SYNTHESIS AND EVALUATION OF ALPHA 7 NICOTINIC AGONISTS AS NEUROPROTECTIVE AGENTS.

by

GURBIR SINGH DOAD

(Under the Direction of JOSEPH W.BEACH)

ABSTRACT

Alzheimer's disease is an irreversible neurodegenerative disease, characterized by cognitive decline and loss of memory. Although a number of neurotransmitters are affected, the loss of cholinergic neurons is considered the hallmark of Alzheimer's disease. Current therapy for Alzheimer's disease involves the use of acetylcholinesterase inhibitors. Although these agents do improve cognition and memory in the Alzheimer's patient, they do not halt the loss of cholinergic neurons and thus, the progression of the disease.

Choline possesses neuroprotective activity via activation of alpha 7 nicotinic receptors. However, choline is a poor drug candidate due to its high polarity, which hinders CNS penetration, and its low potency (ED_{50} = ~1mM). In order to explore the possibility of improving both the potency and the brain penetration of choline, we have synthesized a series of compounds based on the choline structure.

Two series of compounds were synthesized, the N-substituted benzyl-N-Methyl ethanolamines and the N-benzyl-N-hydroxyethyl piperazines. These compounds were evaluated in PC12 cells

for their ability to protect against neuron growth factor (NGF) deprivation (cytoprotection assay) and their ability to interact with the high affinity choline transporter. The 3-fluorobenzyl derivative was the most potent of the N-methyl ethanolamine series, having an ED_{50} for cytoprotection of 30 nM. Nicotine, which also possessed neuroprotection activity, was found to have an ED_{50} of ~60 nM. The piperazine derivatives evaluated were found to be less potent than nicotine, but still showed good overall activity.

INDEX WORDS: Alzheimer's disease, acetylcholinestrase, neuroprotective, alpha 7 nicotinic receptors, N-substituted benzyl-N-Methyl ethanolamines, N-benzyl-N-hydroxyethyl piperazines, PC12 cells, ED_{50}.

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DEDICATION

I dedicate this thesis to my father, Amrik Singh Doad and my mother, Daljit Kaur who always inspired me by setting an example to me. I would not have been able to complete this degree without their love, support, guidance, advice and patience.

I would also like to thank my brother, Kirpal Singh Doad and my friends who believed in me when I no longer believed in myself.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

ALZHEIMER'S DISEASE: PROGRESSION AND TREATMENT

Alzheimer's disease is an irreversible neurodegenerative disease which slowly and progressively destroys brain cells. Alzheimer's disease is neither infectious nor contagious. It is a terminal illness causing general deterioration in health. Dementia is the foremost sign of Alzheimer's disease followed by changes in mood, unusual behavior and disorientation in time and space, loss of memory and mental functioning.

Alzheimer's disease is named after the German neurologist, Dr. Alois Alzheimer,[1] who in 1907, was the first to describe plaques and tangles in the brains of Alzheimer's disease patients. There are two major structural changes in the brains of Alzheimer's disease patients. First, loss of neurons (vital to memory and other mental abilities) and second, deposition of neurofibrillary tangles (intracellular protein deposit) and beta-amyloid (extra cellular protein deposit).[2-5]

Based upon these two changes, two major hypotheses have been postulated: The Cholinergic hypothesis and The Amyloid cascade hypothesis. Neither can explain all of the events which occur at the molecular and cellular level but both in combination are able to explain many of neurological features of Alzheimer's disease.

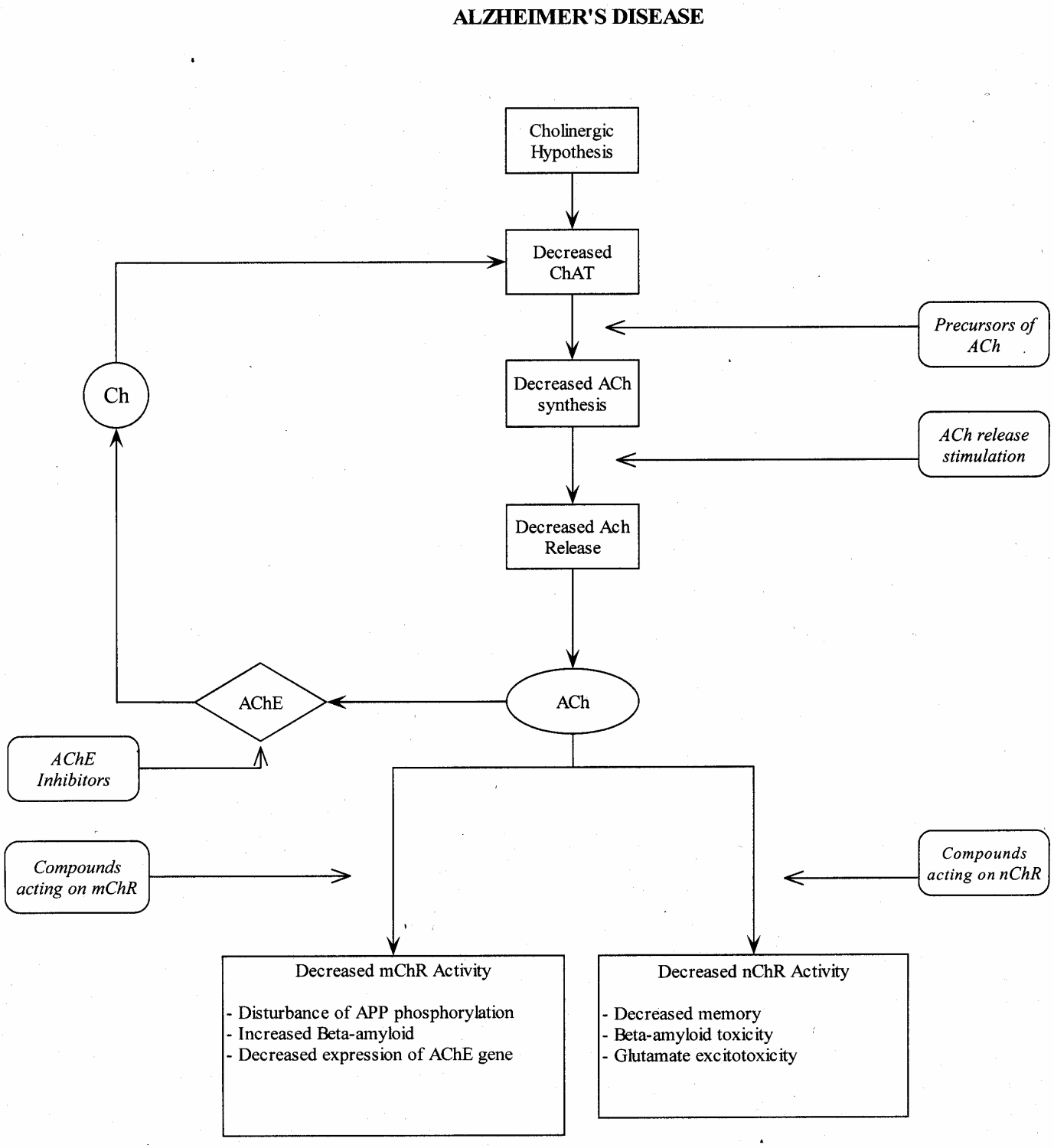


Fig. 1.1 Cholinergic Hypothesis of Alzheimer's disease

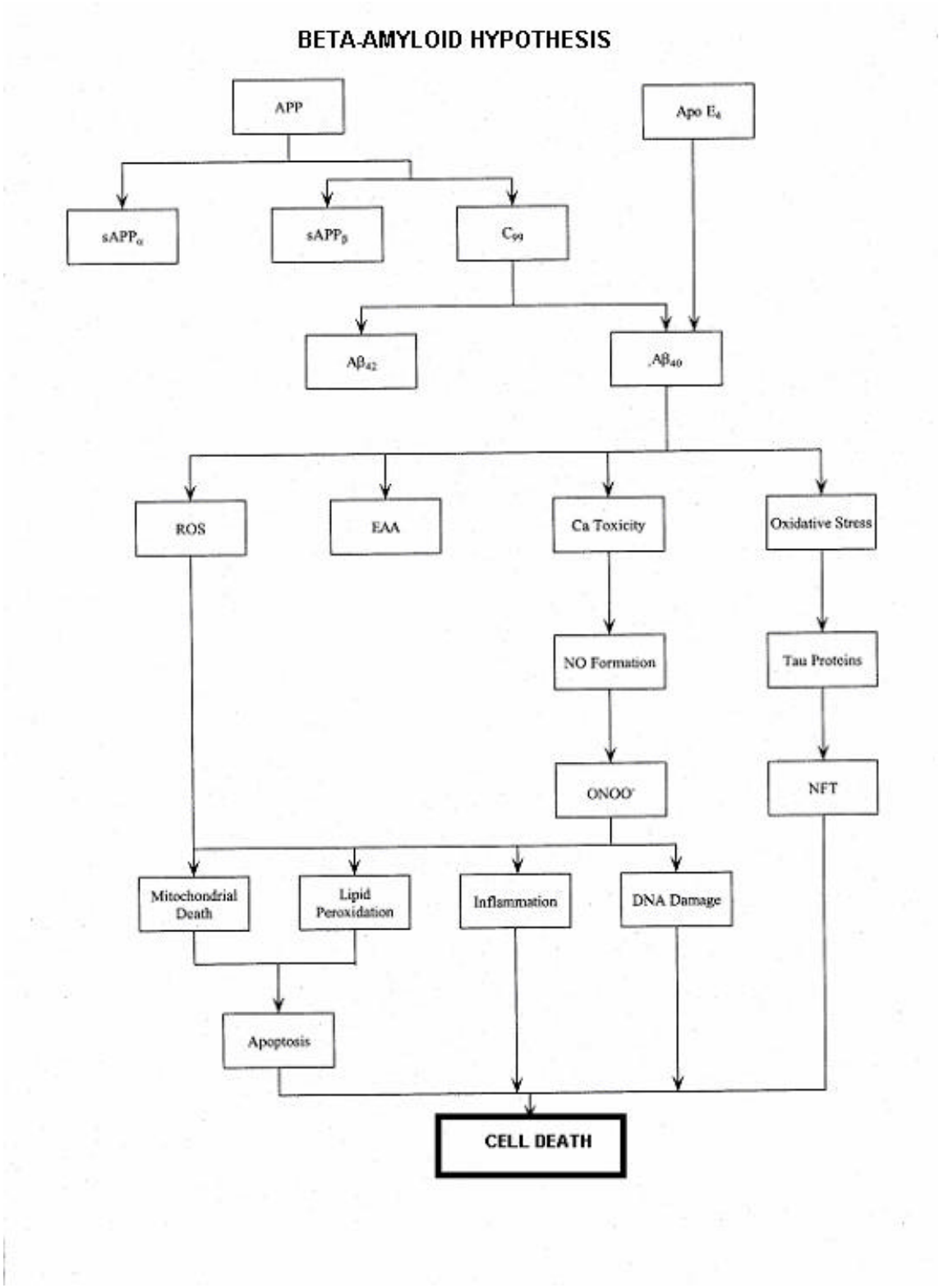


Fig. 1.2 Beta Amyloid Hypothesis of Alzheimer's disease

CHOLINERGIC HYPOTHESES

The cholinergic hypothesis states, "Loss of cholinergic functions in the CNS contributes to the cognitive decline associated with Alzheimer's disease."[6]

It is supported by the fact that many of the cognitive, functional and behavioral symptoms of Alzheimer's disease can be explained, fully or in part, by the following observations:

1. Reduction in Acetylcholine **(Fig.1.3)** synthesis via choline acetyl transferase and reduced choline uptake.[7]
2. Degeneration of cholinergic neurons in the nucleus basilis of Meynert.
3. Decline in basal and rostral forebrain including the projections to the thalamus.[8]
4. Decrease in nicotinic and muscarinic receptors.

The cholinergic hypothesis is the basis of cholinergic replacement therapy being the mainstay of the treatment of the Alzheimer's disease.

AMYLOID CASCADE HYPOTHESES

According to the amyloid cascade hypothesis, the neurodegenerative process in Alzheimer's disease is due to a series of events initiated by the abnormal processing of amyloid precursor protein (APP) which results in beta-amyloid (Aß) production, aggregation, deposition and toxicity.[9]

Amyloid precursor protein (APP) is an integral membrane glycoprotein. APP is cleaved through the amyloid domain by one or more enzymes named a, ß or ? secreatase. One of the fragments formed after the cleavage by ß or ? secreatase is called Aß. These Aß fragments aggregate and form senile plaques.[10] There are a number of proteins such as, amyloid precursor protein, secretase enzymes, presenillens, which play a role in formation of Aß plaques.

