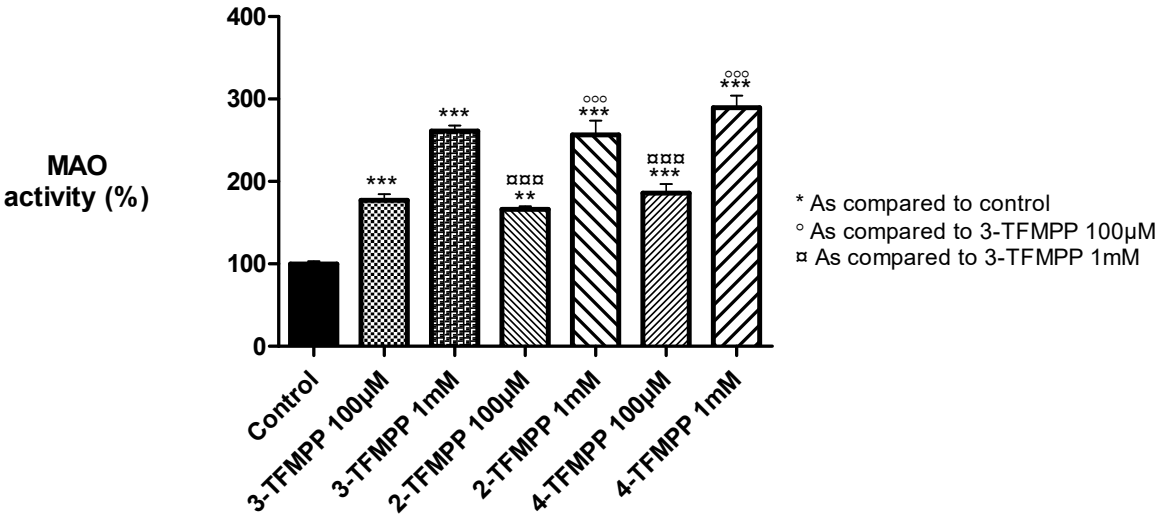


Figure 4.2. Effect of TFMPP derivatives on N27 rat dopaminergic monoamine oxidase activity: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently increased monoamine oxidase activity in N27 cells as compared to the control. (n=6, p< 0.0001). Results are expressed as (%) change as compared to the control, Mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. ***indicates a statistically significant difference when compared to controls.

4.3.3. TFMPP Derivatives Inhibit Tyrosine Hydroxylase Activity

Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of dopamine; it helps in the conversion of L-DOPA to dopamine. Neurotoxins inhibiting tyrosine hydroxylase have shown to exhibit severe dopaminergic neurotoxicity. TFMPP derivatives significantly decreased tyrosine hydroxylase activity as compared to the control (n=6, $p < 0.0001$, Figure 4.3.). 3-TFMPP (the parent drug) showed higher reduction of tyrosine hydroxylase activity (40% at 100µM 70% at 1mM) than 2-TFMPP (38% at 100µM 48% at 1mM) and 4-TFMPP (26% at 100µM 53% at 1mM).

