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SCREENING OF NOOTROPICS: AN OVERVIEW ON PRECLINICAL EVALUATION TECHNIQUES

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ABSTRACT

Nootropics are also referred as smart drugs, memory enhancers, and cognitive enhancers. They are reported to improve mental function such as cognition, memory, intelligence, motivation, attention and concentration. They are thought to be work by altering the availability of brains supply of neurochemicals, by improving the brains oxygen supply or by stimulating nerve growth. Alzheimer's disease (AD) is a progressive neurodegerative disorder which affects older individuals and is the most common cause of dementia. It may progress to a totally vegetative state. Atrophy of cortical and sub-cortical areas is associated with deposition of β-amyloid protein in the form of senile plaques and formation of neurofibrillary tangles. There is marked cholinergic deficiency in the brain, though other neurotransmitter systems are also affected. Various measures to augement cholinergic transmitter in the brain have been tried. The relatively cerebroselective anti-ChEs have been approved for clinical use. There are number screening models available for preclinical evaluation of nootropics drugs. Newer models are developed in accordance with limitations of the earlier one. In-vitro methods inhibition of acetylcholinesterase activity is measured by determining IC50 with the help of Log probit analysis. In ex-vivo cholinesterase inhibition method the dose response relationship determined for drugs such as physostigmine and tacrine. Studies on molecular form of AchEs are carried in rat frontal cortex and striatum for drug such as donepezil, tacrine. Agents which are H_3 receptor agonist are evaluated for [$_3$H] Ach release activity in rat using rat brain slices. The binding affinity of potential nicotinic cholinergic agonist in brain using agonist ligand is determined by [$_3$H]-N- methyl carbamylcholine binding nicotinic cholinergic receptors in rat frontal cortex. In In-vivo methods the inhibitory passive avoidance the test are carried on animals to test the learning and memory capacity of animal by suppressing a particular behavior. It includes step down, step through, two compartment test, up-hill avoidance, scopolamine induced test, and ischemia induced amnesia, memory impairments in basal forebrain. In active avoidance conditioned stimulus is given to the animal, which gives noxious stimulus as a result. It includes runway avoidance, shuttle box avoidance, jumping avoidance. In discrimination learning animals have no choice between the conditioned stimuli. Studies on aged monkeys provides additional advantage for neurobehavioral animal model of aging in that many of behavioral processes thought to be affected by aging.

Keywords: Nootropics, Preclinical evaluation and Memory enhancers

INTRODUCTION

Nootropics are also referred as smart drugs, memory enhancers, and cognitive enhancers. They are reported to improve mental function such as cognition, memory, intelligence, motivation, attention and concentration. They are thought to be work by altering the availability of brains supply of neurochemicals, by improving the brains oxygen supply or by stimulating nerve growth[1]. The concept and definition of a "Nootropic drug" was first proposed by Romanian Dr. Corneliu e. Giurgea. He derived the term nootropics from the Greek words "noos" (=mind) and "tropein" (=to turn towards). The main features of nootropics drug are, the enhancement, at least under same conditions of

learning acquisition as well as resistance of learned behaviors to agents that tend to impair them, the facilitation of inter hemispheric flow of information, partial enhancement of the general resistance of the brain and particularly its resistance to physical and chemical injuries and increase in the efficacy of the tonic cortico sub cortical control mechanisms. During the past 30 years, the PIR nootropics have been used to treat an amazingly broad range of human ailments and condition either alone or with other drugs, with moderate to major benefit. Nootropics have been used to treat various forms of the dementia and "organic brain syndrome".

Hey are used successfully to treat dyslexia, epilepsy and age associated memory impairment. PIR-nootropics have successfully treated vertigo, alcohol withdrawal, cerebrovascular insufficiency and hypoxia. They have shown benefit in normalizing blood flow parameters, decreased platelet aggregation, increased RBC deformability, decreased adherence of damaged and sickle cell RBCs to endothelium and increased prostacyclin production and activity. The racetams (nootropics) are cerebral homeostatic normalizers, neuroprotectants, cerebral metabolic enhancers and brain integrative agents. They enhance brain energy, especially under deficit condition eg. hypoxia, chemical toxicity or impaired cerebral microcirculation. They preserve, protect and enhance synaptic membrane and receptor structure and plasticity. They enhance brain integration horizontally by increased coupling of the cerebral hemisphere and vertically by enhancing cerebral connection with and tonic control of the limbic system through nootropics effect on the hippocampus a major link between cerebrum and limbic system.

In middle aged and older individuals who do not yet suffer any specific neural malady or major mental impairment, nootropics may not only slow down or postpone entropic brain aging but they may even reverse some mild neural or mental decline. Thus a person at 50 might be smarter, have better memory, quicker reflexes and greater vigilance and alertness than when they were 40 the nootropics may literally be safe and effective pharmacologic tools to enhance, protect and optimize truly normal, fully human neuropsychological structures and function, well into old age. However the efficacy of nootropics substances, in most cases has not been conclusively determined. This is complicated by the difficulty of defining and quantifying cognition and intelligence.

Over 30 years have passed since the "Nootropics revolution" quietly began with the development of the Piracetam(PIR) in the late 1960's. The second wave of this pharmacologic revolution occurred in the late 1970's with the development of Oxiracetam(OXR), Pramiracetam(PRM), and Aniracetam(ANR). The action of the PIR nootropics has been studied in a broad range of animals; goldfish, mice, rats, guinea pigs, rabbits, dogs, cats,, monkeys and humans. The toxicity of PIR and its cousins is amazingly low, almost non-existant. PIR nootropics are among the toxicologically safest drug ever developed. In 2006 there were 26.6 million suffers worldwide of Alzheimer's disease (AD) [1-2].

PATHOPHYSIOLOGY OF ALZHEIMER'S DISEASE

Generally nootropics are used to treat dementia and various forms of dementia. The most common form of the dementia is Alzheimer Disease (AD). This incurable, degenerative and terminal disease was 1st described by the german psychiatrist Alois Alzheimer in 1906 and was named after him. Generally it is diagnosed in people over 65 years of age. In 2006 there were 26.6 million suffers worldwide. Although the AD is unique for every individual there are many common symptoms. In the early stages the most commonly recognized symptoms is inability to acquire new memories such as difficulty in recalling recently observed facts. The diagnosis is usually confirmed with behavioral assessments and cognitive test often followed by brain scan. As disease advances symptoms include confusion, irritability, and aggression, mood swings, language breakdown, and long term memory loss. Gradually body functions are lost, leading to death. The duration of disease varies. It developes for an indeterminate period and may remain undiagnosed for many years. Fewer than 3% of individual live more than 14 years after diagnosis. The disease course is divided into 4 stages, with progressive patterns of cognitive and functional impairements, pre-dementia, early dementia, moderate dementia, advanced dementia[2].

Neuropathology: Alzheimer's disease is characterized by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This loss results in gross atrophy of the affected regions, including degeneration in temporal lobe and parietal lobe and parts of the frontal cortex and cingulated gyrus. Studies using MRI and PET have documented reduction in the size of specific brain regions in patients as they progressed from mild cognitive impairment to AD, and in comparison with similar images from healthy older adults. Both amyloid plaques and neurofibrillary tangels are clearly visible by microscopy in brains of those afficated byAD. Plaques are dense; mostly insoluble deposits of amyloid-beta peptide and cellular material outside and around neurons.tangels are aggregates of

mmicrotubule associated protein tau which has become hyperphosphorylated and accumulate inside the cells themselves, although many older individual develop some plaques and tangels as a consequence of aging.

Biochemistry of Alzheimer's disease: Alzheimer's disease has been identified as a protein misfolding disease, caused by accumulation of abnormally folded A-beta and tau proteins in the brain. Plaques are made up of small peptides, 39-43 amino acids in length called beta-amyloid(Aβ). Beta-amyloid is a fragment from a larger protein called amyloid precursor protein (APP), a transmembrane protein that penetrates through the neurons membrane. APP is critical to neuron growth, survival and post injury repair. In AD an unknown process causes APP to be divided into smaller fragments by enzymes through proteolysis. One of this fragments give rise to fibrils of beta-amyloid, which form clumps that deposit outside neurons in dense formation known as senile plaques. And changes in tau protein lead to the disintegration of microtubules in brain cells. Ad is also considered a taupathy due to abnormal aggregation of the tau protein. Tau undergoes chemical changes, becoming hyperphosphorylated; it then begins to pair with other threads, creating neurofibrillary tangels and disintegrating the neurons transport systems.