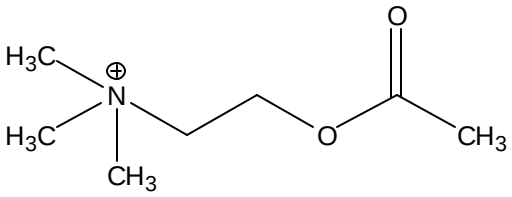


Fig. 1.3 Structure of acetylcholine

APP and Secretase enzymes:

Amyloid precursor protein is a single transmembrane domain protein, which is expressed ubiquitously. Stress, estrogens, endogenous factors like cytokines and some neurotrophic factors stimulate the expression of APP.[11] APP matures in the endoplasmic reticulum and Golgi apparatus and exhibits post translational modifications. Abnormal processing of APP triggers the production and toxicity of Aß, the pathological form of amyloid peptide (AP). Under normal conditions, cleavage of APP by a-secretase results in the production of the soluble form of APP.[12] Aging or mutation of APP gene result in a change in the cleavage site of APP. A total of five mutations have been described in the APP gene that leads purely to Alzheimer's disease.[13] ß-secretase acts on APP and forms soluble APP but it generates a free N-terminus of Aß. This is considered the first step in the production of Aß.[14] By the action of ß and ? secreatases on APP, the secreted form of APPß and Aß are generated. Alternatively, by the action of a and ?-secretases the secreted form of APPa (sAPPa) and P-3(P-340 and P-342) fragments are generated.[15] The P-340 and P-342 are considered to be non-pathogenic. While sAPPa enhances neuronal survival and neurite outgrowth, Aß is known to cause harmful effects on the nervous system. Thus, depending on which pathway (a-secretory vs. ß-secretory pathway) is activated; the same precursor protein can be converted to either a neurotoxic or neuroprotective end product. Different forms of Aß vary in peptide length ie. $Aß_{40}$, $Aß_{42}$ and $Aß_{43}$. Aggregates of these Aß forms leads to the formation of fibrils and then senile plaques.[16]

Processing of APP is regulated through the muscarinic cholinergic receptor (mChR) activation via protein kinases.[17] The mechanism of APP processing with the aid of muscarinic cholinergic receptor may be described as follows. All muscarinic choline receptors act via G-proteins. M1, M3 and M5 muscarinic receptor subtypes stimulate the hydrolyses of

phosphatidylinositol via the activation of phospholipase C (PLC). An activation of the M1 muscarinic receptor subtype can lead to the PLC catalysed hydrolysis of phosphatidylinositol with the formation of diacylglycerol. Diacylglycerol activates protein kinase C (PKC), which phosphorylates APP. This leads to enhanced production of sAPPa. As activation of PKC (or tyrosine kinase) also leads to reduction in Aß production without altering APP expression, it is thought to shift the balance between the a-secretory and ß-secretory pathways of APP processing toward the a-secretory pathway. Therefore, up-regulation of the PKC or tyrosine kinase pathway should produce beneficial effects by reducing Aß and elevating sAPPa.[17]

Presenillins:

The majority of the early onset familial Alzheimer's disease (FAD) cases are attributed to mutations in either of the two related genes, presenillins which code for designated PS-1 and PS-2. PS-1 is located on chromosome 14 and PS-2 is located on chromosome 1, respectively.[18] Potter and colleagues showed that PS-1 and PS-2 proteins are localized in the nuclear membrane and associated with the kinetochores and the centrosomes. Both of these sub cellular structures are involved in cell cycle regulation and mitosis. The exact functions of PS proteins have not to been fully established. However, PS-1 is considered to play an active role in neurogenesis, the development of neurons, in specific brain regions and preventing apoptosis. It is also required for the proper formation of the axial skeleton of neurons. . Initial evidence was provided by the finding that Familial Alzheimer's disease (FAD)-linked PS1 mutations result in increased generation of the highly amyloidogenic $Aß_{42}$.[19]

ApoE:

ApoE gene is associated with late onset Alzheimer's disease cases. ApoE is a serum lipoprotein involved in transport and metabolism of phospholipids and cholesterol. ApoE exists

in 3 allelic variant forms called E2, E3 and E4. ApoE is considered to be associated with the repair of nerve cells in response to damage. Recent studies by Lee et al. have shown that in mixed neuronal glial cell culture, ApoE protects against hydrogen peroxide induced oxidative stress by reducing glutamate toxicity.[20] Earlier, it was thought that ApoE3 and ApoE2 serve a protective role by interfering with Aß and tau binding to itself and thus, inhibited fibril formations. Now, it has been reported that ApoE3 protects neuronal cells against Aß induced toxicity through complexation and internalization of Aß via ApoE receptors. Moreover, this hypothesis of neuroprotection against Aß-induced neurotoxicity by binding and clearing the peptide is also supported by the specificity of the apoE3 protective effect. ApoE3 does not protect against the neurotoxicity of staurosporine, which does not involve a cell surface receptor, or of NMDA, which does not involve an apoE-type receptor. In addition, apoE had no effect on the intracellular Ca^{2+} spikes induced by either KCl or NMDA. This suggests that apoE3 may be interrupting the Aβ toxicity cascade at a point before the initiation of cellular events and is consistent with a mechanism of action in which extracellular apoE3 interacts physically with the peptide to form a complex that is subsequently cleared by apoE receptors.[21]

The precise bases for the toxic effects of apoE4 alone are still a mystery. ApoE4 binds more rapidly to Aß and thus, increases the risk of Alzheimer's disease by promoting Aß polymerization into neurotoxic amyloid filaments.[22,23]

Radical Oxygen Species/Reactive Oxygen Species (ROS):

ROS are generated in the body during various metabolic processes. Accumulation of ROS in the CNS initiate and promote neurodegeneration.[24-27] This forms the basis for the oxidative stress theory of Alzheimer's disease. The brain regularly needs high levels of oxygen, in addition it has a high lipid content and a lower antioxidant concentration.[28,29] This makes the

brain highly vulnerable to oxidative stress. Various in vitro and in vivo studies have shown that there is a strong correlation between the generation of ROS and lipid peroxides, and the formation of senile plaques and Aß fibrils in the brain.[30] Increased levels of intracellular calcium have been correlated with an increased ROS production. Intracellular calcium activates numerous metabolic reactions in mitochondria resulting in the formation of superoxide (O_2^-). It also activates NO^- synthase, generating nitric oxide (NO^-) radicals. O^{2-} and NO^- react with proteins and produce peroxynitrite (NO_3^-), a powerful oxidant, which can cause lipoperoxidation of membranes. Lipid peroxidation products can interact with proteins and nucleic acids through covalent binding, leading to neuronal ATP depletion and death (Poly-ADP-ribose polymerase over activation). NO_3^- can also prevent protein phosphorylation and disturb signal transduction mediated by tyrosine kinase.[31,32] In addition, NO_3^- can cause the over expression of metalloprotease enzymes, especially MMP9 enzymes which causes anoikis.[33,34] Anoikis is apoptosis triggered by the loss of contact between the cells and the extra cellular matrix.

Astrocytes or microglial cells can generate ROS as part of an autoimmune response. Aß activate the microglial cells which in turn produces neurotoxic compounds like glutamate, superoxides and NO.[35-37] The microglia also produce TNF-a and in Alzheimer's disease patients there is an increase in T-lymphocytes, major histocompatibility complex (MHC) glycoproteins, chemokines and interleukins(IL).[38-41] This suggests that inflammatory mediators play a role in the progression of Alzheimer's disease.

Energy Depletion

The mitochondria are responsible for all bioenergetics of the cells and are necessary for the production of sufficient energy for the proper functioning of the cell signaling pathways[42,43]. Mitochondria also provide energy for neuronal ionic pumps used in maintaining the resting

membrane potential. They also contain various nuclease and protease (cytochrome C) enzymes responsible for apoptotic cell death. The primary function of the mitochondria is ATP generation via mitochondrial oxidative phosphorylation.[44] ROS is a detrimental by-product of oxidative phosphorylation. Mitochondria maintain its integrity by maintaining a balance of membrane potential and concentration of nuclease and protease enzymes.[45,46] Whenever mitochondria fail to provide sufficient energy, there is a decrease in the mitochondrial membrane potential leading to partial neuronal depolarization and an imbalance of Ca^{2+} homeostasis which finally leads to apoptosis.[47] The mechanism of apoptosis and role of mitochondria during apoptosis can be explained as follows. When the ROS level in mitochondria exceeds its detoxifying capacity, mitochondrial permeability transition pores are activated, opening a channel across the mitochondrial membrane. There is free diffusion of molecules less than 1500 Da between matrix and cytosol. This results in the collapse of the trans-membrane electrochemical gradient, the loss of matrix solutes and the swelling of mitochondria which causes the release of cytochrome c, procaspases 2, 3, and 9, apoptosis-initiating factor, and caspase activated DNase. Cytochrome c and the cytosolic factor Apaf1 activate the caspases, while apoptosis-initiating factor and caspase activated DNase move to the nucleus initiating apoptosis or programmed cell death.[48]

Due to apoptosis, there is further disruption of normal functioning of mitochondria, thereby, compromising energy production. Moreover, it has been reported that disturbed energy metabolism is an early signs of AD. There is evidence that there is a defective mitochondrial electron transport chain (ETC) in AD.[49] Collectively these studies support that there is a disturbed energy metabolism of cells leading to AD.

Exictatory Amino Acid (EAA)

It has been found that along with the decrease in the cholinergic receptors, there is a significant decrease in the number of glutamate receptors. These glutamate receptors alo ng with NMDA and AMPA subtype receptors play a significant role in learning and memory processes. These findings suggest that the CNS glutamatergic system plays a role in the development of AD.

Glutamic acid, an excitatory neurotransmitter, binds with various amino acid receptors and leads to neuronal excitation. Normally, this neuronal excitation is transitory, but if prolonged, targeted neurons are destroyed. This is referred to as neurotoxicity or excitotoxicity. There are three phases of excitotoxicity :[50]

1. Sodium influx: Activation of AMPA receptors by glutamate causes initial depolarization via voltage dependent sodium channels leading to sodium influx and further depolarization. This change in the membrane potential allows a magnesium ion, which normally blocks the NMDA receptor, to dissociate from the NMDA receptor and glutamate to activate the NMDA receptor. Activation of the NMDA receptors leads to calcium influx. Excessive stimulation can cause increased sodium levels inside the cells leading to the disruption of the osmotic balance of the cell. If left unchecked, the cell can swell and eventually lyse.
2. Calcium influx: Prolonged activation of NMDA receptors leads to an increase in intracellular calcium concentration which triggers a complex cascade of events leading to cell death. Once this process is initiated, it is an irreversible process and is dependent on the presence of calcium ions only.
3. Exocytosis: Cell lysis from the above two events leads to an increase in extracellular

glutamate which will diffuse to neighboring cells causing depolarization and potentially the repeat of the above events. Thus, it spreads and amplifies the necrosis and further cell lysis.

Calcium Toxicity of Aß :

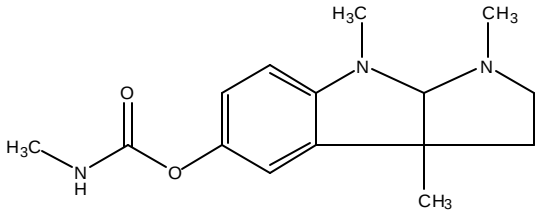
Like the glutamate theory of AD, the calcium hypothesis emphasizes the disturbance in calcium metabolism in the nerve cells.[51-52] The increase in calcium levels is due to a decrease in the calcium binding protein, calbindin and a decrease in the calcium buffering properties of the mitochondria. There are various hypotheses and theories (Mitochondrial apoptosis theory,[53-56] voltage sensitive calcium channels hypothesis,[57-58] generation of endogeneous and exogeneous calcium channels,[59-60] caspase protease theory[61]) explaining the role of calcium toxicity. Briefly, all of them support the fact that there is an increase in calcium ions in the nerve cells and a decrease in the efficacy of nerve cells to balance the calcium ion concentration, which finally leads to apoptosis.

TREATMENT

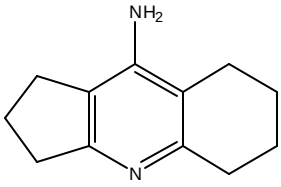
Cholinergic replacement therapy consists of three types of treatment;

1. Acetylcholinestrase inhibitors (AChEI)
2. Compounds acting on nicotinic and muscarinic receptors
3. Compounds promoting the synthesis and release of Ach

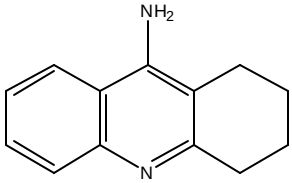
The first generation of AChE inhibitors which include physostigmine **(Fig.1.4)**, amridine and tacrine **(Fig.1.4)** are relatively non selective in their inhibition of AChE- inhibiting both acetyl choline esterase as well as the closely related enzymes like butyl choline esterase. This leads to side effects such as anorexia, bradycardia, nausea, diarrhea, sedation and hepatotoxicity.[62-64] The development of more selective compounds like donepezil, galanthamine, rivastigmine **(Fig.1.5)**



Physostigmine

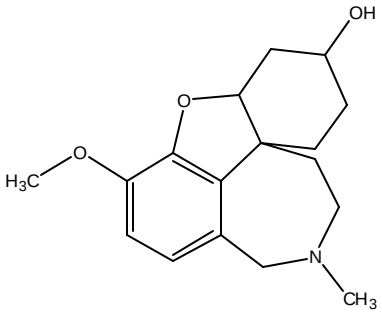


Amiridine

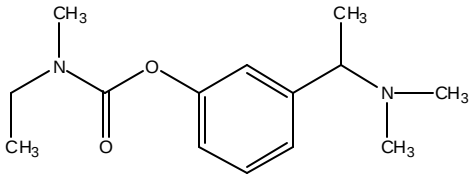


Tacrine

Fig. 1.4 Structure of first generation acetyl cholinesterase inhibitors



Galanthamine



Rivastigmine

Fig. 1.5 Structure of second generation acetyl cholinesterase inhibitors

and eptastigmine which show relatively selective inhibition of brain AChE has lead to compounds having fewer side effects as compared to the first generation of AChE inhibitors[65-68]. However, they still are not fully satisfactory in their efficacy and demonstrate adverse effects. Recently, some traditional medicines were tested as AChEI. Out of these, a plant alkaloid Huperzine A **(Fig.1.6)** extracted from the Chinese herb, Huperzia serrata, that was used for fever, have shown the most promising results.[69-70] Along with AChE inhibitor activity, it shows some neuroprotective activity as well. Despite a number of drawbacks, AChE inhibitors are considered to be the first choice of drug in AD.