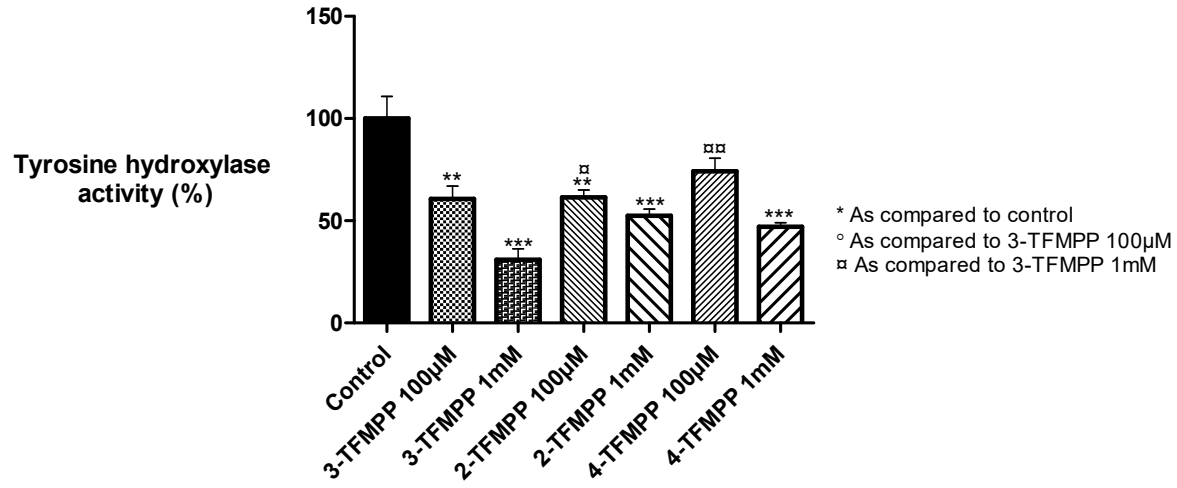


Figure 4.3. Effect of TFMPP derivatives on N27 rat dopaminergic tyrosine hydroxylase activity: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently decreased tyrosine hydroxylase activity in N27 cells as compared to the control. (n=6, $p< 0.0001$). Results are expressed as (%) change as compared to the control, Mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

4.3.4. TFMPP Derivatives Deplete Dopamine

TFMPP derivatives significantly depleted dopamine content as compared to the control in a dose dependent manner (n=3, $p < 0.0001$, Figure 4.4.). Dopamine depletion in N27 cells was as following 3-TFMPP (54% at 100µM 95% at 1mM), 2-TFMPP (6% at 100µM 96% at 1mM) and 4-TFMPP (48% at 100µM 96% at 1mM).

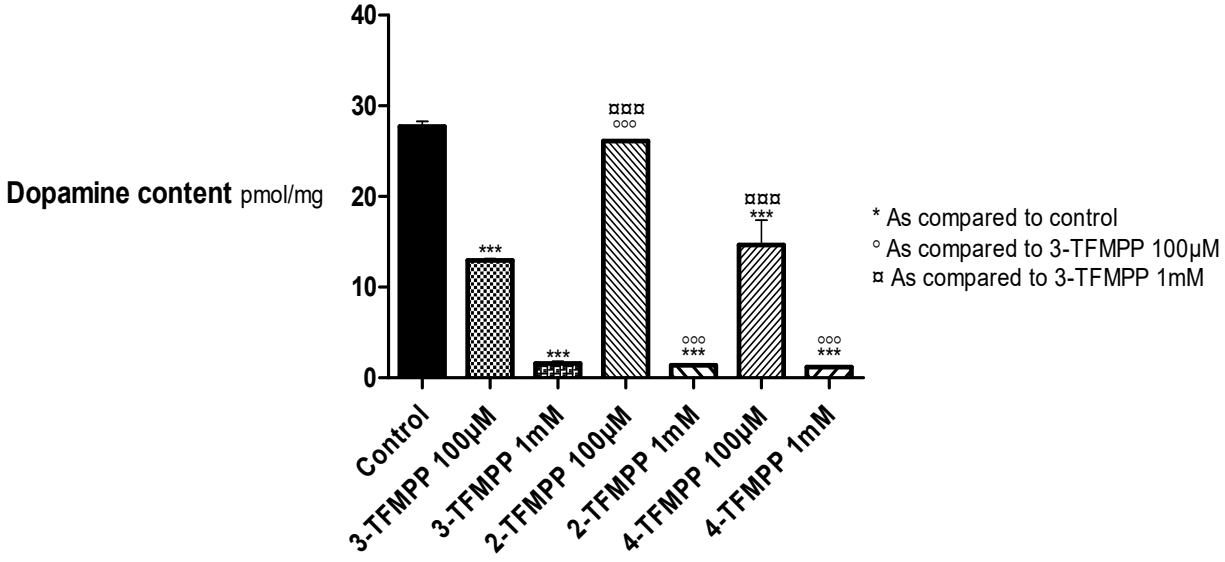


Figure 4.4. Effect of TFMPP derivatives on N27 rat dopaminergic dopamine content: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently decreased dopamine content in N27 cells as compared to the control. (n=3, $p< 0.0001$). Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