Disease mechanism: Exactly how disturbances of production and aggregation of the beta amyloid peptide gives rise to the pathology of AD is not known. The amyloid hypothesis traditionally points to the accumulation of beta amyloid peptides as the central event triggering neuron degeneration. Accumulation of aggregated amyloid fibrils, which are believed to be the toxic form of the protein responsible for disrupting the cell's calcium ion homeostasis, induces programmed cell death (apoptosis). It is also known that Aβ selectively builds up in the mitochondria in the cells of Alzheimer's-affected brains, and it also inhibits certain enzyme functions and the utilisation of glucose by neurons. Various inflammatory processes and cytokines may also have a role in the pathology of Alzheimer's disease. Inflammation is a general marker of tissue damage in any disease, and may be either secondary to tissue damage in AD or a marker of an immunological response. Alterations in the distribution of different neurotrophic factors and in the expression of their receptors such as the brain derived neurotrophic factor (BDNF) have been described in AD.

Diagnosis: Alzheimer's disease is usually diagnosed clinically from the patient history, collateral history from relatives, and clinical observations, based on the presence of characteristic neurological and neuropsychological features and the absence of alternative conditions. Advanced medical imaging with computed tomography (CT) or magnetic resonance imaging (MRI), and with single photon emission computed tomography (SPECT) or positron emission tomography (PET) can be used to help exclude other cerebral pathology or subtypes of dementia. Moreover, it may predict conversion from prodromal stages (mild cognitive impairment) to Alzheimer's disease.

PRECLINICAL EVALUATION TECHNIQUES FOR NOOTROPICS

It is easily understood that behavioral psychopharmacologies faced with the task of dealing with extremely complex behavioral disturbances. This holds true for both patient groups: young people with learning and memory problems and elderly patients with memory deficits. For the elderly, difficulties arise in designing appropriate animal models of human aging or the deficits occurring during human aging. One of the major problems for experimental behavioral pharmacology is whether or not old animals are the appropriate models[4].

IN VITRO METHODS

In vitro inhibition of acetylcholine-esterase activity in rat striatum

***Principle*[3]:** The purpose of this assay is to screen drugs for inhibition of acetylcholine-esterase activity. Inhibitors of this enzyme may be useful for the treatment of Alzheimer's disease. Acetylcholinesterase (AChE), is found in nerve cells, skeletal muscle, smooth muscle, various glands and red blood cells. It is generally accepted that the physiological role of AChE is the rapid hydrolysis and inactivation of acetylcholine. Inhibitors of AChE show marked cholinomimetic effects in cholinergically innervated effector organs and have been used therapeutically in the treatment of glaucoma, myasthenia gravis and paralytic ileus. However, recent studies have suggested that AChE inhibitors may also be beneficial in the treatment of Alzheimer's dementia. Augustinsson (1971) reviewed a number of methods for assaying cholinesterase activity and concluded that the method described by Ellman et al. (1961) was one of the best. The method described is a modification of Ellman's procedure.

Procedure:

Tissue preparation: Male Wistar rats are decapitated, brains rapidly removed, corpora striata dissected free, weighed and homogenized in 19 volumes (approximately 7 mg protein/ml) of 0.05 M NaH2PO4, pH 7.2 using a Potter-Elvejhem homogenizer. A 25 µl aliquot of this suspension is added to 1 ml of the vehicle or various concentrations of the test drug and reincubated for 10 min at 37 °C.

Assay: Enzyme activity is measured with the Beckman DU-50 spectrophotometer. This method can be used for *IC*50 determinations and for measuring kinetic constants. Reagents are added to the blank and sample cuvettes as follows:

Blank: 0.8 ml PO4 buffer/DTNB
0.8 ml buffer/Substrate
Control: 0.8 ml PO4 buffer/DTNB/Enzyme
0.8 ml PO4 buffer/Substrate
Drug: 0.8 ml PO4 buffer/DTNB/Drug/Enzyme
0.8 ml PO4 buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program (Beckman DU-50 series spectrophotometer, kinetics Soft-PacTM module operation instructions: 1–7 also calculates the rate of absorbance change for each cuvette.

Evaluation: For *IC*50 determinations: Substrate concentration is 10 mM diluted 1 : 2 in an assay yielding a final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration.

% Inhibition = (slope control-slope drug/slope control)×100

*IC*50 values are calculated from log probit analysis.

Ex vivo cholinesterase inhibition

***Principle*[4]:** This assay is used to determine the dose-response relationship and duration of action of cholinesterase inhibitors *in vivo*. Cholinesterase inhibitors, including physostigmine and tacrine have been shown to improve cognitive functions in Alzheimer's disease. Physostigmine is a potent, but nonselective inhibitor of cholinesterase and has a short duration of action.

Tacrine also inhibits both acetylcholine- esterase (true) and butyrylcholineesterase (pseudo), but is more potent as an inhibitor of the pseudo-enzyme. Physostigmine is a competitive inhibitor and blocks the active site of the enzyme by carbamylation of a serine hydroxyl group at the esteratic site of the enzyme. This covalently bound carbamyl group then dissociates from the enzyme much more slowly than the acetyl group left by the natural substrate, but the inhibition is not irreversible like that of the organophosphates. The inhibition characteristics of physostigmine, i.e., sub micromolar affinity for the enzyme and covalent binding of the inhibiting group, are ideal for *ex vivo* studies. Tacrine, however, is a mixed competitive inhibitor of cholinesterase, with lower apparent affinity than physostigmine for the enzyme. Tacrine binds to the anionic site of cholinesterase through weak hydrophobic interactions.

Procedure

Drug treatment: Groups of four male Wistar rats are dosed i.p. or p.o. with vehicle or the test drug. For the initial dose response study, the rats are given varying doses of drug based on toxicity reported in primary overt effects testing and sacrificed at either 30 min or 1 h after dosing. The animals are observed and the occurrence of cholinergic signs is noted (piloerection, tremors, convulsions, salivation, diarrhea and chromodacryorrhea).

***Tissue preparation*:** Male Wistar rats are decapitated, brains rapidly removed, corpora striata dissected free, weighed and homogenized in 4 volumes of 0.05 M phosphate buffer, pH 7.2 using a Potter-Elvehjem homogenizer. A 12.5 ml aliquot of the homogenate is added to 1 ml 0.05 M phosphate buffer, pH 7.2/DTNB (reagent 2).

Assay

1. Enzyme activity is measured with the Beckman DU- 50 spectrophotometer. Reagents are added to the blank and sample cuvettes as follows:

Blank: 0.8 ml PO4 buffer/DTNB (reagent 2)
0.8 ml PO4 buffer/Substrate (reagent 3)
Control: 0.8 ml PO4 buffer/DTNB/Enzyme from control animal
0.8 ml PO4 buffer/Substrate
Drug: 0.8 ml PO4 buffer/DTNB/Enzyme from treated animal
0.8 ml PO4 buffer/Substrate

Blank values are determined for each run to control for non-enzymatic hydrolysis of substrate and these values are automatically subtracted by the kindata program available on kinetics soft-pac module. This program also calculates the rate of absorbance change for each cuvette.

2. Substrate concentration is 10 mM diluted 1: 2 in the assay yielding final concentration of 5 mM. DTNB concentration is 0.5 mM yielding 0.25 mM final concentration.

Evaluation

The percent inhibition at each dose or time is calculated by comparison with the enzyme activity of the vehicle control group.

%inhibition = (slope control-slope drug/slope control) × 100

Molecular forms of acetylcholinesterase from rat frontal cortex and striatum

***Principle*[4]:** Different molecular forms of acetylcholinesterase can be isolated from animal tissues after solubilization in buffers containing various detergent and salt concentrations. After solubilization, the molecular forms may be separated according to sedimentation properties by density gradient centrifugation, molecular weight by gel filtration or by electrophoretic mobility. Studies showing selective increases in the 10S form during development and selective loss in Alzheimer's disease suggest that this form of the enzyme may be developmentally and functionally more important. The purpose of this procedure is to determine the effects of various cholinesterase inhibitors on the two major molecular forms of acetylcholinesterase isolated from rat striatum and cerebral cortex.

Procedure: The procedure is divided into three main parts

Preparation and isolation of molecular forms of AChE: Male Wistar rats (200–250 g) are sacrificed, their brains rapidly removed and frontal cortices or corpora striata removed. The brain areas are weighed and homogenized in 5 volumes (wt/vol) of 10 mM phosphate buffer, pH 7.1, containing 1 M NaCl and 1% Triton X-100, except were indicated. The homogenates are centrifuged at 20 000 *g* for 20 min at 4 °C. The supernatant is aspirated and marker enzymes for 16S (*E. coli* β-galactosidase), 11.3S (bovine catalase) and 4.8S (horse liver alcohol dehydrogenase) fractions are added. The supernatant is then centrifuged at 37 000 rpm (140 000 *g* max) for 17.5 h in a Beckman L5-65 ultracentrifuge with a SW-60 rotor. 15-drop fractions are collected for each centrifuge tube and assayed for protein, β-galactosidase, catalase, alcohol dehydrogenase and acetylcholinesterase activity. In addition, butyrylcholinesterase can be measured. A 400 µl sample of the 20 000 *g*-supernatant is carefully layered on top of a 5–20% continuous sucrose gradient. This gradient is made up in a centrifuge tube from 1.65 ml of 20% sucrose and 1.65 ml of 5% sucrose in homogenizing buffer by means of a gradient maker. A 50% sucrose cushion (0.5 ml) is placed at the bottom of the tube. Fractions are collected from the bottom of the tube, i.e, the densest fractions are collected first. Each fraction is 15 drops or approximately 24 fractions are collected per centrifuge tube.