Muscarinic cholinergic receptor agonists are considered potential therapeutic agents, because the muscarinic receptor is involved in the regulation of amyloid precursor protein (APP) processing.[71] It has been proposed that decreases in the activation of the muscarinic receptor leads to deactivation of phospholipase C (PLC) which leads to abnormal processing of APP resulting in increased production of insoluble Aß.[72-73] Various muscarinic receptor compounds such as xanomeline, sabcomeline have been evaluated unsuccessfully.[74] The major drawbacks of these compounds are the lack of selectivity for the M1 receptor subtype and major adverse effects at therapeutically active doses. Moreover, muscarinic receptors activate the AChE gene, increasing the production of esterase and subsequently decreasing acetylcholine levels. Thus, resulting in negative feedback in cholinergic neurotransmission. Recent research has focused on the production of compounds selective for the M1 subtype or compounds which are agonists for the M1 and M4 subtypes and antagonists for M2 and M3 subtype receptors.[75]

Nicotine **(Fig.1.7)** has been shown to possess neuroprotective and cognitive enhancer activity. Brain nicotinic receptors are of two types; a(a2-a7) and ß(ß2-ß4). Various combinations of aß receptors subtypes exist. a7 and a4ß2 receptors have been shown to be active in memory

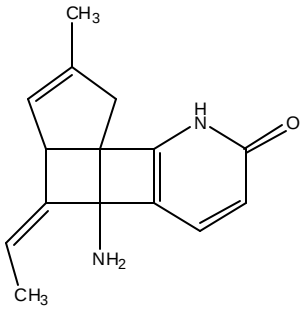


Fig. 1.6 Structure of Huperzine A

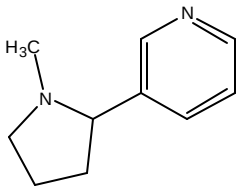
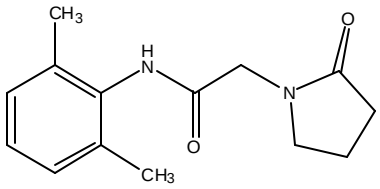


Fig. 1.7 Structure of Nicotine

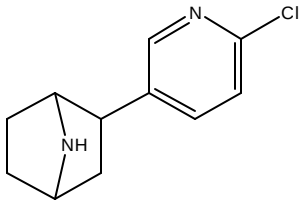
formation and AD patients have been shown to have a reduction in the number of nicotinic receptors.[76] Various experiments have shown that by activating a7 and a4ß2 subtype receptors, Aß toxicity and glutamate excitotoxicity is countered. Various drugs of this class have been tested in animal models of AD, but none have been approved for market, except niferacetam **(Fig.1.8)**. Epibatidine **(Fig.1.8)**, chemical name (chloropyridyl)-azabicycloheptane, is a toxin derived from the skin of the Equadorian poison tree frog, Epipedobates tricolor. Epibatidine is tested with anabasin **(Fig.1.8)** and is considered to be the choice of drug in the near future. The potential for addiction is one of the major drawbacks in this class of drugs. Additionally, fast desensitization of the nicotinic receptors is another problem which must be addressed.

Acetylcholine esterase inhibitors (AChEI) are the first class of drugs available for the treatment of AD. The basic function of these compounds is to increase the duration of action of acetylcholine (Ach) in the synaptic cleft. These agents work only if there is suffcient acetylcholine production. Therefore, cholinomimetic compounds are used as biochemical precursors of acetylcholine. Gliatilin is one of the drugs of this class. Gliatilin is an acetylcholine precursor derived from soy, L-alphaglycerylphosphorylcholine (GPC).[77] The brain converts GPC into acetylcholine bolstering acetylcholine levels in the brain. GPC is considered to be a success after the failures of other natural acetylcholine precursor compounds such as lecithin and choline. Earlier, choline was thought to be a very promising molecule. Choline acts as a precursor to acetylcholine. Additionally, it is one of the building blocks for phosphatidylcholine, a phospholipid that is an important component of brain cell membranes. Without it, membranes lose structural integrity and neurons wither.

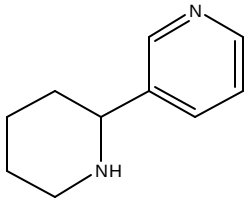
The body partially compensates for acetylcholine deficits by "raiding" existing phosphatidylcholine for conversion to acetylcholine. While it's an intriguing example of the



Niferacetam



Epibatidine



Anabasin

Fig. 1.8 Structure of Nicotinic receptor agonists

body's remarkable adaptability and economy, this reallocation ultimately serves only to weaken cell membrane integrity by depleting phosphatidylcholine stores.

Linopirdine represents a novel class of compounds capable of enhancing potassium-stimulated release of acetylcholine. Linopirdine [DuP996; 3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one], was shown to have memory-enhancing effects in a variety of rodent models of learning and memory. It is currently in Phase III of clinical trials.

THERAPEUTIC STRATEGIES AGAINST "B-AMYLOID CASCADE"

Based on the ß-amyloid cascade hypothesis, several strategies have been investigated against the production of Aß, including ß-sheet dissolvers and agents which prevent the neurotoxic effects of Aß.

Oxidative stress, ROS and Reactive nitrogen species (RNS) are the major factors identified in the progression of AD. Therefore, antioxidant therapy gained some interest. It can be broadly divided into two classes; External antioxidant therapy and stimulation of the endogenous anti-oxidant system. In the last decade, a number of natural and synthetic antioxidants have been tested. For a drug to be an antioxidant useful in AD, three basic requirements should be met. It should be: 1) Able to accept a free electron or radical ion. 2) Have a highly conjugated molecular structure so that molecular charge is diffused. 3) Able to cross the blood brain barrier (BBB).

Vitamin E **(Fig.1.9)** is a well known antioxidant with cytoprotective actions.[79] In studies in AD patients, it has been shown to be neuroprotective and slows the progression of AD. Some synthetic analogues of vitamin E have been recently patented as antioxidants.

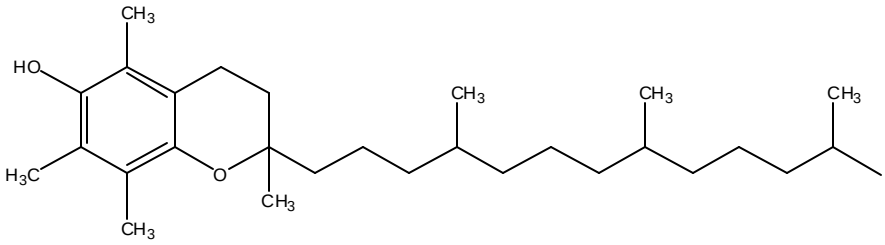


Fig. 1.9 Structure of alpha tocopherol

The tripterine, celastrol, from the Chinese herb, Tripterygium wilfordii shows antiperoxidation activity, by directly scavenging radicals, and is 15 times more potent than a-tocopherol.[80] In addition, Celastrol **(Fig.1.10)** shows anti-inflammatory activity via suppression of the production of TNF-a, IL-1ß and formation of NO from induced Nitric oxide (iNO) synthetase. It has been shown to improve learning, memory and psychomotor activity of animal models of AD.[81]

Ginkgo biloba is another Chinese herbal medicine used as an antioxidant by neutralizing NO^- radicals resulting in the inhibition of the NO induced activity of protein kinase C. Like celastrol, it is also purported to show cognition enhancing properties.[82,83]

Melatonin **(Fig.1.11)**, an endogenous hormone is found to be the most potent free radical scavenger. It scavenges $\cdot OH$ and ONO^{2-}(precursor of $NO\cdot$) efficiently.[84-87] Melatonin also neutralizes ROS that are responsible for the damage of essential neuronal molecules. It stimulates many antioxidant enzymes like superoxide dismutase(SOD), glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase which detoxify or reduces peroxides and NO^{3-}. Additionally, melatonin strongly inhibits the formation of ß-sheets and amyloid fibrils. This is because of structural interactions of the hormone with A3. The His and Asp residues play important roles in 3-amyloid fibril production and stability. The imidazole-carboxylate salt bridges between the side chains of the His^+ and the Asp^- residues are critical to the formation of the amyloid 3-sheet structures.[88] Melatonin disrupts these salt bridges and promotes fibril dissolution. Indole-3-propionic acid (IPA), is closely related structurally to naturally occurring melatonin, and was found superior to melatonin in its antioxidant properties. N-acetylserotonin (NAS), a melatonin precursor, was found to have better radical scavenging and high anti-amyloid activity.[89,90]

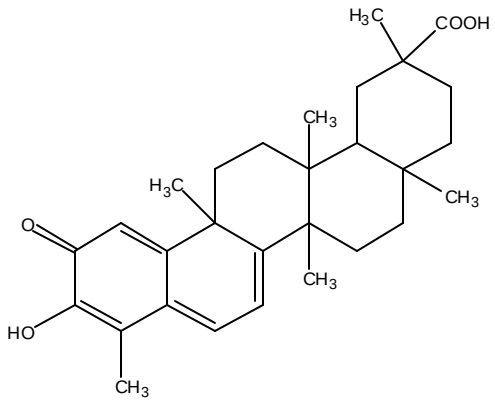


Fig. 1.10 Structure of Celastrol

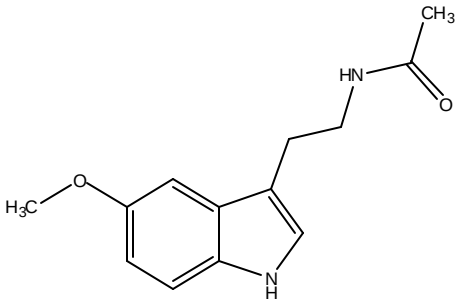


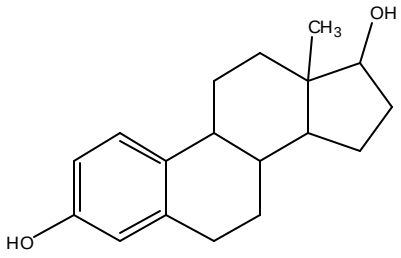
Fig. 1.11 Structure of Melatonin

The steroid hormones, estradiol **(Fig.1.12)** and its derivatives were the first steroids to show neuroprotective properties.[91] Estradiol (17ßE) activates protein kinase C (PKC) in neurons which modulate cell viability pathways resulting in neuronal and non neuronal cells. Estradiol along with 17aE blocks the intracellular accumulation of ROS, protecting the neurons from the toxic effects of oxidative stress.[92] Additionally, estradiol and its derivatives prevent production of Aß40 and Aß42. Recently, androgens, such as testosterone **(Fig.1.12)** were evaluated for their antioxidant and neuroprotective properties. Testosterone increased the production of soluble amyloid precursor protein (sAPP) and had anti apoptotic activity.[93] No other steroid hormones have shown any antioxidant nor neuroprotective properties.

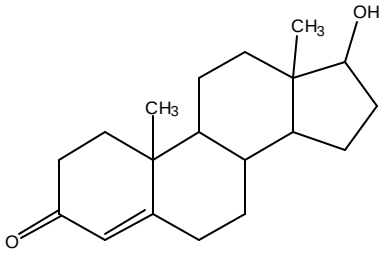
Stimulation of formation of NF-kB has recently become associated with the endogenous activation of the internal anti-oxidant system.[94] It is based on the observations that NF-kB levels are high in neuronal cells resistant to Aß.