4.3.5. TFMPP Derivatives Increases DOPAC Content

At the dose of 1mM, 3-TFMPP, 2-TFMPP and 4-TFMPP significantly increased DOPAC content as compared to the control by (1805%, 1597% and 768%) respectively (n=3, $p < 0.0001$, Figure 4.5.). Likewise, 3-TFMPP (100µM) resulted in significant increase in DOPAC content by (481%) (n=3, $p < 0.0001$). However, 2-TFMPP and 4-TFMPP did not induces statistically significant effect at dose of 100µM.

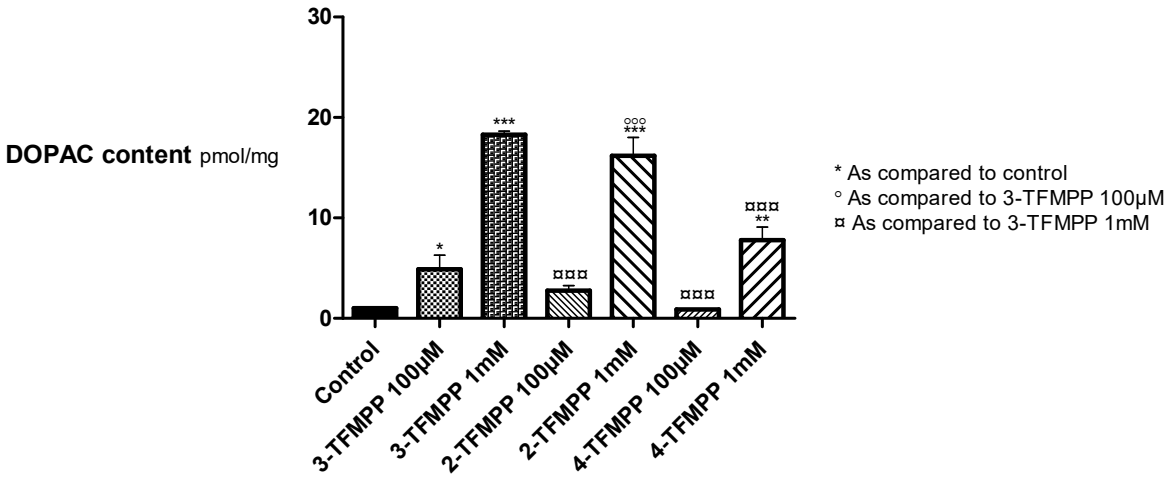


Figure 4.5. Effect of TFMPP derivatives on N27 rat dopaminergic DOPAC content**:** 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently increased DOPAC content in N27 cells as compared to the control. (n=3, $p< 0.0001$). Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

4.3.6. TFMPP Derivatives Increases HVA Content

At the dose of 1mM, 3-TFMPP and 2-TFMPP significantly increased HVA content as compared to the control by (314% and 329%) respectively (n=3, $p < 0.0001$, Figure 4.6.). While, 4-TFMPP did not show statistically significant effect at the same dose. All the three TFMPP had no effect on HVA at the dose of 100µM.