Assays for marker enzymes

A) Equine liver alcohol dehydrogenase (ADH), sedimentation coefficient 4.8 S.

1. Enzyme: alcohol dehydrogenase from equine liver, crystallized and lyophilized.
2. Requirements:

a) β-nicotinamide adenine dinucleotide (NAD)
b) 0.1 M glycine-NaOH buffer, pH 9.6
c) Absolute ethanol
d) buffer-substrate-NAD mixture: 875 µl NAD + 875 µl ethanol + 18.75 ml 0.1 M glycine-NaOH buffer, pH 9.6

3. Assay: 10 µl enzyme fraction and 850 µl mixture (reagent 2d) are incubated for 5 min at room temperature. The reaction is stopped by adding: 300 µl 1.5 M ZnSO4. Absorbance is read at 340 nm and enzyme units are determined from a standard curve using values of 1.25, 2.5, 5, 10 and 20 mU of ADH.

B) Bovine liver catalase, sedimentation coefficient 11.3 S.

1. Enzyme: catalase from bovine liver, purified powder (Sigma Chem. Co., C-10)
2. Requirements:

a) 30% hydrogen peroxide
b) 0.05 M sodium phosphate buffer, pH 7.0
c) Mixture: 111 µl 30% hydrogen peroxide + 100 ml buffer, yielding 0.033% peroxide.

3. Assay: 10 µl enzyme fraction 2990 µl peroxide-buffer mixture (reagent 2c)

Wavelength is set to 240 nm, absorbance is adjusted to 0.480 units. The amount of time is recorded for absorbance to decrease from 0.450 to 0.400. This corresponds to 3.45 µmol of hydrogen peroxide in 3 ml solution. Total catalase activity in 3 ml is 3.45 µMol/min.

C) *E. coli* β-galactosidase, sedimentation coefficient 16.0 S

1. Enzyme: β-galactosidase from *E. coli*, grade VI, partially purified, lyophilized.
2. Requirements:

a) Substrate: 15 mg/ml O-nitrophenyl-β-D-galacto-pyranoside (Sigma Chem. Co.) in water
b) 0.6 M Na2CO3, pH 7.25
c) 1 M NaCO3

3. Assay: 10 µl enzyme fraction, 150 µl 0.6 M phosphate buffer, pH 7.25, and 50 µl O-Nitrophenyl-β-D-galactopyranoside (12 mM in assay) are incubated for 25 min at 30 °C. The reaction is stopped by adding: 500 µl 1 M Na2CO3, 1.75 ml water. Absorbance is read at 420 nm and enzyme

units are determined from a standard curve using values of 0.015, 0.030, 0.525, 0.125 and 0.250 U of β-galactosidase.

Enzyme inhibition studies

For the enzyme inhibition studies, 25 µl aliquots of the enzyme preparation are preincubated with varying concentrations of the inhibitor for 10 min at 25 °C and acetylcholinesterase activity is determined as previously described.

***Evaluation*:** Values for the *IC*50 are determined by log-probit analysis of the inhibition data using six to seven concentrations of the inhibitor and represent the means of 3 separate experiments.

Release of [$_3$H]ACh and other transmitters from rat brain slices

***Principle*[5-6]:** Electrically stimulated release of [$_3$H] ACh is used as a biochemical screen for agents which may possibly enhance or inhibit release of [$_3$H]ACh through a direct muscarinic interaction or other indirect interactions. Muscarinic autoreceptors have been shown to have a role in the regulation of ACh release in several areas of the CNS. Direct stimulation of muscarinic receptors with muscarinic agonists or indirect stimulation with acetylcholinesterase inhibitors decreases ACh release evoked by either increased potassium concentration or electrical stimulation. Muscarinic antagonists can either block their inhibition, or, under certain conditions, enhance ACh release. This technique measures only presynaptic effects of test compounds.

Procedure

Tissue preparation: Male Wistar rats (100–150 g) are decapitated, and cortical, striatal, or hippocampal tissue removed on ice and 0.4 mm slices are prepared with a McIlwain tissue slicer. The slices are made individually, placed in cold, oxygenated buffer (10–20 ml) and incubated at 35 °C for 30 min under oxygen. After this incubation, the buffer is decanted, leaving the slices behind. Then 2.5 ml of cold oxygenated buffer is added, and enough [$_3$H] choline to bring the final concentration to 100 nM. This is then incubated and shaken for 60 min at 35 °C under oxygen. After this step, the buffer is decanted and the "loaded" slices are immediately placed on nylon mesh in the stimulation chambers.

Assay: Control buffer is pumped through the chamber for 42 min at a flow rate of 0.7 ml/min, to establish a stable baseline. Under these conditions released [$_3$H] Ach is subject to hydrolysis by acetylcholinesterase. The perfusion buffer is changed to fresh KHBB containing 20 µM HC-3. The potent choline uptake inhibitor HC-3 is included to prevent the re-uptake of any [$_3$H] choline formed from the hydrolysis of released [$_3$H] ACh. This maintains the stoichiometry of the stimulated release. The evoked release has been shown to be mostly [$_3$H] ACh rather than [$_3$H] choline, whereas spontaneous release under control, drug-free conditions is mostly [$_3$H] choline.

Evaluation: After conversion of dpm, percent fractional release is calculated for each fraction, using a Lotus program. Percent fractional release is defined as the amount of radio labeled compound released divided by the amount present in the tissue. "Spontaneous Release" (SP) values are the average of the two fractions preceding and the first fraction in that range after the stimulation period. "Stimulated" (S) values are the summed differences between the percent fractional release during stimulation and the appropriate SP value. The effects of drugs can be reported as S2/S1 ratios. To normalize the data, drug effects can be estimated by first calculating S2/S1 values for control and drug treated slices and then expressing the S2/S1 value for the drug-treated slices as a percentage of the S2/S1 value for the control slices for each experiment. Each condition should be tested in slices from the same animal.

[$_3$H] Oxotremorine-m binding to muscarinic cholinergic receptors in rat forebrain

***Principle*[7]:** The muscarinic receptors are members of the super family of G-protein-coupled receptors. They are relatively abundant and mediate the diverse action of acetylcholine in the CNS, as well as throughout non-nervous tissues innervated by the parasympathetic nervous system. The purpose of this assay is to determine the binding affinity of potential cholinomimetic drugs for muscarinic receptors in brain, using an agonist ligand. Oxotremorine is a potent centrally and peripherally acting muscarinic cholinergic agonist, which has been shown to be active in isolated tissue preparations as well as *in vivo*. Both central and peripheral effects of oxotremorine are blocked by antimuscarinic drugs such as atropine. Structural modification of the oxotremorine molecular yields compounds which are full agonists, partial agonists and antagonists at muscarinic receptors. Oxotremorine-M (oxo-M), a quaternary nitrogen analog of oxotremorine, is a full agonist for the phosphatidyl-inositol response. Oxotremorine and oxo-M are full agonists for inhibition of adenylate cyclase of the muscarinic agonists, oxotremorine is the most potent inhibitor of [$_3$H] QNB binding, and however, the *IC*50 is still only in the micromolar range. The apparent low affinity of agonist competition for [$_3$H]-antagonist

binding sites is a common phenomenon and is due to the existence of multiple agonist affinity states of the receptor, as described by Birdsall et al. (1978). For this reason, it is desirable to use an agonist ligand to measure the binding affinities of potential agonists.

Procedure

Tissue preparation: Male Wistar rats are decapitated and their brains rapidly removed. The forebrains are weighed (400–500 mg each) and homogenized in 10 volumes of 0.05 M Tris buffer, pH 7.4 (reagent 2) using a Potter- Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 1 000 *g* for 10 min. The supernatant is then centrifuged at 50 000 *g* for 60 min. The supernatant from this centrifugation is discarded and the pellet resuspended in the original volume of 0.05 M Tris buffer, pH 7.4, on the Polytron to 100 mg/ml (wet weight). Specific binding is roughly 1% of total added and 50% of total bound.

Binding assay:

Tubes are vortexed and incubated at 30 °C for 45 min. Bound [3H]-oxotremorine-M is captured by filtration under reduced pressure. The incubation mixture is diluted with approximately 4 ml ice-cold 0.05 M Tris buffer, pH 7.4 (reagent 2), then exposed to vacuum, and tubes washed once more with approximately 5 ml of reagent 2. The filters (GFC in 0.5% polyethylene-imine for more than 3 h, reagent 5) are then counted in 10 ml Liquiscint scintillation fluid.