Bafilomycin **(Fig.1.13)** and Concanamycins, both members of the plecomacrolide antibiotics were shown to block the formation of APß by an indirect inhibition of ß-secretase activity which results from the prevention of lysosomal acidification due to blockage of V-type ATPases.[95] APP selective ?-secretase inhibitors are the next target as the drugs inhibiting the formation of APß. Peptide leupeptine and E-64 stabilize the C-terminal fragment of APß by inhibiting ?-secretase.[96]

Recently, some studies have shown the neuroprotective role of statins by their effects on nitric oxide formation, anti-inflammatory effects and anti-oxidant effects.[97] Statins competitively inhibit HMG-CoA reductase (3-hydroxy-3methylglutaryl coenzyme A), an early step in the biosynthesis of cholestrol, thus decreasing the production of mevalonate and isoprenoids (derivates of intermediates in cholesterol biosynthesis affecting G-proteins, adhesion molecules



17-a-estradiol



Testosterone

Fig. 1.12 Structure of Steroid hormones

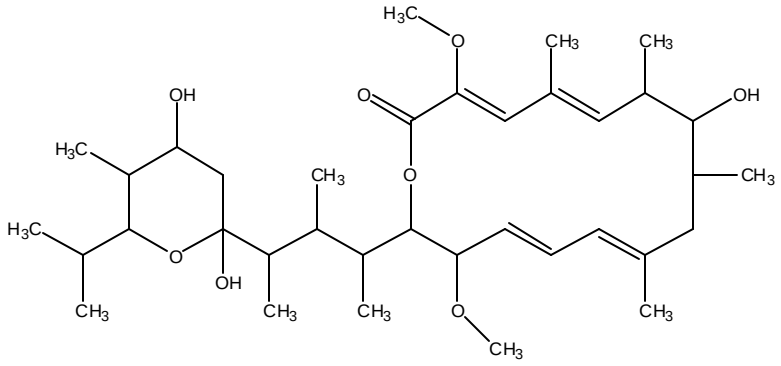
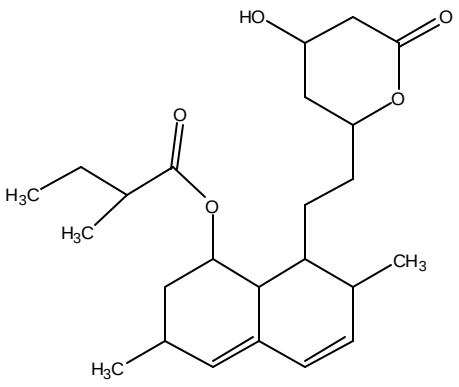


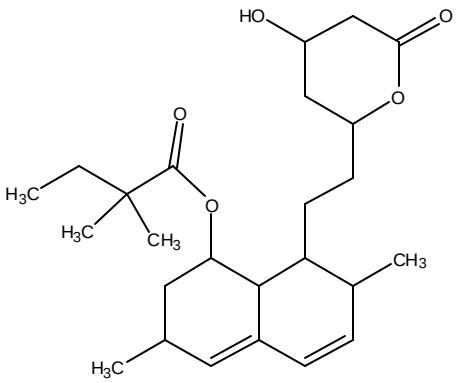
Fig. 1.13 Structure of pleomacrolide antibiotic

and cell proliferation), isopentenyl pyrophosphate (involved in transfer-RNA synthesis), dolichol (plasma membrane fluidity), ubiquinone (mitochondrial respiration) and geranyl- and farnesyl-pyrophosphate (involved in post-translational modification of number of intracellular regulatory proteins).[98] Thus, inhibition of HMG-CoA has multiple effects. Statins increase the up-regulation of endothelial nitric oxide (eNO) synthase with increased bioavailability of NO (protective physiological role), decrease toxic production of NO via inducible nitric oxide (iNO), block the ability of cytokine interferon-gamma to activate T-cells, decrease the formation of pro-inflammatory isoprenoids, decrease leukocyte-endothelial interactions and thus, decreases inflammation.[99,100] Statins do not contain any anti-oxidant centers. However, by inhibiting the isoprenoid reaction during the activation of NADPH oxidase, they may affect the generation of oxygen radicals. The exact mechanism by which statins act to help in the treatment of Alzheimer's disease is not known. It is also unclear why certain statins (lovastatin and pravastatin, **Fig.1.14**) should lower the risk of Alzheimer's disease while another (simvastatin, **Fig.1.14**) with similar central nervous system penetration does not.

Prevention of fibrilization of APß is one potential but unexplored area of drug discovery. Inhibition of ß-fibrillogenesis is important because Aß-induced neurotoxicity is associated with ß-sheet conformation of the peptide and soluble ß-sheet conformation is a precursor of insoluble ß-sheet conformations. Moreover, peptide solutions that contain a lot of ß-sheet structures are resistant to proteolytic degradation.[101] There are several low molecular weight synthetic peptides, modified analogues of beta amyloids that possess strong Aß anti-aggregation properties and protective effects against the amyloid fibril formation. Five residue peptide KLVFF, glycoprotein Laminin, antibiotic Rifampicin and Melatonin are examples of these ß-sheet



Lovastatin



Simvastatin

Fig. 1.14 Structure of statins

breakers.[102-104] Weak penetration through blood brain barrier and allergic reactions are the major drawbacks of these drugs.

In the last few years, immunization has gained a great deal of attention as a future drug therapy for AD. Several studies have been performed in mice and humans. Although interesting results have been seen in mice,[105] active immunization against ß-amyloid has not been very encouraging in human studies. Central nervous system infections were the major side effects in these studies. However, no explanation of the cause of these infections was given. Passive immunization by administration of anti Aß monoclonal antibodies has also been evaluated. Peripheral injection of an Aß monoclonal antibody increased plasma Aß levels by 1000 fold (probably antibodies may act as a peripheral sink for central Aß) and also decreased Aß deposition in the brain. [106] In addition, there was an increase in the formation of ABAPß (antibodies against ß-amyloid peptide). Transgenic PDAPP mice (a hybrid genomic DNA construct of human amyloid precursor protein gene), that have increased levels of both APß42 production and deposition of amyloid plaques, were used for this study. Administration of ABAPß prevented the deposition of amyloid plaques and decreased the existing plaques.[107] In addition, the mice were shown to be protected from memory decline. Recently, during clinical trials in France, several cases of CNS inflammation in patients receiving the vaccine were reported.[108] The reasons were again unknown. Kraszpulski et al. have shown that Aß plaques in man are highly dense, compact and insoluble as compared to that in mice, which may be one of the reasons for failure of this technique.

Much effort has been spent on evaluating the muscarinic agonists, without any concrete results. AChEIs were also promising in curing AD. This forced researchers to explore some other plausible drug classes. The glutamatergic system has been considered a valid target for the drug

discovery due to the decrease in glutamatergic receptors seen in Alzheimer's disease. The excitatory amino acid, glutamate, is responsible for excitatory transmission via both ionotropic and metabotropic receptors.[109] The ionotropic glutamatergic receptor family is divided into two subfamilies; NMDA receptors and non-NMDA receptors. Non-NMDA receptors have been further subdivided into AMPA receptor subtypes and Kianate receptors subtypes. A number of different NMDA and AMPA receptor agonists and antagonists have been evaluated. However most were failures because of their behavioral side effects and excitotoxicity. Recently introduced Memantine **(Fig.1.15)**, an uncompetitive NMDA antagonist and an agonist for AMPA receptor, has shown promising results.[110] In comparision to cholinergic compounds, memantine shows less side effects and better neuroprotective activity. Cycloserine **(Fig.1.16)**, an antibiotic, has also been shown to interact at the NMDA receptors and shows cognitive effects. D-Cycloserine (DCS) is an antituberculous drug and has good bioavailability in the brain. In low doses, it exhibits partial agonist properties at the NMDA associated strychnine insensitive glycine-binding site, and noncompetitively enhances NMDA neurotransmission. At high doses, it exhibits NMDA antagonist activity. The fact that it targets the glycine-binding site allows for enhancement of NMDA neurotransmission while presumably avoiding excitotoxicity that can be induced by agonists at the glutamate binding site.[111] Speculation about the role of the AMPA receptor in learning and memory resulted in the introduction of "ampakines", a class of drug which acts at a specific site on the AMPA receptor.[112] Some positive allosteric modulators of AMPA are proposed to be cognitive enhancers, but none has completed phase III clinical trials. Some drugs like Sabeluzole (antagonist of GluR) and Dimebon (anti-NMDA) were evaluated as neuorprotective agents due to their ability to block glutamate induced calcium influx.[113,114] Taurine, an amino acid, and Gastrodin, a natural glycoside, also block glutamate induced

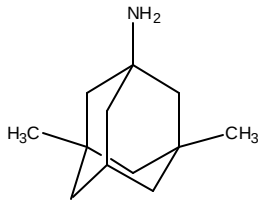
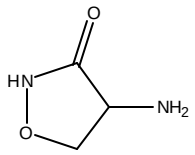
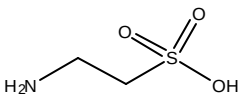


Fig. 1.15 Structure of Memantine



Cycloserine



Taurine

Fig. 1.16 Structure of muscaranic receptor agonists

calcium influx.[115,116] Unlike induced glutamatergic receptors (iGluR), muscarinic glutamatergic receptors (mGluR) act via secondary messenger systems and G-proteins. There are three groups of mGluR; I, II and III. These are highly heterogeneous with respect to their location and activity. Due to this mGluR show mixed neuroprotective and neurotoxic effects. Agonists of Group II and III show neuroprotective actions while agonists of Group I show excitotoxicity. Antagonists of Group I show neuroprotection. Nootropic drugs are those compounds which are capable of enhancing learning and memory with excellent safety and tolerability.[117] Pyrrolidones (Piracetam, levetiracetam, aniracetam, nefiracetam **Fig.1.17**) are the first class of drugs in this category. Cerebrolyzin, *N*-methyl-D-glucamine, centrofenoxine and pyritinol are other important drugs in this class. But there is a little acceptability of these drugs because of their variable results. There is no single predominant mode of action that is shared by the whole drug class. Most, however, influence cholinergic function, but these cholinergic effects are complex.[118]

From the study of the pathophysiology of AD, it can be recognized that its treatment is multidimensional. There are many unexplored regions in the understanding of the progression of the disease. There is a need to establish the cause of the disease, based on which some permanent cure for the disease could be found.

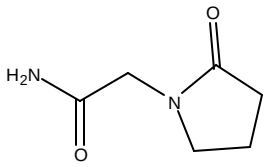


Fig. 1.17 Structure of Piracetam

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CHAPTER II

SYNTHESIS OF CHOLINE AND PIPERAZINE DERIVATIVES

NICOTINIC RECEPTORS

Alzheimer's Disease (AD), characterized by cognitive decline and loss of memory, is a complex neurodegenerative disorder affecting multiple neurotransmitters such as acetylcholine, serotonin and norepinephrine, with acetylcholine being the most affected. The hallmark of AD is the loss of cholinergic neurons in the basal forebrain, which has led to the cholinergic hypothesis. Based on this hypothesis, the current therapy for AD involves the use of acetylcholinesterase inhibitors that decrease the breakdown of the available acetylcholine. Although this approach does relieve some of the symptoms of AD, it does not prevent the progression of the disease, (ie. loss of cholinergic neurons).[1,2]

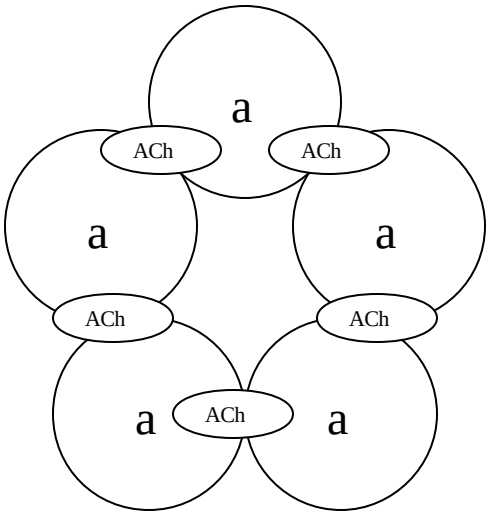
Acetylcholine is one of the major neurotransmitters in the central nervous system. Receptors for acetylcholine can be divided into two groups, the muscarinic receptors and the nicotinic receptors. Central muscarinic receptors outnumber (50-100 times) central nicotinic receptors. This, as well as the heavy loss of high affinity nicotinic cholinergic receptors in AD compared to muscarinic receptors, has lead some researchers to investigate the utility of muscarinic agonist (M1) and antagonists (M2) for the treatment of AD.

Nicotinic receptor agonists have also received a considerable amount of attention.[3-7] This is based on the observed loss of nicotinic cholinergic neurons as well as the fact that nicotine and other nicotinic agonists have been shown to enhance learning and memory and possess neuroprotective properties. A number of these compounds have entered preclinical and clinical evaluation.[8-10]

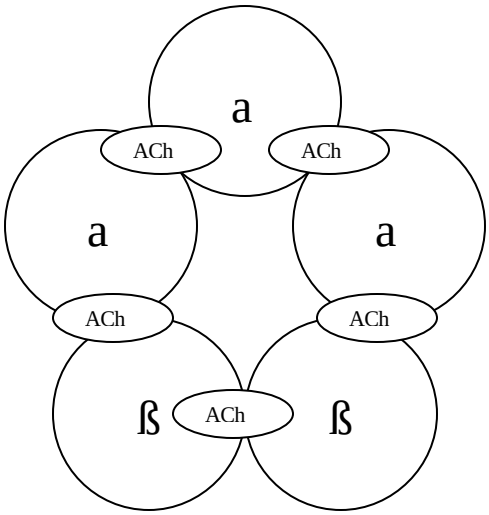
Several problems have been encountered in the development of nicotinic agonists as therapeutic agents, Selectivity of the central nicotinic receptor verses the peripheral receptor is one major hurdle that has been addressed.[11] Without this selectivity, the drug candidate would have an unacceptable side effect profile. Another major goal in this area is to gain some selectivity for specific central nicotinic cholinergic receptors subtypes. Accomplishing this goal would give us a better understanding of not only the location of the subtypes but also their function.