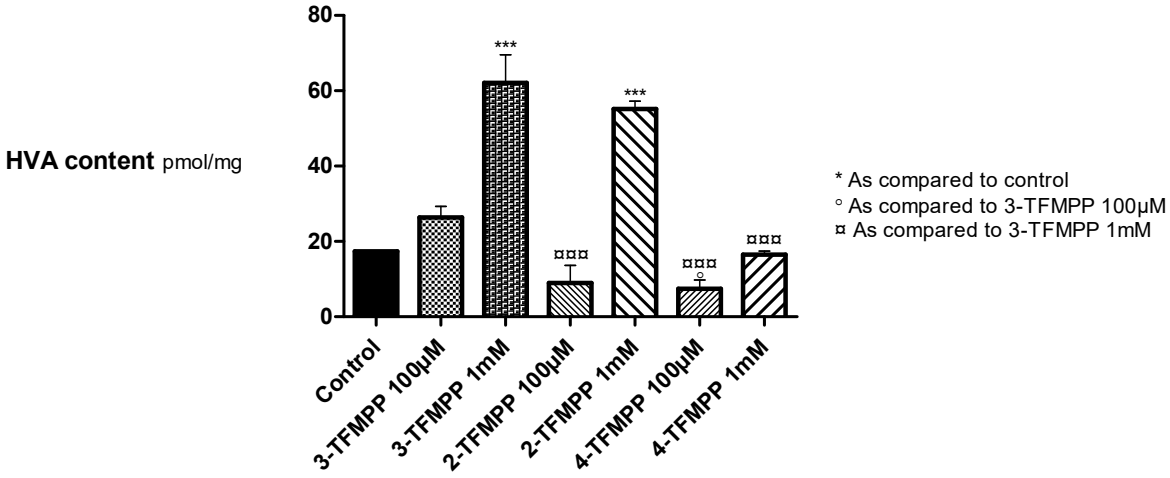


Figure 4.6. Effect of TFMPP derivatives on N27 rat dopaminergic HVA content**:** 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently increased HVA content in N27 cells as compared to the control. (n=3, $p< 0.0001$). Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

4.3.7. TFMPP Derivatives Increases Dopamine Turnover

At the dose of 1mM, 3-TFMPP, 2-TFMPP and 4-TFMPP significantly increased dopamine turnover as compared to the control by (3925%, 3588% and 1320%) respectively (n=3, $p < 0.0001$, Figure 4.7.).

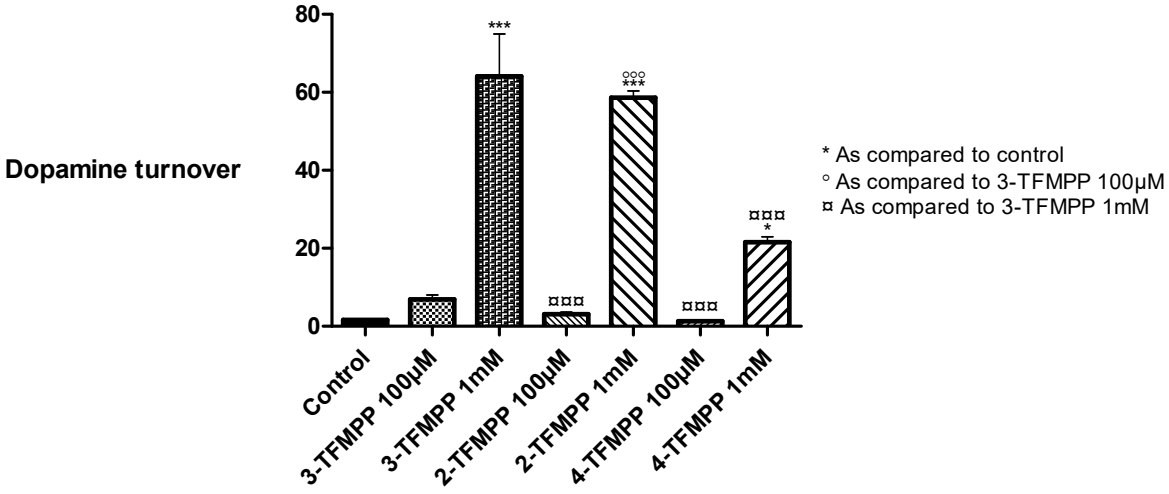


Figure 4.7. Effect of TFMPP derivatives on N27 rat dopaminergic dopamine turnover: 3-TFMPP, 2-TFMPP and 4-TFMPP dose-dependently increased dopamine turnover in N27 cells as compared to the control. (n=3, $p< 0.0001$). Results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA/Turkey's multiple comparison test. *indicates a statistically significant difference when compared to controls.