Evaluation: Specific binding is the difference between total bound (in presence of vehicle) and that bound in the presence of 40 μM atropine. Percent inhibition of specific [3H]-oxotremorine-M is calculated for each concentration of test drug and *IC*50 values determined by computer- derived log probit analysis. The percent inhibition at each drug concentration is the mean of duplicate or triplicate determinations. Some day-to day variability is present in this assay, and *IC*50 values should be confirmed by repeat analysis.

[H]-N-methylscopolamine binding in the presence and absence of GPP(NH)p

***Principle*[8]:** G-protein-linked muscarinic receptors are converted by guanine nucleotides from a high-affinity binding state to a low-affinity binding state for muscarinic agonists, while the binding of muscarinic antagonists to the receptor is not affected. The effects of guanine nucleotides on muscarinic agonist affinity are brain region- and temperature-dependent. Therefore, incubation of cerebellar membranes with 50 μM 5'-guanylylimidophosphate (Gpp(NH)p), the non-hydrolyzable analog of GTP, causes a shift to the right (decreased affinity) of the muscarinic agonist inhibition curves when 3H-NMS is used as the ligand. The assay differentiates the interaction of muscarinic agonists and muscarinic antagonists with 3H-N-methylscopolamine (3H-NMS)-labeled receptors in cerebellar tissue based on the selective effect of guanine nucleotides on the affinity of muscarinic agonists for the receptor.

Procedure: The procedure is based on 3H-NMS rat brain binding assay described by Aronstram and Narayanan (1988).

Tissue Preparation: Male Wistar rats are decapitated and their brains rapidly removed. The cerebella are dissected, weighed and homogenized in 10 volumes of 0.05 M Tris-HCl buffer, pH 7.4 + 2 mM MgCl2 + 100 μM phenylmethylsulfonyl fluoride (buffer 4), using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate is centrifuged at 20 000 *g* for 20 min. The pellet is resuspended in 10 volumes of 0.05 M Tris-HCl buffer + 2 mM $MgCl_2$ (buffer 3).

Binding Assay: Tubes are incubated at 20 °C for 90 min. Bound [3H] NMS is captured by vacuum filtration. The filters are washed three times with 5 ml aliquots of 0.05 M Tris buffer, pH 7.4. Filters are counted in 10 ml Liquiscint scintillation fluid.

Evaluation: Specific binding of [$_3$H] NMS is the difference between total bound (in the presence of vehicle) and that bound in the presence of 1 mM atropine. Percent inhibition of specific [$_3$H] NMS binding is calculated for each concentration of test drug and *IC*50 values are determined by computer-derived log-probit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

[$_3$H]N-methylcarbamylcholine binding to nicotinic cholinergic receptors in rat frontal cortex

***Principle*[9]:**

The purpose of this assay is to determine the binding affinity of potential nicotinic cholinergic agonists in brain, using an agonist ligand. Nicotinic cholinergic receptors are classified as ligand- gated ion channels, and are found in skeletal muscle, autonomic ganglia and brain tissue. Due to its rapid desensitization of the receptor, both stimulatory and depressant effects may result. Also, many of nicotine's effects are thought to be associated with release of neurotransmitter substances. Nicotine functions as a

nicotinic cholinergic receptor agonist in the CNS and is thought to play a role in learning and memory. Reductions in nicotinic binding sites were found in post-mortem tissues from Alzheimer's patients. Therefore nicotinic agonists may prove beneficial; however, clinical data are still quite limited. N-Methylcarbamylcholine (NMCC) is a nicotinic agonist which binds specifically and with high affinity to central nicotinic receptors and, like nicotine, causes an increase of acetylcholine release from certain cholinergic nerve terminals. This is due to the loss of presynaptic nicotinic autoreceptor function. Pharmacological results reveal that along with its specificity and high affinity, [$_3$H] NMCC is selectively displaced by agonists, making it a desirable ligand to screen for potential agonistic compounds.

Procedure

Tissue preparation: Male Wistar rats are decapitated, their frontal cortices removed, weighed and homogenized in 40 volumes of ice-cold 0.05 M Tris buffer, pH 7.7 (l b). The homogenate is centrifuged at 48 000 *g* for 10 min. The pellet is rehomogenized in fresh buffer and recentrifuged at 48 000 *g* for 10 min two more times. The final pellet is resuspended in the original volume of buffer, but with physiological salts (2 b). This yields a final tissue concentration of 20 mg/ml in the assay.

Assay:The tubes are incubated at 0 °C for 60 min. The assay is stopped by rapid filtration through Whatman GF/B filters which are then washed 4 times with 3 ml of ice-cold 0.05 M Tris buffer, pH 7.7. The filters are then counted in 10 ml of Liquiscint scintillation cocktail.

Evaluation: Specific binding is defined as the difference between total binding and binding in the presence of the 10 µM (–) nicotine ditartrate. Specific binding is about 1% of the total added ligand and 60–70% of the total bound ligand. *IC*50 calculations are performed using logprobit analysis. The percent inhibition at each drug concentration is the mean of triplicate determinations.

Cultured neurons/astroglial cells

***Principle*[4]:** Cultured brain cells can be used for many purposes such as investigation of synthesis and secretion of nerve growth factor or for testing neuroprotective drugs. Nerve growth factor is required for the development and maintenance of peripheral and sensory neurons. Nerve growth factor prevents neuronal death after brain injury, especially in basal forebrain nuclei involved in memory processes. Drug induced increase in nerve growth factor secretion may be beneficial in primary degenerative dementia.

Procedure: Whole brains of 8-day-old mice (ICR) are dissected out and cut into small pieces. The pieces are washed with calcium- and magnesium-free phosphate-buffered saline, treated with 0.25% trypsin at 37 °C for 30 min, and triturated with a Pasteur pipette. The excess trypsin is removed by centrifugation at 200 *g* for 5 min. The cells or cell clumps from one brain are cultured in a culture bottle with Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS), 50 milli units/ml penicillin, and 50 mg/ml of streptomycin at 37 °C in a humid atmosphere of 5% CO_2 for 1–2 weeks with medium changes every 3 days. After confluence is reached, the cells in each bottle are dissociated by trypsin treatment and recultured in new bottles. This procedure is repeated 3 times. The culture becomes composed of morphologically uniform cells. Preparation of quiescent cells is performed by inoculating into 96-well plates and culture in FCS-containing DMEM until confluence is reached. Then, the cells are cultured for an additional week in FCS-free DMEM containing 0.5% BSA, with medium changes every 3 days. Because the cells never proliferate in FCS free medium, most of the cells are arrested in the quiescent phase. Then, the medium is changed to DMEM containing 0.5% BSA with or without drugs, and the cells are cultured for 24 h. Nerve growth factor (NGF) content in the culture medium is determined by a two-site enzyme immunoassay. Mouse β NGF isolated from male mouse sub maxillary glands is purified by CM-Sephadex C-50 chromatography. Antiserum to this mouse β NGF is produced in New Zealand White rabbits by repeated subcutaneous injections of an emulsion in complete Freund's adjuvant over 18 months. Immunoglobin G is prepared from Anti-mouse β NGF antiserum by Sepharose chromatography. Antibody IgG is incubated with pepsin and chromatographed. Fab' fragments are coupled to β-D-galactosidase. IgG-coated solid phase is prepared in polystyrene tubes. The IgG-coated polystyrene tubes are incubated with 0.25 ml buffer containing various amounts of NGF with gentle shaking. After incubation for 18–24 h at 4 °C, each tube is washed twice with 1 ml of buffer and 0.13 milliunits of the Fab'-β-D-galactosidase complex in 0.25 ml buffer is added. After incubation for 18–24 h at 4 °C with gentle shaking each tube is washed as described above and β-D-galactosidase activity bound to the tube is assayed. The enzyme reaction is started by addition of 60 mM 4-methylumbelliferyl-β-D-galactoside and 0.1% Triton

X-100 in 0.25 ml buffer. After 1-h incubation at room temperature, the enzyme reaction is stopped by the addition of 1.25 ml 0.1 M glycine-NaOH buffer (pH 10.3). The amounts of 4-methylumbelliferone formed are measured by fluorometry (Excitation wavelength 360 nm, emission wavelength 450 nm).

Evaluation: Time-response curves of release of NGF into the medium are established after addition of drug and compared with controls. Dose-response curves can be prepared after addition of various amounts of test drug.

INVIVO METHODS

Inhibitory (passive) avoidance[10]

One of the most common animal tests in memory research is the inhibition to imitate activities or learned habits. The term "passive avoidance" is usually employed to describe experiments in which the animal learns to avoid a noxious event by suppressing a particular behavior. Netto and Izquierdo (1985) have discussed this term in a brief paper.

1. Step-down

***Principle[4]*:** An animal (mouse or rat) in an open field spends most of the time close to the walls and in the corners. When placed on an elevated platform in the center of a rectangular compartment, it steps down almost immediately to the floor to explore the enclosure and to approach the wall.