Nicotinic receptors are one of the members of the super family of ligand-gated ion channels which includes excitatory receptors such as nicotinic receptors (NRs), and 5-HT_3 serotonin receptors and inhibitory receptors such as glycine or ?-aminobutyric acid A ($GABA_A$).[12-18]

The nicotinic receptors are composed of five polypeptide subunits (**Fig. 2.1**) arranged around a central water filled pore[19] where they act both as a receptor via ligand binding and as effector via the opening of an ionic pore. Nicotinic receptors can be divided into three subfamilies :[20-25] 1) Nicotinic muscle subunits (a, ß, ?, e and d subunits) present on skeletal muscles; 2) Heteromeric neuronal subunits (a2- a6 and ß2-ß4) that form nAChRs in aß combination; these are a-bungarotoxin insensitive; 3) Homomeric neuronal subunits (a7-a9) forming homomeric nAChRs which are a-bungarotoxin sensitive.[26] The distribution of neuronal nAChRs is not consistent in central nervous system, although a number of different subunit combinations exist in the brain, $a_4ß_2$ and a7 nAChRs are most abundant.[27-30]



Top view of homomeric nicotinic acetylcholine receptor



Top view of heteromeric nicotinic acetylcholine receptor

Fig 2.1 Structure of nicotinic acetylcholine receptors

The nicotinic receptors (nACh) have three main functional states; closed, open and desensitized. In the resting state, the receptors are non-conducting because the ion channels are closed. Upon binding with acetylcholine or other agonist, the nAChR ion channel is stabilized in the open conformation for several milliseconds. This allows the passage of permeant cations such as Ca^{2+} through the membrane via the water filled pore. This leads to membrane depolarization and closure of the pore of the channel to a desensitized state that is unresponsive to acetylcholine or other agonists for many milliseconds. The complete kinetic behavior of nAChRs can not be predicted by these three states alone.[31,32]

Nicotinic receptors are involved in the induction of release of a variety of neurotransmitters, including dopamine, noradrenaline, serotonin, and especially glutamate and GABA, which are considered to be involved in the development of Alzheimer's disease.[33-36]

Nicotinic receptors are involved in a variety of different complex and cognitive functions such as learning, attention, memory and sensual perceptions. Various animal and human studies support this fact. Levin and colleagues have performed a number of studies with rats using nicotine and nicotinic agonists such as dimethylethanolamine, epibatidine, isonicotine, norisonicotine and AR-R17779, all of which produce a significant improvement in memory performance in the eight-arm radial maze test in rats.[37-42] In support of the nicotinic receptor's involvement in learning and memory, the nicotinic antagonist mecamylamine impaired radial-arm maze choice accuracy.[43-44] Chronic nicotine infusion induced memory improvement was blocked on chronic administration of mecamylamine.[45] In addition, other studies have shown that nicotine weakens the amnestic effects of dizocilpine, an N-methyl-D-aspartate (NMDA) type glutamatergic receptor antagonist. In animal studies, dizocilpine and other NMDA type

glutamatergic receptor antagonists have been shown to cause mnemonic deficits in both working memory and reference memory.

In vitro studies show that nicotine protects cultured neurons from neurotoxicity induced by various agents.[46] Glutamate plays an important role in neurodegeneration.[47] In the brain, N-methyl-D-aspartate (NMDA) glutamate receptor subtypes play a crucial role in glutamate neurotoxicity. NMDA receptor stimulation induces Ca^{2+} influx in the cell through ligand-gated ion channels. This triggers the production of nitric oxide (NO), which is toxic to the cells.[48-50] Various studies have shown that nicotine is neuroprotective by reducing nitric oxide production induced by Ca^{2+} influx. The a7 receptors play an important role in this.[51] Other studies have shown that blockers of a7 nicotinic receptors, blockers of protein kinase C and the absence of extracellular Ca^{2+} abolish the neuroprotective effects of nicotinic receptors.[52-53]

Despite various studies showing the efficacy of nicotine as a cognitive enhancer and as a neuroprotective agent, the use of nicotine as a therapeutic agent is still under debate. There are some studies which have shown that nicotine can be neurotoxic, especially for developing neurons.[54] Nicotine mediated neurotoxicity in developing neurons may be due to the extra calcium load in immature cells, which lack calbindin, a calcium binding protein.[55] A recent study has shown that the neuroprotective effect of nicotine follows an inverted U-shaped dose response curve with higher doses resulting in either neurotoxicity or no effect.[56] Finally, nicotine and nicotinic agonists can exert neuroprotective actions against various neuronal insults, by the activation of multiple nicotinic receptor subtypes, including a7 and $a_4ß_2$ nAChRs.

MECHANISM OF NEUROPROTECTION

There are various mechanisms to explain the neuroprotective role of nicotine and nicotinic receptors. These mechanisms involve direct or indirect blockade of the toxic agent

itself, activation of intracellular antiapoptotic cascades and induction of neurotropic agents. The selective $a_4ß_2$ nicotinic receptor agonist, cystine, and a selective a7 nicotinic receptor agonist, 3-(2,4)-dimethoxybenzylidene anabasine (DMXB) have been shown to reduce ß-toxicity.[57] Nicotine directly binds to more soluble a-helical structures and slows down or prevents an a-helical to ß-sheet conversion[58] which inhibits ß-amyloid formation. In vivo studies have shown that Transthyretin inhibits Aß aggregation by binding to Aß protein. Administration of nicotine has been shown to increase the levels of transthyretin.[59] Calcium is one of the major ions that initiates excitotoxicity and cell death. Nicotine has been shown to regulate the effect of calcium indirectly through an increase in calcium in hippocampal cells.[60] Nicotine has been shown to increase the level of phosphorylated Akt and Bcl-2, two cell survival proteins. Moreover, the a7 nAChR antagonist, a phophotidylinositol-3-kinase (PI3K) inhibitor, suppresses the nicotine induced neuroprotection against Aß.[61]

Nicotine has been shown to decrease or inhibit the activity of various proteins involved in the cell death cascade. Nicotine decreases cytochrome C release and activation of caspases 3, 8, and 9 which are involved in cell apoptosis. Nicotine inhibits neuronal nitric oxide synthase activity.[62] Nicotine has been shown to increase the level of growth factor signals, FGF-2 and brain derived nicotinic factor (BDNF) in the striatum. Injection of nicotine to the hippocampal region elevates the neurotrophic receptors trkA and trkB. Although different mechanisms of nicotine mediated neuroprotection are proposed, it is not clear which pathway is critical for these neuroprotection effects of nicotine and nicotinic agonists.

ALZHEIMER'S DISEASE AND ALPHA 7 RECEPTORS

A number of therapeutic targets have been investigated for the treatment of AD. These include acetylcholinesterase inhibitors, muscarinic agonists (M1 agonists), muscarinic

antagonists (M2 antagonists) and nicotinic agonists (non-selective, α4β2 selective and α7 selective). Of these, the α7 selective agonists are an interesting and relatively unexplored target for drug development. Several lines of evidence validate the choice of the α7 nicotinic receptor as a target. The α7 nicotinic receptor is found in abundance throughout the brain at presynaptic, perisynaptic and postsynaptic locations[63,64] and remains in high concentrations even as other nicotinic receptors are lost during the progression of AD. Therefore, these receptors are still available as a drug target.[65-68] The α7 nicotinic receptor has a much higher permeability to calcium ion as compared to α4β2 nicotinic receptors. This increased calcium influx can produce metabotropic effects leading to neurotransmitter release, stimulation of gene transcription and protein biosynthesis.[69-71]

The α7 receptor agonists such as DMXBA have not shown signs of receptor up-regulation that would lead to ever increasing doses, nor have they shown signs of drug dependence. These α7 receptor agonists have been shown to possess cognitive enhancing properties in both animals and human studies even after chronic nicotine administration.[72,73] In addition, the neuroprotective properties of nicotine and other nicotinic agonists against β-amyloid and glutamate induced toxicity is mediated via the α7 receptor.[74]

The α7 receptor, like other cholinergic receptors, is activated by acetylcholine. However, choline is also a full and selective agonist at the α7. Choline is about 10 times less potent than acetylcholine in its ability to activate or desensitize the α7 receptor[75-77] and to modulate GABAergic synaptic transmission in CA1 interneurons in rat hippocampus.[78]

As stated previously, nicotine (a nonselective agonist) and DMXBA (a selective α7 agonist) have been shown to possess neuroprotective activity. Choline has also been shown to protect nerve cells from growth factor deprivation induced cytotoxicity[79] as well as

glutamate(AMPA) induced cytotoxicty.[80] Choline itself, however, has limited utility as a drug candidate. It is a quaternary ammonium compound that shows poor oral bioavailability and requires transport into the CNS via the choline transporter at the blood brain barrier. Analogs of choline as neuroprotective agents have also been investigated. Jonnala et al. showed that the choline analog, pyrrolidinecholine, possessed neuroprotective properties (EC50 = 20μM) which were more potent than choline (EC50 = ~1mM), but still less than nicotine (EC50 = 0.7μM). Pyrrolidinecholine, being a quaternary ammonium compound, also still suffers from the same absorption and distribution problems as choline, making it a poor drug candidate but a good lead compound.

The aim of this research was to design choline like compounds which would have greater potency than pyrrolidine choline and possess a favorable absorption and distribution profile. These compounds will also provid better understanding of the structure requirements for agonist binding to the α7 nicotinic receptor.

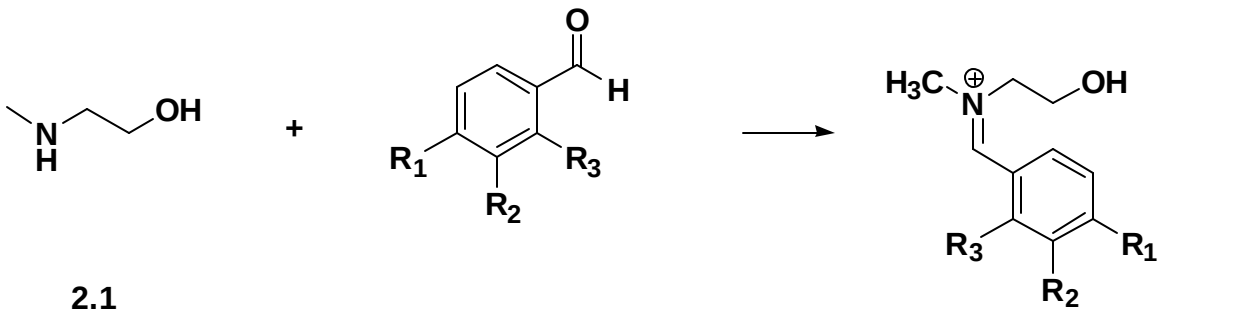
CHEMISTRY

Reductive amination that involves the reaction of carbonyl compounds (aldehyde or ketones) with ammonia, primary amines or secondary amines in the presence of reducing agents, gives primary, secondary or tertiary amines respectively. This reaction is a very important tool in the synthesis of amines. The reductive amination reaction can be run in two ways: either through the formation of an intermediate imine or iminium, (ie. isolation of the intermediate followed by reduction of the imine) or by reduction of the intermediate in situ as it forms. The reductive amination is called direct amination if no intermediate is isolated. It is called indirect or a stepwise reaction if it the intermediate is isolated.

A number of reducing agents can be used for the reductive amination reactions. Catalytic hydrogenation with the platinum, palladium, or nickel catalysts is one of the most economical and effective reductive amination methods. The drawback of these reducing agents is mixture of products and low yield. Additionally, hydrogenation can not be used with compounds containing multiple bonds. Sodium cyanoborohydride ($NaBH_3CN$) is another widely used reducing agent. This reducing agent is most active at low pH, which is conducive to imine formation. At higher pH, it is slow in action and contamination of final product with toxic cyanide can be problem. Reducing agents like $NaBH_4$ and $LiAlH_4$ are generally too strong for reductive amination leading to a reduction of carbonyl before imine formation resulting in multiple competing side reactions.

Abdel-Magid et al. has reported the use of sodium triacetoxyborohydride for reductive amination. Its advantage is that it does not require aqueous buffered conditions and it is active in organic solvents such as tetrahydrofuran and dicloroethane. In addition, there are no cyanide disposal problems. The boron-hydrogen bond is stabilized by both the stearic and the electronic effects of the three acetoxy groups, giving a very mild and selective reducing agent.

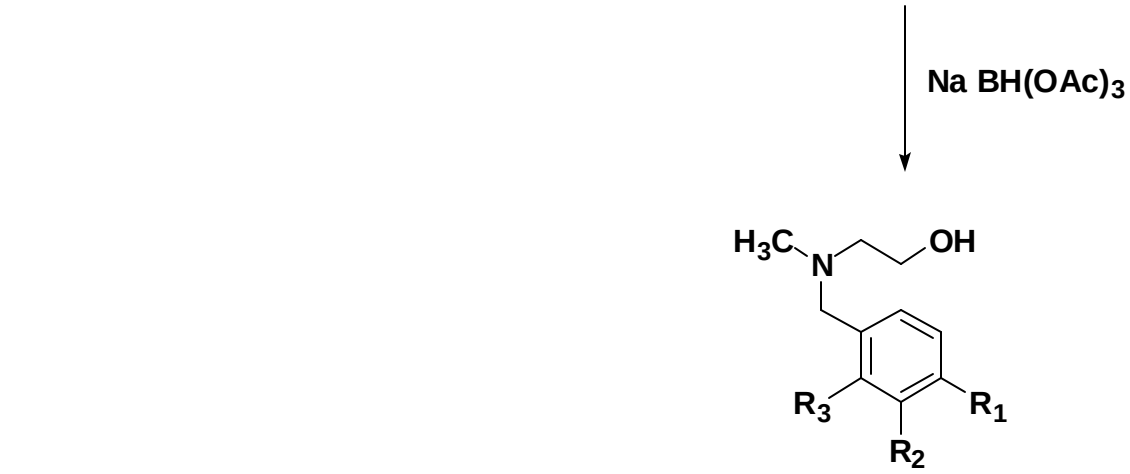
For the synthesis of the choline derivatives(2.8, 2.9, 2.10, 2.17, 2.18, 2,19) and the piperazine choline derivative(2.27, 2,28, 2.29, 2.38, 2.39, 2.40, 2.41), N-methyl ethanolamine or N-Hydroxypiperazine, respectively was dissolved in solvent followed by addition of the various aromatic aldehydes. Although tetrahydrofuran and dichloromethane were tried, dichloroethane was found to give the best results. After stirring for approximately one hour at room temperature, sodium triacetoxyborohydride was added and the reaction was monitored by TLC. On completion, the reaction was quenched with aqueous sodium bicarbonate. On work-up, fairly high yields (77-97%) of the desired product were obtained. The crude free bases were further purified by salt formation.