4.4. Discussion

Various neurotoxins have shown to affect the *in vitro* dopamine content[59,60]. These studies have shown to elucidate the dopaminergic neurotoxic mechanisms of these endogenous or exogenous substances. *In vitro* models can be used as a pilot study before conducting the in vivo studies. Furthermore, there are several advantages of this model including the ability to maintain the specific cells of interest in completely controlled environmental conditions, permitting the study of precise cellular and molecular pathways and saving time and resources as well. Thus, the current work is one of the first research studies to investigate the comparative effect of TFMPP derivatives on dopamine metabolism. N27 cells are valid in vitro model to illuminate the monoaminergic toxicity[61,62], elucidate the dopaminergic neuronal signaling/pathway[63,64], and illustrate the neuroprotective effects of synthetic and herbal/botanical compounds[65,66].

Dopamine is a catecholamine and phenethylamine neurotransmitter that transfers signals between neuronal cells. It has a crucial role in the brain where it contributes in cognition, motor control, motivation, arousal, lactation, sexual drive and reward. Dopamine is also present outside the central nervous system where it is produced locally to serves in cell signaling. Dopamine induces vasodilation by acting on dopamine and beta-adrenergic receptors and vasoconstriction by acting on Alpha-adrenergic receptors. While in the kidneys it enhances urine output. Dopamine also decreases insulin production in the pancreas and lessens gastrointestinal motility in the digestive system. The first step in dopamine synthesis is the conversion of the amino acid

phenylalanine to tyrosine by phenylalanine hydroxylase. Then, in the presence of the essential co-factor BH_4, tyrosine hydroxylase which is the rate-limiting enzyme in dopamine synthesis converts tyrosine to L-Dopa. Subsequently, DOPA decarboxylase converts L-Dopa to yield dopamine[67,68]. With respect to dopamine metabolism, it can be metabolized by two enzymes MAO and COMT to result in the production of the main end product HVA that does not have any identified biological effect. Generally, MAO is an intracellular enzyme and *COMT* is an extracellular enzyme[69]. MAO metabolizes dopamine to DOPAC only to be further metabolized by COMT to HVA. Another possibility is the metabolism of dopamine by COMT to 3-MT which is converted by MAO to HVA[70].

Neurotoxicity of TFMPP can be attributed to its action on the monoaminergic (dopamine, noradrenaline and serotonin) neurotransmitters[71]. Designer drugs can alter dopamine metabolism and result in abuse followed by neurodegeneration[72,73]. Endogenous neurotoxins (glutamate, hydrogen peroxide) and exogenous neurotoxins (MPTP, 6-hydroxydopamine) can impair dopaminergic neurons resulting in higher risk for several dopaminergic diseases (PD bipolar disorder, schizophrenia and depression) [30,74]**.** Stimulants have a biphasic effect on the dopamine metabolism, low dose and/or acute exposure to toxins trigger upsurge in dopamine release and high doses and/or chronic exposure to toxins result in vesicular depletion and higher breakdown of dopamine[54,75]**.** Dopaminergic neurotoxins are known to be able to inhibit tyrosine hydroxylase which is the rate limiting enzyme in the synthesis of dopamine leading to neurotoxicity[76–80]. In our previous study, 2-TFMPP, 3-TFMPP and 4-TFMPP induced oxidative stress and apoptosis, decreased mitochondrial function and inhibited tyrosine hydroxylase expression in N27 cells[55].

4.5. Conclusion

Various endogenous and exogenous neurotoxins have shown to induce dopaminergic neurotoxicity. As compared to both the endogenous (hydrogen peroxide) and exogenous neurotoxins (glutamate, MPTP), Piperazine designer drugs have shown to significantly affect the dopaminergic neuronal viability. Various hallmarks of Parkinson's disease are decreased dopamine content, increased dopamine turnover, decreased mitochondrial Complex-I activity, increased iron content, increased caspase-3 expression/activity, augmented oxidative stress substantia nigra. Similar to well-known dopaminergic neurotoxins such as MPTP, hydrogen peroxide piperazine derivatives also exhibited significant dopaminergic neurotoxicity.