Procedure: Mice or rats of either sex are used. A rectangular box (50 x 50 cm) with electrifiable grid floor and 35 cm fits over the block. The grid floor is connected to a shock device which delivers scrambled foot shocks. The actual experiments can be performed in different ways. A typical paradigm consists of three phases: (1.) Familiarization: The animal is placed on the platform, released after raising the cylinder, and the latency to descend is measured. After 10 s of exploration, it is returned to the home cage. (2.) Learning: Immediately after the animal has descended from the platform an unavoidable footshock is applied (Footshock: 50 Hz; 1.5 mA; 1 s) and the animal is returned to the home cage, (3.) Retention Test: 24 h after the learning trial the animal is again placed on the platform and the step-down latency is measured. The test is finished when the animal steps down or remains on the platform (cut-off time: 60 s).

Evaluation: The time of descent during the learning phase and the time during the retention test is measured. A prolongation of the step-down latency is defined as learning. The variability of this method is relative high; therefore, it is necessary to test large groups of animals (minimum 10 animals per group).

2. Step-through[4]

Principle: This test uses normal behavior of mice and rats. These animals avoid bright light and prefer dim illumination. When placed into a brightly illuminated space connected to a dark enclosure, they rapidly enter the dark compartment and remain there. The standard technique was developed for mice by Jarvik and Kopp (1967) and modified for rats by King and Glasser (1970). It is widely used in testing the effects of memory active compounds.

Procedure: Mice and rats of either sex are used. The test apparatus consists of a small chamber connected to a larger dark chamber via a guillotine door. The small chamber is illuminated with a 7 W/12 V bulb. The test animals are given an acquisition trial followed by a retention trial 24 h later. In the acquisition trial the animal is placed in the illuminated compartment at a maximal distance from the guillotine door, and the latency to enter the dark compartment is measured. Animals that do not step through the door within a cut-off time: 90 s (mice) or 180 s (rats) are not used. Immediately after the animal enters the dark compartment, the door is shut automatically and an unavoidable footshock (Footshock: 1 mA; 1 s – mice; 1.5 mA; 2 s – rat) is delivered. The animal is then quickly removed (within 10 s) from the apparatus and put back into its home cage. The test procedure is repeated with or without drug. The cut-off time on day 2 is 300 s (mice) or 600 s (rats), respectively.

Evaluation: The time to step-through during the learning phase is measured and the time during the retention test is measured. In this test a prolongation of the step-through latencies is specific to the experimental situation. An increase of the step-through latency is defined as learning.

3. Two compartment test

Principle:A rodent in an open field tends to enter any recesses in the walls and to hide there. When placed into a large box, connected through a narrow opening with a small dark compartment, the animal rapidly finds the entrance into the small chamber, enters it and spends most of its time there. The times spent in the large and small compartments are measured. The latency of the first entrance into the dark chamber and the number of crossings from one compartment into the other can be used as auxiliary criteria. The

technique described was developed by Kurtz and Pearl (1960) and modified by Bures and Buresova (1963).

Procedure: Mice and rats of both sex and a rectangular box with a 50 x 50 cm grid floor and 35 cm high walls are used. In the centre of one wall is a 6 x 6 cm opening connecting the large compartment to a small 15 x 15 cm box with dark walls, electrifiable grid floor and removable ceiling. The connection between the two compartments can be closed with a transparent sliding door. Illumination is provided with a 100 W bulb placed 150 cm above the centre of the large compartment.

Evaluation: The times the animal spends in the large and the small compartment are measured.

4. Up-hill avoidance[4]

Principle: Many animal species exhibit a negative geotaxis, i.e. the tendency to orient and move towards the top when placed on a slanted surface. When placed on a tilted platform with head facing down-hill, rats and mice invariably turn around and move rapidly up the incline.

Procedure: Rats of both sex were used and maintained under standard conditions. The experimental apparatus is a 50 x 50 cm box with 35 cm high opaque plastic walls. The box can be inclined at different angles. The floor consists of 10 mm diameter stainless steel grid bars placed 13 mm apart. To deliver the tail-shock, a tail electrode is constructed, consisting of a wire clip connected to a constant current shock source. The animal is first fitted with the tail-electrode and then placed onto the grid with its nose facing down. During baseline trials the animal's latency to make a 180° turn and initiate the first climbing response is measured. Thereafter the animal is returned to its home cage. During the experimental trials the latencies are measured and additionally a tail-shock (1.5 or 2 mA) was administered contingent on the first climbing response after the 180° turn. Immediately after the shock the animal is placed in its home cage. Retest is performed 24 h later.

Evaluation: The latencies are measured.

5. Trial-to-criteria inhibitory avoidance[4]

Principle: As animals experience different sensitivity to the footshock punishment applied in the dark area, immediately after the first trial the animal is returned to the lighted area to evaluate if the task has been acquired. A criteria is established to determine the learning of the test, usually requiring the animal to remain in the lighted area for a period of 30–60 s. In this way, all the animals have a similar degree of learning independently of the amount of trials needed to attain it.

Procedure: Mice or rats are generally used. The animals are trained in the same way as in the step-through version. They are placed in the lighted compartment and after they entered with the four paws into the dark area, the door is closed and a mild footshock is delivered. Immediately after the shock they are placed back in the lighted area for another trial. Training would continue this way until the animal remains in the lighted area for a certain period of time (30 or 60 s), a time at which the training is considered to be acquired by all the animals. The numbers of trials to attain criteria are counted as an indication of the speed of acquisition.

Evaluation: Retention of the test is measured 24 or 48 Hs later. The animals are placed in the lighted area, the door opened and the latency to step with the four paws into the dark area is recorded. A cut-off latency of 180 or 300 s is usually imposed.

6. Scopolamine induced amnesia in mice[10]

***Principle*:** The administration of the antimuscarinic agent scopolamine to young human volunteers produces transient memory deficits. Analogously, scopolamine has been shown to impair memory retention when given to mice shortly before training in a dark avoidance task. The ability of a range of different cholinergic agonist drugs to reverse the amnesic effects of scopolamine is now well documented in animals and human volunteers. However, the neuropathology of dementia of the Alzheimer type is not confined to the cholinergic system.

Procedure: The scopolamine test is performed in groups of 10 male NMRI mice weighing 26–32 g in a one-trial, passive avoidance paradigm. Five min after i.p. administration of 3 mg/kg scopolamine hydrobromide, each mouse is individually placed in the bright part of a two-chambered apparatus for training. After a brief orientation period, the mouse enters the second, darker chamber. Once inside the second chamber, the door is closed which prevents the mouse from escaping, and a 1 mA, 1-s foot shock is applied through the grid floor. The mouse is then returned to the home cage. Twenty four hours later, testing is performed by placing the animal again in the bright chamber. The latency in entering the second darker chamber within a 5 min test session is measured electronically. Whereas untreated control animals enter the darker chamber in the second trial

with a latency of about 250 s, treatment with scopolamine reduces the latency to 50s. The test compounds are administered 90 min before training. A prolonged latency indicates that the animal remembers that it has been punished and, therefore, does avoid the darker chamber.

Evaluation: Using various doses latencies after treatment with test compounds are expressed as percentage of latencies in mice treated with scopolamine only. In some cases, straight doses-response curves can be established whereas with other drugs inverse U-shaped dose-responses are observed.

7. Memory impairment by basal forebrain lesions in rats

***Principle*:** Memory impairment can be produced by lesions caused by bilateral injections of ibotenic acid into the basal forebrain of rats. Water maze tasks, habituation tasks, passive avoidance tasks with a light/dark compartment apparatus, and the inhibition of the decrease of choline acetyltransferase activity in the cortex can be used to evaluate the effect of drugs.

Procedure: Male Wistar rats weighing 270–310 g are anesthetized with sodium pentobarbital (45 mg/kg i.p.) and placed in a stereotaxic apparatus. Neurotoxic lesions of the basal forebrain are produced by injection of ibotenic acid. An injection needle connected to a 5-µl microsyringe is inserted into the basal forebrain. Ibotenic acid is dissolved in 50 mM Na phosphate buffer at a concentration of 12 µg/ml, and then 0.5 ml (6 µg per side) is infused for 5 min. The injection needle is left in place for an additional 5 min to allow the toxin to diffuse away from the needle tip. One week later, the contralateral side is treated in the same manner. The same procedure is used to administer microinjections of 50 mM Na phosphate buffer into the basal forebrain of sham-operated rats. Three to 5 weeks after the first lesion, the animals are tested on the acquisition of a task in a Morris water maze (Morris 1981), on a habituation task in a novel situation, and in a passive avoidance task with light and dark compartments. The rats are treated once a day during the experiment. After the behavioral experiments, the animals are sacrificed for determination of choline acetyltransferase activity in the brain. The tissue is homogenized (4% w/v) in cold 50 mM Na phosphate buffer (pH 7.4), and Triton X-100 (0.55, v/v) is added to homogenates to ensure enzyme release. To 75 µl of enzyme solution, 125 µl of substrate mixture (0.4 mM [14C]acetyl- Co A (50.6 mCi/mmol), 300 mM NaCl, 50 mM Na phosphate buffer (pH 7.4), 8 mM choline chloride, 20 mM EDTA-2Na, and 0.1 mM physostigmine) is added in a scintillation vial and the mixture is incubated at 37 °C for 30 min. After the incubation, 0.8 ml of cold 50 mM phosphate buffer, 0.5 ml of acetonitrile containing 2.5 mg of tetraphenylborate and 2.0 ml toluene are added to the scintillation vial. The vials are shaken lightly and allowed to stand overnight before radioactivity is determined.