2.1

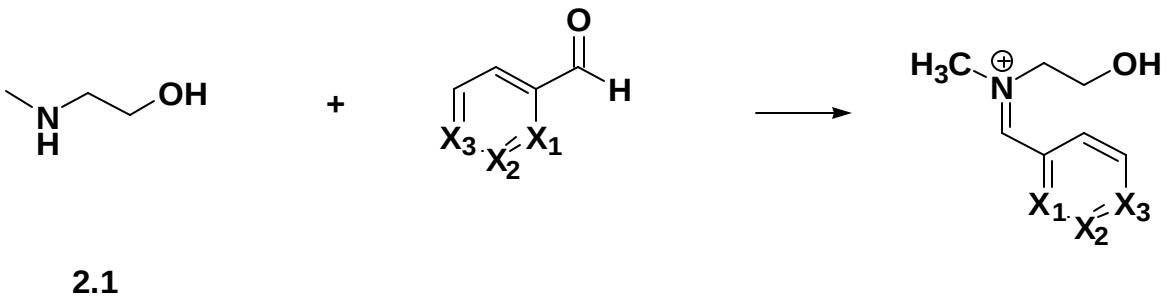
	R_1	R_2	R_3
2.2	F	H	H
2.3	H	F	H
2.4	H	H	F

	R_1	R_2	R_3
2.5	F	H	H
2.6	H	F	H
2.7	H	H	F



	R_1	R_2	R_3
2.8	F	H	H
2.9	H	F	H
2.10	H	H	F

Scheme 1

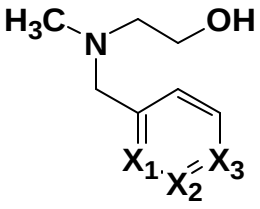


2.1

	X_1	X_2	X_3
2.11	N	C	C
2.12	C	N	C
2.13	C	C	N

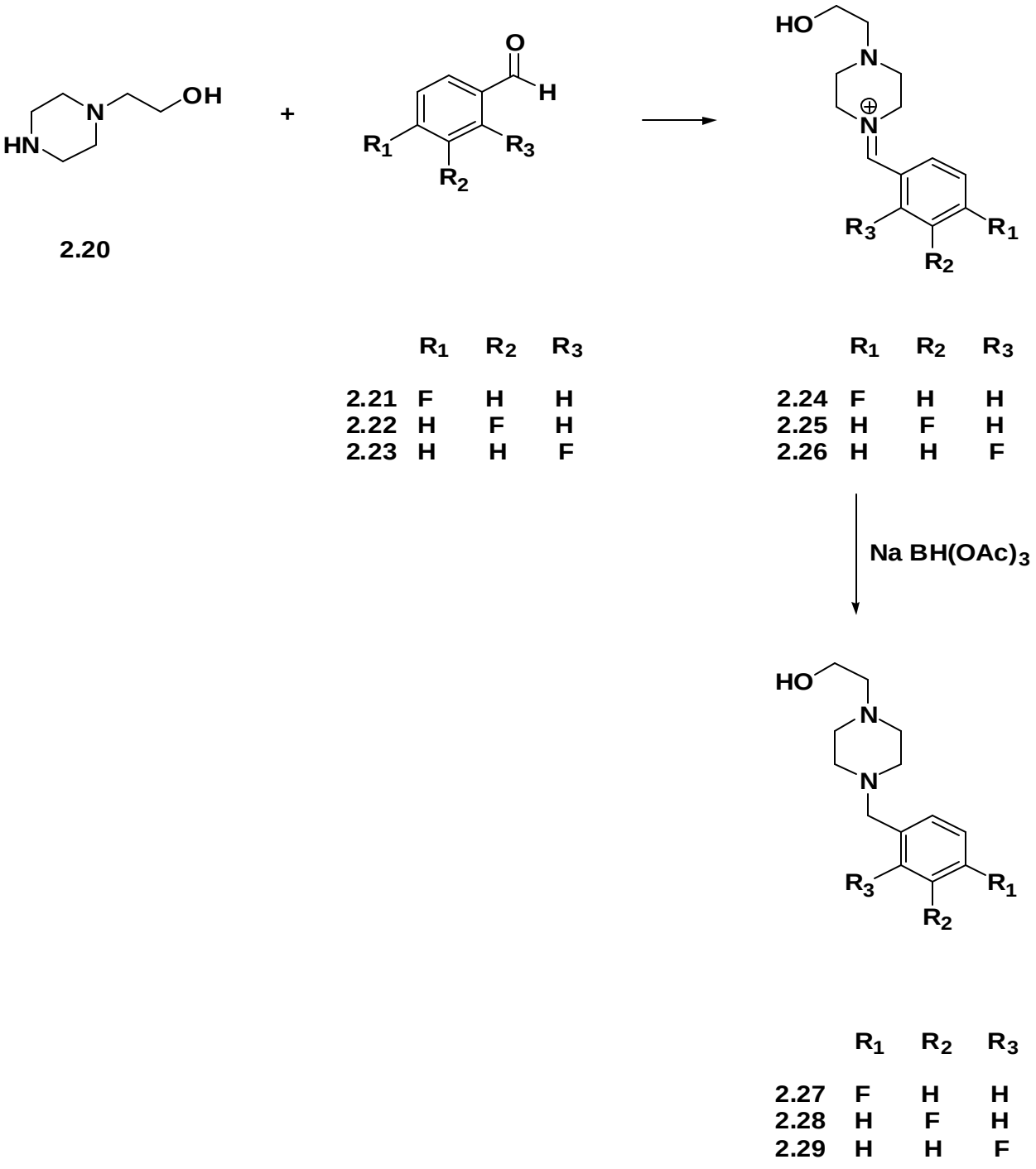
	X_1	X_2	X_3
2.14	N	C	C
2.15	C	N	C
2.16	C	C	N

$Na\ BH(OAc)_3$



	X_1	X_2	X_3
2.17	N	C	C
2.18	C	N	C
2.19	C	C	N

Scheme 2



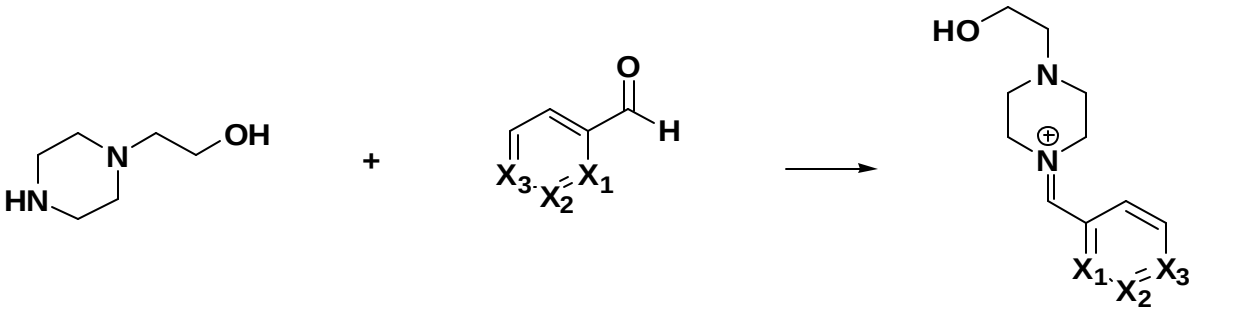
2.20

	R_1	R_2	R_3
2.21	F	H	H
2.22	H	F	H
2.23	H	H	F

	R_1	R_2	R_3
2.24	F	H	H
2.25	H	F	H
2.26	H	H	F

	R_1	R_2	R_3
2.27	F	H	H
2.28	H	F	H
2.29	H	H	F

Scheme 3

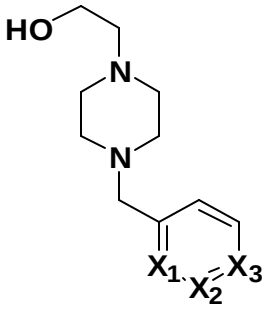


2.20

	X_1	X_2	X_3
2.30	**H**	**H**	**H**
2.31	**N**	**H**	**H**
2.32	**H**	**N**	**H**
2.33	**H**	**H**	**N**

	X_1	X_2	X_3
2.34	**H**	**H**	**H**
2.35	**N**	**H**	**H**
2.36	**H**	**N**	**H**
2.37	**H**	**H**	**N**

Na BH(OAc)$_3$



	X_1	X_2	X_3
2.38	**H**	**H**	**H**
2.39	**N**	**H**	**H**
2.40	**H**	**N**	**H**
2.41	**H**	**H**	**N**

Scheme 4

BIOLOGICAL EVALUATIONS

MATERIALS AND METHODS

Cell Culture

PC12 cells were maintained in 150-cm^2 tissue-culture flasks in Dulbecco's modified Eagles medium containing 7% horse serum, 7% fetal calf serum, 1% non-essential amino-acids and 1% streptomycin (DMEM). The cells were incubated at 37°C in a 5% CO_2-enriched, humidified atmosphere. To achieve maximum differentiation, the cells were maintained in DMEM plus NGF (DMEM-NGF) media for 5 days, with the media being changed every 2 or 3 days. A second cell line, NIH3T3, was transfected to express both choline acetyltransferase and the high affinity choline transporter (CHT1). NIH3T3 cells were released by trypsin treatment and seeded in 24-well culture plates at an initial density of 2×104 cells/well. The medium for NIH3T3 cells included: DMEM containing pencillin (105 U/L), streptomycin sulfate (100 mg/L), Blasticidin S hydrochloride 5 mg/ml), geneticin (G418, 0.2 mg/ml) and 10% calf serum was used for cell culture.

Choline Transport

On the day of the experiment, the culture media was replaced by an equal volume of uptake buffer. The uptake buffer in most experiments was 25 mM Hepes/Tris (pH 7.5), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. When the influence of Na^+ on the uptake process was investigated, the concentration of NaCl in the uptake buffer was adjusted, as desired, by iso-osmotically replacing NaCl with *N*-methyl-D-glucamine chloride. The medium was removed by aspiration and uptake buffer containing [^{3}H]choline was added to the cells to initiate uptake. After incubation at 37°C for the desired time, uptake was terminated by the removal of the medium and washing of the cells twice with

ice-cold uptake buffer. The cells were then dissolved in 1% SDS in 0.2 M NaOH and prepared for measurement of radioactivity. Saturation kinetics were analysed by fitting the data to the Michaelis–Menten equation, wherein Kt, was calculated by linear regression of the transformed data. Each experiment was performed in triplicate.

Assay for Cell Viability

Cells were dissociated by trituration and plated (5,000 – 10,000 cells per well) on poly-L-lysine-coated 96 well plates containing DMEM-NGF media maintained at 37^{o}C. The cells were maximally differentiated within 5 days. Sets of replicate cells (wells) were incubated for 24 hr with DMEM-NGF containing one of several concentrations of a study compound. An equivalent set of cells were not drug treated, but were maintained in DMEM-NGF for 24 hr at 37^{o}C. At the termination of the incubation period, both drug-treated and non-treated cells were deprived of NGF and serum over the next 24 hr. A separate control set of cells were maintained in DMEM-NGF throughout the experiment. These cells received no drug treatment and were never deprived of growth factor. This set of cells controlled for cell viability during the 24 hr of incubation after differentiation. Data derived from these cells were normalized to 100% cell viability. Cell viability was determined by using the Cell Titer 96 non-radioactive cell proliferation/ cytotoxicity assay kit (Promega). The assay is based on the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a formazan product that can be detected spectrophotometrically. At the completion of the growth factor deprivation component of the experiment, culture medium was aspirated from each cell and 15 µl of dye solution dissolved in 100 µl Dulbecco's modified Eagles medium (115 µl) was added. After 4 hr at 37^{o}C, 100 µl of solubilization/stop solution was added and the absorbance of the solubilized MTT formazan products was measured at 570 nm. Each experiment was performed in

replicates of 6 percent protection values were calculated as the ratio of ELISA-based absorbance values for [protected cells – deprived cells (no analog): control (non-deprived) cells – deprived cells] x 100.

All biological experiments were performed in the laboratory of Dr. J.J. Buccafusco, Alzheimer's Research Center, Medical College of Georgia, Augusta, GA.