Declined dopaminergic neurotransmission can be instigated from decreased dopamine synthesis[81,82] and increased dopamine metabolism[83,84] leading to the development of several movement disorders. Our results display that 3-TFMPP, 2-TFMPP and 4-TFMPP increased monoamine oxidase activity, inhibited tyrosine hydroxylase activity, depleted dopamine content, increased DOPAC content and increased HVA content. The decrease in tyrosine hydroxylase activity by TFMPP derivatives can decrease the synthesis of dopamine leading to a reduced amount of dopamine content. Likewise, enhanced monoamine oxidase activity by TFMPP derivatives can boost dopamine metabolism resulting in the depletion of dopamine content, increase in DOPAC content and increase in the content of the end product of dopamine metabolism HVA. The difference between 3-TFMPP, 2-TFMPP and 4-TFMPP is in the position

of substitution of the trifluoromethyl-group on the aromatic ring. Our results demonstrate that the position of the trifluoromethyl group is a main factor in the experimental differences in the effect of TFMPP derivatives. The parent designer drug 3-TFMPP which has the trifluoromethyl group at the meta-position of the aromatic ring exhibited superior dopaminergic neurotoxicity when compared with of 2-TFMPP and 4-TFMPP.

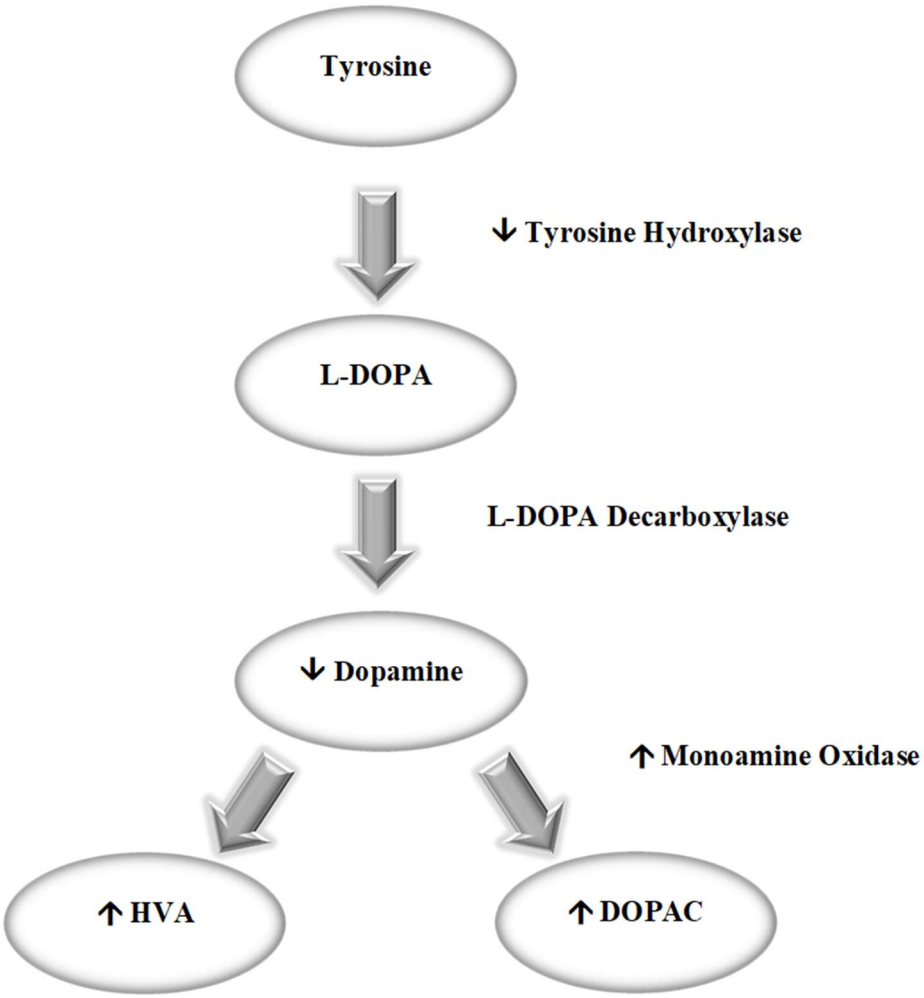


Figure 4.8. The effect of TFMPP derivatives on dopamine synthesis and metabolism

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5. Conclusion

Exposure to various endogenous and exogenous neurotoxins have shown to increase the risk for neurodegeneration leading to dementia or Parkinson's disease. Numerous endogenous and exogenous neurotoxins include metabolites of tryptophan (kynurenine, quinolinic acid[1], soluble oligomers of Aβ[2–4], glutamate, Ammonia[5], infection-pneumonia[6], chronic alcohol and nicotine exposure[7], hyperglycemia[8], Aluminum[9], Granulins (cysteine-rich proteolytic products of progranulin)[10], Cyanobacterial β-N-methylamino-l-alanine-BMAA[11], Salsolinol[12], MPTP[13], 6-OHDA[14], rotenone[15], alpha-synuclein[16], isoquinolines (IQs) and beta-carbolines[17], paraquat / diquat[18], amphetamine / methamphetamine[19] and metal (iron, zinc)[20] have demonstrated to significantly increase the risk for hippocampal and dopaminergic neurodegeneration. These neurotoxins have displayed to alter the neurotransmission by altering protein degradation, inducing abundant reactive oxygen species leading to oxidation of lipids (lipid peroxides) and proteins (protein carbonyls), decreasing ATP production, increasing pro-inflammatory mediators and decreasing anti-inflammatory mediators, increasing calcium influx by NMDA receptor activation, and augmenting programmed cell death. Furthermore, these neurotoxins also affect the synthesis, storage, release, reuptake, degradation of various neurotransmitters.