Evaluation: Data are evaluated by usual statistical means. All analyses are followed by a Bonferroni's test.

***8. Ischemia induced amnesia in gerbils*[4]**

***Principle*:** Impairment of cerebral metabolism induced by reduced blood supply is known to induce cognitive deficits. Because of the absence of posterior communicating arteries in the brain of Mongolian gerbils, complete forebrain ischemia can be produced by occluding both common carotid arteries resulting in amnesia.

Procedure: Male Mongolian gerbils weighing 50–70 g are anesthetized by i.p. pentobarbital injection. Both common carotid arteries are exposed through a ventral neck incision and occluded for 5 or 10 min with miniature aneurysm clips. In sham-operated controls, the common carotid arteries are exposed but not occluded. Twenty-four hours after occlusion, each animal is placed in the bright part of a light/dark-chambered apparatus for training. After a brief orientation period, the gerbil enters the second, dark chamber. Once inside the second chamber, the door is closed which prevents the animal from escaping, and a 100 V, 2sec foot shock is applied through the grid floor. The gerbil is then returned to the home cage. Twenty-four hours later, testing is performed by placing the animal again in the bright chamber. The latency in entering the second dark chamber within a 5 min test session is measured electronically. The latency compared with sham-operated controls is decreased depending on the duration of ischemia. After drug treatment, an increase of latency in entering the dark compartment indicates good acquisition.

Evaluation: Using various doses a dose-dependent increase of latency can be found after active drugs, sometimes resulting in inverse U-shaped dose-response curves.

ACTIVE AVOIDANCE

Active avoidance learning is a fundamental behavioral phenomenon. As in other instrumental conditioning paradigms the animal learns to control

the administration of the unconditioned stimulus by appropriate reactions to the conditioned stimulus preceding the noxious stimulus. The first stage of avoidance learning is usually escaped, whereby a reaction terminates the unconditioned stimulus.

1. Runway avoidance[26]

***Principle*:** A straightforward avoidance situation features a fixed aversive gradient which can be traversed by the animal. The shock can be avoided when the safe area is reached within the time allocated.

Procedure: Mice or rats of either sex are used and maintained under standard conditions and handled for several days before the experiment. The same box as used in the step-through model can be used in this experiment. The apparatus is uniformly illuminated by an overhead light source. A loudspeaker, mounted 50 cm above the start-box, serves for presenting the acoustic conditioned stimulus. The footshock is employed by the same source as in the step-through avoidance. The animal is allowed to explore the whole apparatus for 5 min. The guillotine door is then closed and the animal is placed into the light starting area. After 10 s the acoustic CS is applied and the door is simultaneously opened. Shock is turned on after 5 s. The CS continuous until the animal reaches the safe area. It is left there for 50–70 s before returned to the same area again. The procedure starts again. The training is continued until the animal attains the criterion of 9 avoidances in 10 consecutive trials. On the next day the procedure is repeated until the same learning criterion is reached. The time needed to reach the safe area is measured.
Evaluation: The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) is recorded.

2. Shuttle box avoidance (two- way shuttle box)[10]

***Principle*:** Compared to runway avoidance, shuttle box avoidance (two-way-shuttle-box) is a more difficult task. Since the animal is not handled between trials, the shuttlebox can be easily automated.
Procedure: Rats of both sexes are used and maintained under standard conditions. The apparatus used consists of a rectangular box 50 × 15 cm with 40 cm high metal walls, and an electrifiable grid floor. The box is divided by a wall with a manually or solenoid-operated guillotine door (10 × 10 cm) into two 25 × 15 cm compartments. Each compartment can be illuminated by a 20 W bulb mounted in the hinged Plexiglas lids. A fixed resistance shock source with an automatic switch (0.5 s on 1.5 s off) is used. Simple programming equipment provides for automatic delivery of the conditioned stimulus (CS) and the unconditioned stimulus (US). The apparatus is placed in a dimly light room with a masking noise background (white noise) of 60 dB. The animal is allowed to explore the apparatus for 5 min with the connecting door open and the compartment lights switched off. The guillotine door is then closed. After 20 s the light is switched on in the compartment containing the animal, and the door is opened. A tone (CS) is presented and 5 s later the floor shock is applied in the illuminated compartment and continued until the animal escapes to the dark side of the compartment, the connecting door is closed and the shock discontinued. After a variable inter trial interval (30–90 s) the light is switched on in the previous dark compartment, the door is opened and the animal is required to cross to the other side. The training is continued until the animal reaches the criterion of 9 avoidances in 10 consecutive trials. Retention is tested at different intervals after the original training by retraining the animal to the same criterion again.
Evaluation: The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors (not reaching the safe area) is recorded.

3. Jumping avoidance (one-way shuttle box)

Principle: Since a high degree of automation and minimum handling are additional requirements for this model, the obvious solution is a simplified one-way avoidance, allowing for the spontaneous or forced return of the animal to the start. In order to enhance the start-goal distinction a vertical gradient is introduced which requires the animal to perform a discrete response of an all-or-none character, such as the jump, which clearly differs from the more continuous translational movements required in the usual avoidance tasks.

Procedure:Rats of both sex are used and maintained under standard conditions. The apparatus used consists of a rectangular box 40 × 25 cm with 40 cm high metal walls, an electrifiable grid floor and a Plexiglas ceiling. A 12 × 12 × 25 cm opaque plastic pedestrial, mounted onto one of the narrow walls of the box provides the isolated goal area. Flush with the horizontal surface of the pedestal moves a vertical barrier, which can either be retracted to the rear wall of the apparatus to expose the goal area or pushed forward to block access to the goal completely. The animal is placed into the apparatus for 5 min with the

goal area exposed (barrier re-tracted). The barrier is then moved forwards and the goal is blocked for 2 s. The first trial starts by exposing the goal area and applying an acoustic CS (1 000 Hz, 85 dB). Electric shocks – US (1.0 mA; 50 Hz; 0.5 s) – are applied 5 s later (once per 2 s), and continued together with the CS until the animal jumps onto the platform. After 30 s the barrier pushes the animal off the platform onto the grid floor. The sequence is repeated until the criterion of 10 consecutive avoidances is reached. Retention is tested on the next day until the animal reaches criterion.

Evaluation: The time the animal needs to reach the safe area on both days is measured. In addition, the number of errors is recorded.

Discrimination learning [4, 10]

In the experiments described above the animals have no choice between the conditioned stimuli. They have only one conditioned stimulus. The following examples illustrate the special techniques employed for discrimination among different stimulus modalities. The experiments can be classified either as simultaneous or successive discrimination paradigms.

1. Spatial habituation learning

Principle: The open-field test utilizes the natural tendency of rodents to explore novel environments in order to open up new nutrition, reproduction and lodging resources. The rate of exploratory behaviors exhibited in an unfamiliar environment is limited through the inherent necessity to avoid potential dangers. The observed behavior therefore is always a compromise between these conflicting interests and is regulated in part by the momentary physiological needs. Spatial habituation learning is defined as a decrement in reactivity to a novel environment after repeated exposure to that now familiar environment. This reduction in exploratory behaviors during re-exposures is interpreted in terms of remembering or recognition of the specific physical characteristics of the environment. The test can be used to examine short-term spatial memory and/or long-term spatial memory.

Procedure: The open-field apparatus is a rectangular chamber (rats: 60 × 60 × 40 cm, mice: 26 × 26 × 40) made of painted wood or grey PVC. A 25 W red or green light bulb is placed either directly above or beneath the maze to achieve an illumination density at the centre of approximately 0.3 lx. Masking noise is provided by a broad spectrum noise generator (60 dB). Prior to each trial, the apparatus is swept out with water containing 0.1% acetic acid. Housing room and the testing location are separated and animals are transported to the testing room 30 min before testing. The digitized image of the path taken by each animal is stored and analyzed post hoc with a semi-automated analysis system. In aged or hypoactive rodents testing is performed during the animal's dark phase of day. The rodent is placed on the center or in a corner of the open-field for 5–10 minute sessions. The animals are re-exposed to the open-field 24 and 96 h after the initial trial.

Evaluation: The exploratory behaviors' registered are: (1) Rearing or vertical activity: the number of times an animal was standing on its hind legs with forelegs in the air or against the wall. (2) The duration of single rearing as a measure of non-selective attention Locomotion or horizontal activity: the distance in centimeters an animal moved.