RESULTS

Growth factor withdrawal (removal of NGF and serum from the culture medium) for 24 hr resulted in cytotoxicity in 30-40% of differentiated PC-12 cells. The effects of pre-incubation (24 hr) with 6 novel tertiary amine choline analogs, compound 2.20 (commercially available) and nicotine, in differentiated PC-12 cells is presented in **Figure 2.2**. All compounds produced some level of cytoprotection, though there were clear differences in potency and efficacy. The ED50 values of the various compounds are given in **Table 2.1**. Compound 2.9 (3-fluorobenzyl choline) and 2.18 (3-pyridyl choline) showed similar degrees of potency and efficacy as that of nicotine. In fact, 2.9 (3-fluorobenzyl choline) was the most potent with an ED50 of approximately 30 nM. Relative potency of compounds in descending order is 2.9, 2.18 = Nicotine, 2.20, 2.8, 2.40, 2.39, benzyl choline and acetate of benzyl choline. It is interesting to note that the unsubstituted piperazine is more potent than 4-substituted piperazine derivatives. However, due to a lack of sufficient data on all of the synthesized compounds no structure activity relation can be inferred at this time.

The graphical representation of the ability of choline, and each of the analogs to inhibit [^{3}H]choline transport into the transfected NIH3T3 cells is described in **Figure 2.3**. Compound 2.9 (3-fluorobenzyl choline) and 2.8 (4-fluorobenzyl choline) were the most effective of the

compounds evaluated in inhibiting [^{3}H]choline transport into the transfected NIH3T3 cells (**Table 2.3**).

It is not known at the present whether the compound competing for the transport are themselves transported or block the transporter. Further experiments are needed in order to determine which of these two potential explanations of the data are valid.

EXPERIMENTAL

Melting points were determined on an Electrotherm apparatus and are uncorrected. NMR spectra were obtained in CDCl3 or DMSO-d6 or D2O with tetramethylsilane as an internal standard on a Varian (500 MHz) instrument. The chemical shifts (d) are given in parts per million (ppm), and coupling constants are in Hertz. Splitting patterns are designated as follows: s, singlet; bs, broad singlet; t, triplet; q, quadruplet; and m, multiplet. Elemental analyses were performed by Atlantic Microlabs, Atlanta, Georgia, and were within 0.4% of the theoretical values.

2-[(4-Fluoro-benzyl)-methyl-amino]-ethanol. Hydro bromide salt (2.8):

One equivalent each of N-methylethanolamine **(2.1,** 1.0 g/1.069 ml) and 4-fluorobenzaldehyde **(2.2**, 1.65 g/1.428 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 1.10g of **2.8** as bromide salt, 81.0%

m.p. 101-102^{o}C. *Anal.* Calcd. for $C_{10}H_{14}FNO.HBr$: C, 45.45; H, 5.68; N, 5.30. Found: C, 45.46; H, 5.75; N, 5.30. ^{1}H NMR (500 MHz, $CDCl_3$): d 2.53-2.64 (m, 10H); 3.52 (s, 2H); 3.59-3.68 (m,

Serial No.	Compound	EC-50 cytoprotection
1	Nicotine	58 nM
2	2.8 (4-fluoro choline)	110 nM
3	**2.9 (3-fluoro choline)**	**30 nM**
4	2.18 (3-pyridyl choline)	58 nM
5	2.20 (piperazine)	89 nM
6	2.39 (2-pyridyl piperazine)	316 nM
7	2.40 (3-pyridyl piperazine)	260 nM
8	*Benzyl choline acetate	1 µM
9	*Benzyl choline	>1 mM

* These compounds were synthesized earlier.

Table 2.1: EC-50 cytoprotection data

S.No.	Compound	EC-50 cytoprotection
1	2.10 (2-fluoro choline)	In Process
2	2.17 (2-pyridyl choline)	In Process
3	2.19 (4-pyridyl choline)	In Process
4	2.27 (4-floro piperazine)	In Process
5	2.28 (3-fluoro piperazine)	In Process
6	2.29 (2-fluoro piperazine)	In Process
7	2.38 (benzyl piperazine)	In Process
8	2.41 (4-pyridyl piperazine)	In Process

Table 2.2: EC-50 cytoprotection data (results awaited)

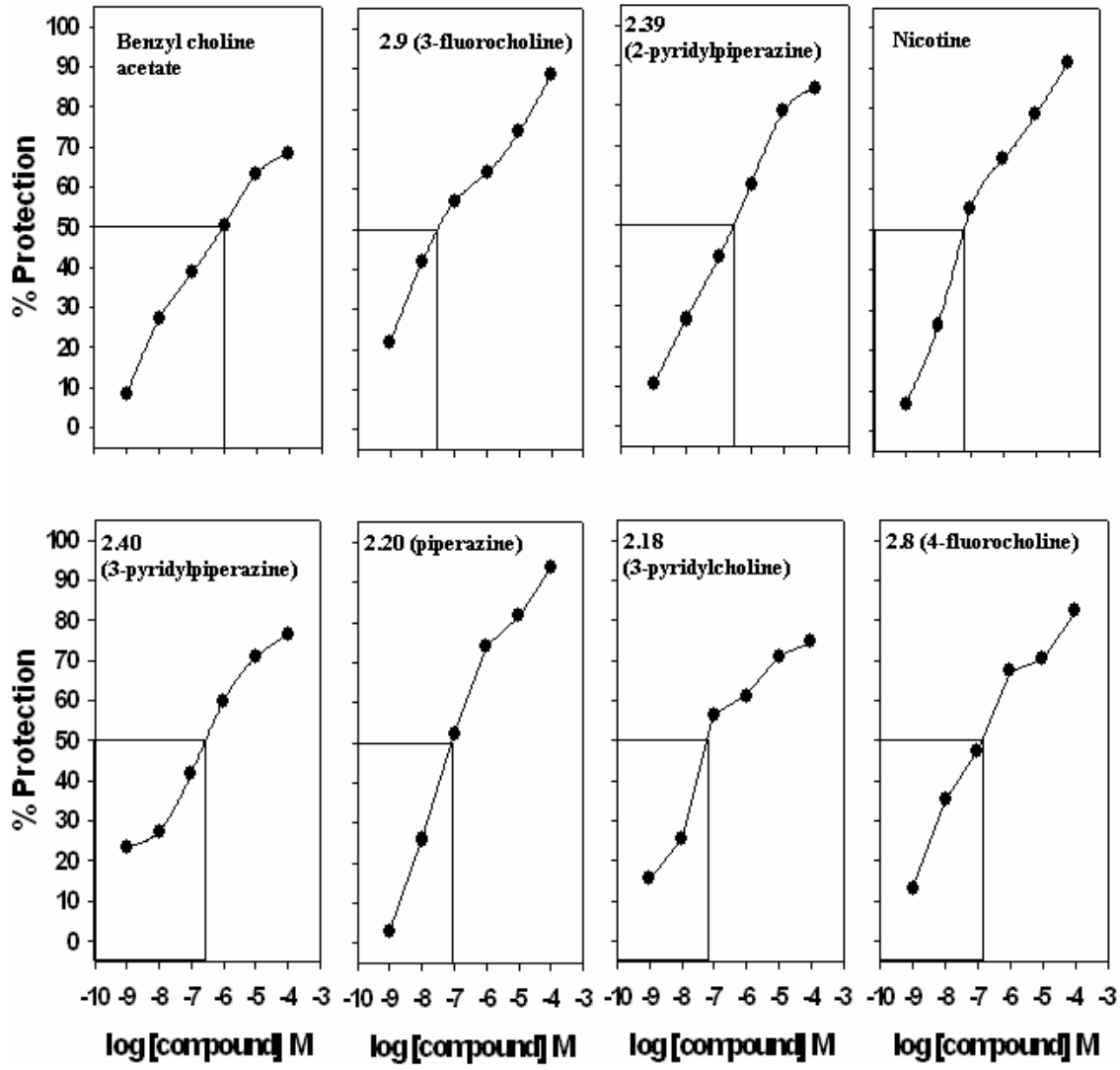


Fig.2.2: The ability of tertiary amine analogs of choline and nicotine to protect differentiated PC-12 cells from the cytotoxicity associated with growth factor withdrawal.

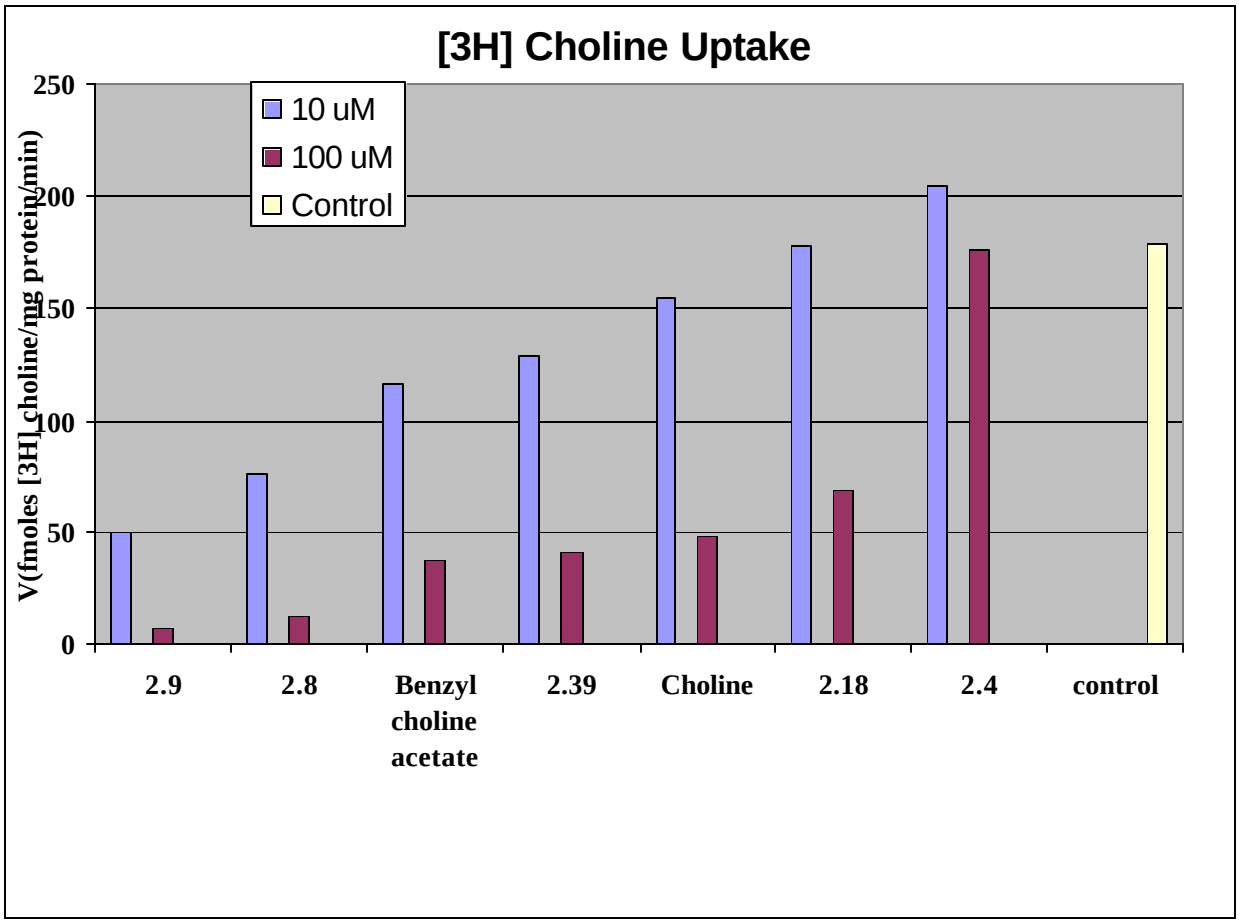


Fig. 2.3: Inhibition of [^{3}H]choline transport into the transfected NIH3T3 cells

S.No.	Compound	Velocity Fmoles [^{3}H]choline transported/mg protein/min	%of control
1	Control	.782	100.0
2	2.8	.307	39.26
3	2.9	.264	33.86
4	2.18	.571	72.98
5	2.39	.560	71.68
6	2.40	.692	88.48
7	Benzyl choline acetate	.611	78.12

Table 2.2: %Inhibition of [^{3}H]choline transport with compared to control

2H); 6.96-6.99 (m, 2H); 7.25-7.28 (m, 2H). ^{13}C NMR (500 MHz, D_2O): d 47.88, 48.81, 54.93, 58.08, 59.75, 116.31, 116.52, 127.17, 133.52, 133.53, 164.75.

2-[(3-Fluoro-benzyl)-methyl-amino]-ethanol. Maleate salt (2.9):

One equivalent each of N-methylethanolamine **(2.1,** 1.0 g/1.069 ml) and 3-fluorobenzaldehyde (**2.3**, 1.65 g/1.41 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added.

Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. The reaction was quenched by adding aqueous bicarbonate and stirred for 15 minutes. The solvent was concentrated with a rotary evaporator and then 10% H_2SO_4 was added to the remaining oil. This was then extracted with the ethyl acetate and the organic layer was discarded. The pH of the water layer was changed to a slightly basic pH with potassium carbonate (K_2CO_3) and extracted again with ethyl acetate. This pH change was necessary for the removal of any excess benzaldehyde. The organic layer was dried over sodium sulfate and concentrated to a small volume. The resulting free base was converted to a maleate salt (**2.9**) and recrystallized from 200% proof ethyl alcohol. 3.22 g, 81.0%

m.p. 67-68^{o}C. *Anal.* Calcd. for $C_{14}H_{18}FNO_5$: C, 56.18; H, 6.06; N, 4.68. Found: C, 56.20; H, 6.04; N, 4.49. ^{1}H NMR (500 MHz, DMSO): d 2.73 (s, 3H); 3.12-3.14 (t, J = 5 Hz, 2H); 3.74-3.76 (t, J = 5 Hz, 2H); 4.33 (s, 2H); 6.04 (s, 2H); 7.31-7.56 (m, 4H). ^{13}C NMR (500 MHz, D_2O): d 39.76, 55.12, 56.73, 59.12, 116.99, 117.19, 117.19, 117.67, 117.84, 127.03, 131.16, 134.29, 170.46, 181.66.

2-[(2-Fluoro-benzyl)-methyl-amino]-ethanol. Oxalate salt (2.10):

One equivalent each of N-methylethanolamine (**2.1,** 1.0 g/1.069 ml) and 2-fluorobenzaldehyde (**2.4**, 1.65 g/1.40 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. The reaction was quenched by adding aqueous bicarbonate and stirred for 15 minutes. The solvent was concentrated with a rotary evaporator and then 10% H_2SO_4 was added to the remaining oil. This was then extracted with the ethyl acetate and the organic layer was discarded. The pH of the water layer was changed to a slightly basic pH with potassium carbonate (K_2CO_3) and extracted again with ethyl acetate. This pH change was necessary for the removal of any excess benzaldehyde. The organic layer was dried over sodium sulfate and concentrated to a small volume. The resulting free base was converted to an oxalate salt (**2.10**) and recrystallized from 200% proof ethyl alcohol, 2.87 g, 79.06%.

m.p. 111°C. *Anal.* Calcd. for $C_{12}H_{16}FNO_5$: C, 52.75; H, 5.90; N, 5.13. Found: C, 52.76; H, 5.85; N, 5.08. ^{1}H NMR (500 MHz, $CDCl_3$): d 2.24 (S, 3H); 2.60-2.62 (t, J = 5 Hz, 2H); 2.83 (brs, OH); 3.61-3.64 (m, 4H); 7.02-7.33 (m, 4H).), ^{13}C NMR (500 MHz, D_2O): d 39.91, 53.35, 55.22, 57.15, 116.0, 116.17, 125.13, 132.91, 133.31, 165.63, 181.63.

2-(Methyl-pyridin-2-ylmethyl-amino)-ethanol. HCl salt (2.17):

One equivalent each of N-methylethanolamine **(2.1,** 1.0 g/1.069 ml) and 2-pyridine carbaldehyde (**2.11**, 1.27 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued

until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 1.3 g of **2.17** as chloride salt, 73.77%

m.p. 184-188^{o}C. *Anal.* Calcd. for $C_9H_{16}Cl_2N_2O$: C, 45.20; H, 6.74; N, 11.71 Found: C, 45.24; H, 6.82; N, 11.50. ^{1}H NMR (500 MHz, DMSO): d 2.86 (S, 3H); 3.22-3.24 (t, J = 5 Hz, 2H); 3.84-3.86 (t, J = 5 Hz, 2H); 4.79 (s, 2H); 7.66-7.69 (t, J = 9.5 Hz, 1H); 7.88-7.90 (d, J = 7.5 Hz, 1H); 8.1-8.21 (t, J = 9.5 Hz, 1H); 8.73-8.74 (d, J = 7.5 Hz, 1H). ^{13}C NMR (500 MHz, D_2O): 39.9, 55.73, 57.61, 62.45, 126.08, 127.78, 141.40, 147.37, 148.55.

2-(Methyl-pyridin-3-ylmethyl-amino)-ethanol. HCl salt (2.18):

One equivalent each of N-methylethanolamine **(2.1,** 1.0 g/1.069 ml) and 3-pyridine carbaldehyde (**2.12**, 1.26 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.353 g of **2.18** as chloride salt, 95.0%

m.p. 158-160^{o}C. *Anal.* Calcd. for $C_9H_{16}Cl_2N_2O$: C, 45.18; H, 6.69; N, 11.71 Found: C, 45.16; H, 6.79; N, 11.59. ^{1}H NMR (500 MHz, DMSO): d 2.76 (S, 3H); 3.24-3.26 (t, J = 5 Hz, 2H); 3.84-3.86 (t, J = 5 Hz, 2H); 4.78 (brs, 2H); 8.16-8.19 (t, J = 9.5 Hz, 1H); 8.82-8.84 (d, J = 7.5 Hz, 1H); 8.97-8.99 (t, J = 9.5 Hz, 1H); 9.21 (s, 1H). ^{13}C NMR (500 MHz, D_2O): 39.8, 55.62, 57.73, 59.02, 126.08, 127.78, 144.40, 146.0, 148.05.

2-(Methyl-pyridin-4-ylmethyl-amino)-ethanol. Maleate salt (2.19):

One equivalent each of N-methylethanolamine **(2.1,** 1.0 g/1.069 ml) and 4-pyridine carbaldehyde (**2.2**, 1.27 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and

then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 2 hours. Work up of this was done as described above. Yield: 2.353 g of **2.19** as chloride salt, 95.0%

m.p. absorbed moisture°C. *Anal.* Calcd. for $C_{17}H_{22}N_2O_9$: C, 51.26; H, 5.57; N, 7.03 Found: C, 51.44; H, 5.62; N, 7.01. ^{1}H NMR (500 MHz, DMSO): d 2.76 (S, 3H); 3.11-3.13 (t, J = 5 Hz, 2H); 3.74-3.76 (t, J = 5 Hz, 2H); 4.36 (s, 2H); 6.05 (s, 2H); 7.54-7.55 (d, J = 6 Hz, 2H); 8.69-8.71(d, J = 6 Hz, 2H). ^{13}C NMR (500 MHz, D_2O): 39.8, 55.62, 57.73, 59.02, 128.10, 127.21, 132.02, 135.30, 136.80, 170.46, 181.66.

2-[4-(4-Fluoro-benzyl)-piperzin-1-yl]-ethanol. HCl salt (2.27):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and 4-fluorobenzaldehyde (**2.22**, 1.1318 g/0.978 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.3 equivalents (4.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.42 g of **2.27** as chloride salt, 86.64%

m.p. 260-261°C. *Anal.* Calcd. for $C_{13}H_{21}Cl_2FN_2O$: C, 50.17; H, 6.80; N, 9.00 Found: C, 50.29; H, 6.86; N, 8.90. ^{1}H NMR (500 MHz, DMSO): d 2.86 (s, 3H); 3.22-3.24 (m, 2H); 3.45 (brs, 1H); 3.84-3.86 (t, J = 5 Hz, 2H); 4.40 (m, 2H); 6.96-6.99 (m, 2H); 7.25-7.28 (m, 2H). ^{13}C NMR (400 MHz, DMSO): 39.9, 55.26, 56.88, 59.30, 117.14, 117.85, 127.17, 131.27.

2-[4-(3-Fluoro-benzyl)-piperzin-1-yl]-ethanol. H Cl salt (2.28):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and 3-fluorobenzaldehyde (**2.22**, 1.1318 g/0.967 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature

for one hour and then approximately 1.3 equivalents (4.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 1.85 g of **2.28** as chloride salt, 66.0%

m.p. 260-262^{o}C. *Anal.* Calcd. for $C_{13}H_{21}Cl_2FN_2O$: C, 50.17; H, 6.80; N, 9.00 Found: C, 50.25; H, 6.77; N, 9.01. ^{1}H NMR (500 MHz, DMSO): d 2.48-2.49 (t, J = 3.5 Hz, 2H); 3.2-3.78 (m, 10H); 4.37 (brs, 2H); 7.27-7.60 (m, 4H). ^{13}C NMR (500 MHz, D_2O): d 47.84, 48.81, 54.83, 58.18, 59.65, 116.31, 116.42, 128.17, 132.52, 134.53, 165.75.

2-[4-(2-Fluoro-benzyl)-piperzin-1-yl]-ethanol. HCl salt (2.29):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and 2-fluorobenzaldehyde (**2.23**, 1.138 g/0.960 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.3 equivalents (4.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.3 g of **2.29** as chloride salt, 82.14%

m.p. 240-241^{o}C. *Anal.* Calcd. for $C_{13}H_{21}Cl_2FN_2O$: C, 50.17; H, 6.80; N, 9.00 Found: C, 50.28; H, 6.85; N, 8.95. ^{1}H NMR (500 MHz, DMSO): d 2.48-2.49 (t, J = 3.5 Hz, 2H); 3.2-3.78 (m, 10H); 4.31 (brs, 2H); 7.27-7.33 (m, 2H); 7.49-7.53 (s, 1H); 7.72-7.73 (m, 1H). ^{13}C NMR (500 MHz, D_2O): d 48.05, 48.73, 53.81, 54.88, 58.08, 116.11, 116.27, 125.31, 133.43, 160.49, 161.0, 181.56, 181.87.

2-(4-Benzyl-piperazin-1-yl)ethanol. HCl salt (2.38):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and benzaldehyde (**2.30**, 0.964 g/0.923 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour

and then approximately 1.3 equivalents (4.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.2 g of **2.38** as chloride salt, 82.70%

m.p. 242°C. *Anal.* Calcd. for $C_{13}H_{22}Cl_2N_2O$: C, 53.25; H, 7.56; N, 9.55 Found: C, 53.15; H, 7.69; N, 9.54. ^{1}H NMR (500 MHz, $CDCl_3$): d 2.52-2.63 (m, 10H); 3.19 (brs, 1H); 3.60-3.69 (m, 4H); 7.23-7.35 (m, 5H). ^{13}C NMR (500 MHz, D_2O): d 47.52, 47.99, 48.83, 54.97, 58.15, 60.62, 127.52, 129.56, 131.34, 135.12.

2-(4-Pyridin-2-ylmethyl-piperazin-1-yl)-ethanol . HCl salt (2.39):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and 2-pyridine carbaldehyde (**2.31**, 0.973 g/0.864 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.5 equivalents (5.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.56 g of **2.39** as chloride salt, 85.3%

m.p. 156-158°C. *Anal.* Calcd. for $C_{12}H_{22}Cl_3N_3O.H_2O$: C, 41.33; H, 6.94; N, 12.05 Found: C, 41.32; H, 6.93; N, 11.81. ^{1}H NMR (500 MHz, DMSO): d 2.48-2.49 (t, J = 3.5 Hz, 2H); 3.14-3.62 (m, 8H); 3.77-3.79 (t, J = 10.5 Hz, 2H); 4.37 (s (2H); 8.18-8.19 (d, J = 3.5 Hz, 2H); 8.94-8.95 (d, J = 6 Hz, 2H). ^{13}C NMR (500 MHz, D_2O): d 48.75, 49.76, 55.09, 56.46, 58.12, 125.58, 127.02, 141.17, 142.33, 150.32.

2-(4-Pyridin-3-ylmethyl-piperazin-1-yl)-ethanol . HCl salt (2.40):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and 2-pyridine carbaldehyde (**2.32**, 0.973 g/0.857 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature

for one hour and then approximately 1.3 equivalents (4.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.56 g of **2.40** as chloride salt, 85.3%

m.p. 249-250°C. *Anal.* Calcd. for $C_{12}H_{22}Cl_3N_3O$: C, 43.59; H, 6.71; N, 12.71 Found: C, 43.57; H, 6.71; N, 12.49. ^{1}H NMR (500 MHz, $CDCl_3$): d 2.50-2.56 (m, 10H); 3.52 (s, 2H); 3.60-3.62 (t, J = 10.5 Hz, 2H); 7.24-7.27 (m, 1H); 7.65-7.68 (m, 1H); 8.49-8.51 (m, 2H);.; ^{13}C NMR (500 MHz, D_2O): d 48.78, 49.78, 55.01, 56.47, 58.17, 128.10, 132.02, 142.49, 142.95, 149.25.

2-(4-Pyridin-4-ylmethyl-piperazin-1-yl)-ethanol . HCl salt (2.41):

One equivalent each of 1-(2-hydroxyethyl) piperzine **(2.20,** 1.0 g/0.942 ml) and 4-pyridine carbaldehyde (**2.33**, 0.973 g/0.831 ml) were added to a 250 ml round bottomed flask containing 25 ml of 1,2-dicholoroethane (DCE). The reactants were stirred at room temperature for one hour and then approximately 1.3 equivalents (4.0 g) of $NaBH(OAc)_3$ were added. Stirring was continued until TLC showed the absence of all starting materials, about 3 hours. Work up of this was done as described above. Yield: 2.30 g of **2.41** as chloride salt, 76.67%

m.p. 244-246°C. *Anal.* Calcd. for $C_{12}H_{22}Cl_3N_3O$: C, 43.59; H, 6.71; N, 12.71 Found: C, 43.61; H, 6.71; N, 12.52. ^{1}H NMR (500 MHz, DMSO): d 2.48-2.49 (t, J = 3.5 Hz, 2H); 3.14-3.62 (m, 8H); 3.77-3.79 (t, J = 10.5 Hz, 2H); 4.37 (brs (2H); 8.18-8.19 (d, J = 3.5 Hz, 2H); 8.94-8.95 (d, J = 6 Hz, 2H). ^{13}C NMR (500 MHz, D_2O): d 49.03, 49.79, 54.85, 58.01, 58.45, 128.61, 145.84, 150.23.

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