Historically, Substances of abuse have been used by priests to perform pious rituals; for healing / curative cause and for social interaction. Traditionally common substances of abuse include

alcohol, cannabis, opium, cocoa and nicotine[21]. In the recent past, designer drugs are being abused regularly by people around the world. Designer drugs induce drastic behavioral changes, alteration in sanitation or appearance, deterioration in performance (work or school), decrease in interest in regular activities (hobbies or relationships), Inexplicable changes in body weight (increase or loss), or disturbed sleep schedule and delirium. Interestingly, chronic use of these new designer drugs has shown to induce mental, movement or memory related disorders. Hazardously, Piperazine designer drugs are one of the newest designer drugs and has shown to exhibit several toxicological effects. However, the molecular mechanisms associated with piperazine-induced neurotoxicity is not well elucidated. Therefore, in this study, we explored the effects of piperazine derivatives on dopaminergic and hippocampal neurons and elucidated the mechanism of action. Piperazine derivatives increased the turnover of acetylcholine and dopamine by decreasing the synthesis (inhibit the synthesizing enzymes) and increasing the breakdown (activating the degrading enzymes) which can lead to the depletion of these neurotransmitters. Moreover, piperazine derivatives altered the antioxidants (SOD, Catalase, GSH) which induced the generation of ROS and NO leading to lipid peroxidation. Subsequently, piperazine derivatives also decreased the mitochondrial functions, increased caspase activities and increased the formation of pro-inflammatory mediators. All the above markers can affect the neuronal signaling mechanisms leading to neuronal death. Thus, our current study depicts the potent neurotoxicity of piperazine derivatives. Future, *in vivo* studies have to be conducted in control and valid animal models of Parkinson's / Alzheimer's disease to substantiate the neurotoxic effects of piperazine derivatives. Thus, our result coupled with the future studies can highlight the toxic effects of the piperazine derivatives which can result in decreased abuse.