2. Spatial discrimination

Principle: In the simplest case of discrimination learning the animal distinguishes between two symmetric stimulus response sets, the equal probability of which has been changes by differential reinforcement events. Position of the cues with respect to the animal's body defines the CS+ and CS– . Usually left-right discrimination is employed, while axial orientation of the body is ensured by the construction of the apparatus.

Procedure: Rats and mice of both sexes are used and maintained under standard conditions. The apparatus used is usually a simple T- or Y-maze, with an electrifiable grid floor. The last 10 cm of each arm are separated from the rest of the apparatus by a swing-door which prevents the animal from seeing the food cup or the plastic sheet covering the grid in the goal area. A fixed resistance shock source is connected to an automatically operated switch. In an aversively motivated spatial discrimination learning the animal is trained to escape and/or to avoid foot shocks by always going to the right. Training starts by allowing the animal to explore the apparatus. Then the animal is placed on the start and after 5 s electric shocks (0.5 s, 50 Hz, 1.0 mA) are applied at 3 s intervals. The animals are trained to a criterion. On the following day the animal is retrained to the same criterion. After a 60 min interval the safe goal area is shifted to the other arm of the maze and the discrimination is reversed.

Evaluation: Errors are scored. An error means that the animal enters the wrong arm with all four legs.

During retention the number of trials until the animal makes correct choices is counted.

3. Spatial learning in the radial arm maze

Principle: Olton and co-workers have developed a spatial discrimination task for rodents that has been extensively used in learning and memory studies, and that has served as the basic task for one of the most important theories on the role of the hippocampus. The rat uses spatial information provided by the distal cues in the room to efficiently locate the baited arms. The radial arm-maze allows the study of spatial reference and working memory processes in the rat. In reference memory procedures, information is useful for many sessions/days and may usually be needed during the entire experiment. On the contrary, working memory procedures have a major temporal component as the information presented in the maze is useful for one session but not for subsequent ones; the rat has to remember the information during a delay interval. Correct choices in the radial arm-maze are rewarded by food.

Procedure: The apparatus is a wooden elevated eight-arm radial maze with the arms extending from a central platform 26 cm in diameter. Each arm is 56 cm long and 5 cm wide with 2 cm high rails along the length of the arm. The maze is well illuminated and numerous cues are present. Food pellets (reward) are placed at the end of the arms. During the test, rats are fed once a day and their body weights maintained at 85% of their free feeding weight to motivate the rat to run the maze. Animals are trained on a daily basis in the maze to collect the food pellets. The session is terminated after 8 choices and the rat has to obtain the maximum number of rewards with a minimum number of errors.

Evaluation: The number of errors is counted during the session.

4. Visual discrimination

Principle: Vision is better than any other sensory system for the analysis of spatial relationships in the environment of the animal. From the retina to the cerebral cortex, the organization of the visual system ensures processing of visual information according to simple principles i.e. by fitting the distribution of light over the receptive surface to elementary geometrical concepts and by comparing these patterns with images stored in the memory.

Procedure: Rats and mice of both sexes are used and maintained under standard conditions. The apparatus consists of a square 10x10 cm start area separated by a Plexiglas sliding door from the choice area, which is connected by swing doors to the goal compartment. The grid floor in the starting and the choice areas is electrifiable. The stimulus can be attached to the swing doors. The patterns are black on a white background and have different forms. The apparatus is illuminated by a dim light. The animal is placed into the apparatus with all doors open and allowed to explore it. Then it is placed in the start and after 5 s released by raising the Plexiglas door. After another 5 s, electric shocks (1 mA, 50 Hz, 0.5 s, 1/3 s) are applied until the animal escapes through either of the open doors to the safe goal compartment where it is left for some seconds. As soon as this preliminary step is mastered, the stimulus cards are inserted, the negative door is locked and the grid section in front of this door is electrified. The animal is trained to a criterion. On the next day the animal is retrained to the same criterion and retention is expressed in savings. Another parameter which can be used to evaluate the savings is the cumulative number of errors until the criterion is reached.

Evaluation: The number of correct answers as well as the number of trials until the criterion is reached is counted.

5. Spatial learning in the water maze[11]

***Principle*:** A task was developed where rats learn to swim in a water tank to find an escape platform hidden under the water. As there are no proximal cues to mark the position of the platform, the ability to locate it efficiently will depend on the use of a configuration of the cues outside the tank. Learning is reflected on the shorter latencies to escape and the decrease on the length of the path to find the platform. Although rodents can find the platform by using non-spatial strategies, the use of a spatial strategy is the most efficient way to escape and young animals develop the spatial strategy after a small number of trials.

***Procedure*:** Different strains of rats are generally used. The apparatus is a circular water tank filled to a depth of 20 cm with 25 °C water. Four points equally distributed along the perimeter of the tank serve as starting locations. The tank is divided in four equal quadrants and a small platform (19 cm height) is located in the centre of one of the quadrants. The platform remains in the same position during the training days. The rat is released into the water and allowed 60–90 s. to find the platform. Animals usually receive 2–4 trials per day for 4–5 days until

they escape onto the platform. Well trained rats escape in less than 10 s.

Evaluation: The latency to reach the escape platform is measured during the training days. A free-swim trial is generally performed after the training days where the escape platform is removed and the animal is allowed to swim for 30 s. With the help of a video system, the latency to reach the previous position of the platform, the number of annulus crossings as well as the time the rat spent in the training quadrant is measured. Well-trained rats show short latencies, a large number of annulus crossings and bias to the quadrant where the escape platform was located during the training sessions.

6. Olfactory learning

Principle: Odors provide rodents with important information on the environment and the learning of successive olfactory discrimination problems in rats is closely related to the acquisition rules of higher primates. Odor-reward associations are learned in few trials as odors exert more discriminative control over other sensory modalities like tones or lights. Animals have to learn to discriminate an arbitrary designated positive odor (i.e., banana) from a negative one (i.e., orange) to receive a reward.

Procedure: Rats are generally used. Animals are deprived of water for 48 h before the training and during the test they receive ad libitum water for only 30 min. The olfactory apparatus is a rectangular box (30 x 30 x 55 cm) with a photosensitive cell mounted on top of the water spout/odor outlet. Rats are trained to approach the water spout and to break the light beam. Responses to the positive odor are rewarded with water while responses to the negative odor results in the presentation of a light flash. The inter trial interval before the presentation of a new odor is usually 15 s. and the sessions last 30 min per day. Sessions are terminated when the rat makes 90% correct choices or after 400 trials.

Evaluation: The animal is rewarded with 0.05 ml of water when it breakes the beam to the positive odor or when it does not respond to the negative odor. Results are reported as the % correct responses or as a logit transformation of the % correct/incorrect response ratio.

7. Hole board discrimination learning[12]

***Principle*:** Here, we aimed at developing a higher throughput mouse spatial learning task using a holeboard chamber, an apparatus that has primarily been used to look at exploratory behaviors, not complex spatial learning. Holeboard-learning paradigms for rats have been described. A few studies also showed that mice could learn to retrieve a food pellet located in one hole out of four in a holeboard task setup. However, the simplicity of the task used in these mouse studies precluded a direct assessment of reference and working memory performances. Thus, as a starting point, we chose to adapt to mice a protocol similar to that described by vander Stay et al. (1990). The holeboard apparatus consists of an open-field chamber with a 16-hole floor insert. Across trials, animals have to learn that the same four holes of 16 are always baited. After habituation to the apparatus, mice undergo four to six trials day across multiple days. Moreover, daily pretreatment with 0.1 and 1 mg/kg of scopolamine induces significant learning impairment compared with vehicle or 0.01 mg/kg. Thus, the present results suggest that this learning task could be used as an alternative approach to assess spatial discrimination performance in mice and may be useful for future pharmacological or behavioral phenotyping studies.

Apparatus: The holeboard discrimination task was conducted in test chambers corresponding to the rat open-field activity system provided by MED Associates Inc. (St. Albans, VT) and housed in sound-attenuated cubicles. The large test chambers (44.5x44.5 x30.5 cm) were originally developed for rats and were modified by the manufacturer with our input to allow for monitoring of mice. IR beam intensity was increased to reduce the spread of the beam, which increased activity monitoring precision. Two arrays of IR beams were used to track activity on the floor and nosepokes into the holes. The beams were adjusted so that the mice had to dip their head 8–9 mm to break an IR beam. A nosepoke was recorded when the IR beams were broken for three consecutive 50-ms samplings. The holeboard chambers contained a 16-hole holeboard floor insert. This three-piece assembly was placed in the bottom of the holeboard chamber. A specially made stainless steel floor with 0.95-cm legs was then placed on top of the three-piece assembly in the holeboard chamber. The stainless steel floor has four rows of four holes that are equidistant apart. The holes are 1.5 cm in diameter and spaced 6 cm apart. To preclude animals to use olfactory clues, all 16 holes were filled with 45-mg food pellets that were inaccessible and that were changed daily.