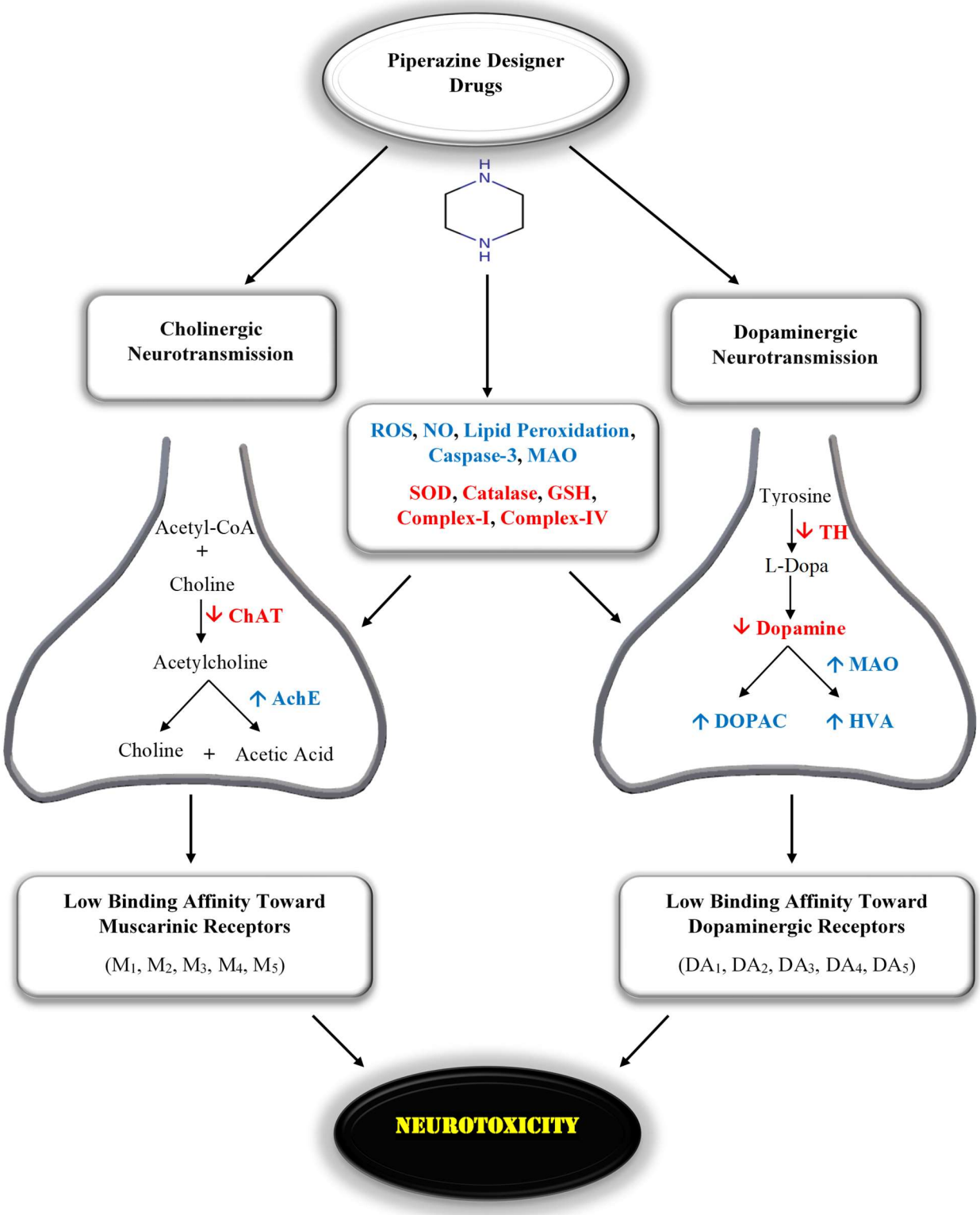

Figure 5.1. Effect of piperazine derivatives on hippocampal and dopaminergic neurotransmission

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