Procedure: In this test, an animal's ability to remember which four of 16 equidistant holes are baited with food was measured. On the first day, food-restricted mice received a single 15-min habituation session to acclimate to the apparatus.

During this session, mice had to collect all the food pellets that were placed in every hole. The MED Associates ACTIVITY MONITOR software (version 4.0) automatically ended the session after all the pellets had been collected or after 15 min had elapsed, whichever came first. The training period began the following day; depending on the experiment, mice were trained for at least 4 consecutive days. Each daily session consisted of four or six 3-min trials with an intertrial interval of 3 min. The start position of a mouse was randomly changed across trials. The same four holes were baited with a 20-mg food pellet during each trial. The software automatically ended a trial when all four pellets had been collected or after 3 min had elapsed. After each trial, mice were returned to the home cage, and the holeboard floor insert was cleaned with alcohol to homogenize potential olfactory traces.

Evaluation: The average number of errors per trial an animal made each day was used as a measure of cognitive performance. Errors consisted of entering a hole that was never baited, re-entering a hole (working-memory error) or missing a baited hole (error of omission). When appropriate, we also analyzed the task completion time and the number of head entries. Learning curves were analyzed utilizing repeated-measures ANOVAs. Also, one-way ANOVAs followed by posthoc analyses (Student Newman–Keuls test) were used to compare overall performances between groups. Statistical analyses were performed utilizing the STATVIEW5 software package.

Conditioned nictitating membrane response in rabbits[4]

***Principle*:** The rabbit's classically conditioned eye blink response has become a widely used model system for studying associative learning in mammals and to find drugs potentially useful in the treatment of age-related memory disorders.

Procedure: A small loop of surgical nylon is sutured into the right nictitating membrane, and the surrounding hair is removed. One day later, the rabbit is placed in a Plexiglas restrainer, and two stainless-steel wound clips are applied to the skin over the parietal region. The rabbit is fit with a headmount that supports a photoresistive assembly for recording the nictitating membrane response by physical coupling with a length of thread to the nylon loop in the nictitating membrane. The transducer assembly converts nictitating membrane movements into electrical signals that are subjected to an analogto digital conversion using a 5-ms sampling rate and a resolution of 0.06 mm actual membrane extension. The animal is then positioned in a ventilated, sound-attenuated chamber facing a stimulus panel containing an 11.4 -cm speaker and two 6-W, 24-V DC house lights, one mounted at each side of the speaker. During the course of the experiment, two stimuli are employed as conditioned stimulus: a) a 1000-ms, 1-kHz, 84-dB tone; b) a 1000-ms, intermittently presented light produced by interruption of the house lights at 10 Hz to yield a change in illumination, measured at the eye level of the rabbit from 32.11 to 8.01. The unconditioned stimulus is a 100-ms, 3-mA, 60Hz shock delivered to the wound clips by a constant current shock generator. Drug solutions or saline are injected subcutaneously into the cervical area of the rabbit via an infusion pump at a rate of 3 ml/min, 30 min before behavioral testing. Experimentally naive rabbits are randomly assigned in equal numbers to each of the treatments (n = 10 per treatment). The experiment consists of two phases: Phase 1 is an adaptation day followed by 9 days of acquisition training. No stimuli are presented during the 60-min adaptation session. Subjects are injected with their assigned treatment 30 min before each acquisition session. Each acquisition session consists of 30 tone-shock and 30 light-shock trials presented in a randomized sequence within 10 trial blocks, with the restriction that no more than three consecutive tone or light trials can occur. On each conditioned stimulus unconditioned stimulus trial, the offset of the 1 000- ms tone or light conditioned stimulus occurs simultaneously with the onset of the 100-ms unconditioned stimulus. The inter-trial interval is about 60 s. A response is defined as at least a 0.5-mm extension of the nictitating membrane. Responses occurring during the tone or light conditioned stimulus, but before the unconditioned stimulus are recorded as conditioned response; those occurring after the unconditioned stimulus onset are recorded as unconditioned response.

Evaluation: The data are analyzed by repeated measures analyses of variance and Tukey tests. The significance level is set at $p < 0.05$.

Studies in aged monkeys[4]

***Principle*:** Nonhuman primates offer additional advantages for neurobehavioral animal models of aging in that many of the behavioral processes thought to be affected by aging (e.g. reaction time, attention, learning and memory) can be studied easily in nonhuman primates. Evidence is beginning to accumulate suggesting that certain neurological and behavioral deficits are observed in aged human and in nonhuman primates.

***Procedure*:** The apparatus developed specifically for the series of studies used to develop the primate model was the Automated General Experimental Device (AGED). The AGED is a totally automated, computer-controlled testing system, whose prominent feature consists of a 3 x3 matrix of stimulus response (SR) panels. Each SR panel is hinge mounted directly in front of the reinforcement well so that when a panel is pushed, a red switch is magnetically activated and reinforcement well is exposed. Both colored and patterned stimuli can be projected onto the SR panels. A plastic partition with a stimulus window and armholes separates the monkey from the SR matrix. The stimulus observation window is equipped with a photocell and an infrared light source to detect when the monkey's head is oriented toward the stimuli.

Evaluation: The monkey must remember the stimulus location to get reinforcement. Number of correct answers will be counted as well as the time until the monkey answers correctly.

Electrophysiological method

***1. Long-term potentiation in-vivo*[13]**

***Principle*:** Placing recording and bipolar stimulating electrodes in the granule cell layer of the dentate gyrus and angular bundle allows the evaluation of long-term-potentiation *in vivo*, even in freely moving animals, and the comparison with effects on learning.

Procedure: Female Sprague Dawley rats weighing 225–250 g are anesthetized with 1.5 g/kg urethane (i.p.) and placed in a Kopf stereotaxic instrument. The recording and bipolar stimulating electrodes are placed in the granule cell layer of the dentate gyrus and angular bundle, respectively. The recording electrodes are pulled from thin-walled glass capillary tubes, filled with 150 mM NaCl, and adjusted to resistances ranging from 2.0 to 4.0 M□ . Stimulating electrodes are made from twisted nichrome wire with Teflon insulation, and approximately 0.75 mm separated each tip. The recording electrode is first lowered into area CA1 of the dorsal hippocampus. The stimulating electrode is then placed into the ipsilateral angular bundle. As the recording electrode is lowered further, field potentials are evoked to determine when the recording electrode enters the dentate granule cell layer. All rats are maintained at 37 °C. Responses are evoked using a Grass S-88 stimulator and a Microprobe System M-7 070A amplifiers, and are recorded on a Nicolet 310 oscilloscope. Once the electrodes are appropriately placed, field potentials are generated over a range of stimulus intensities to generate an input/output (I/O) curve. The field potentials are quantified in two ways: The population spike (PS) is expressed as the distance from the deepest point (in mV) of negativity to the preceding highest positivity on the left and right side of the response, and then averaged. The slope of the rising phase of the population excitatory postsynaptic potential (pESP) is measured in mV/ms. The amplitude of the test pulse is based is based on the I/O curve being 25% of the current intensity which evokes the maximal population spike. Long-term-potentiation is induced using three theta-burst stimulus trains, each delivered 1 min apart. Each train consists of five groups of four pulses at 100 Hz, separated by an interval of 150 ms. Each train is delivered at the stimulus intensity which evokes the maximal population spike at the I/O curve. At the end of the experiment, the electrode placements are verified using standard histological techniques.

Evaluation: Student's *t*-tests are used to assess the significance of differences between the means of both the population spike amplitudes and the pESP slopes.

***2. Long latency averaged potentials*[4]**

Principle: Wirtz-Brugger et al. (1986, 1987) studied long latency averaged evoked potentials (P300) in anesthetized rats as a possible model for detecting memory-enhancing drugs. The P300 waveform is described as a positive long-latency (~300 ms) potential believed to reflect endogenous cognitive processes rather than exogenous physical parameters of the stimulus. Compounds that have been shown clinically to enhance cognitive ability also significantly increase the integrated area under the P300 wave.

Procedure: Male Wistar rats weighing 300–500 g are anesthetized with 120 mg/kg i.p. Inactin. Stainless steel screws serve as recording electrodes located at the surface of the cortex of seven distinct brain areas: posterior: Pz (midline), P3, P4 (lateral); central: Cz (midline); and frontal: Fz (midline, F3, F4 (lateral). A linked reference is provided with platinum needle electrodes behind the ears. All leads are fed into a digital averaging computer. Event-related potentials are elicited in response to an oddball paradigm of two tones (500 Hz frequent and 3 KHz rare) randomly presented with a probability of 10% for the rare tone. The intensity of the tones is 95 bB, pulse duration 100 ms, rise/fall 9.9 ms. The auditory stimuli are delivered bilaterally at a rate of 0.3/s through special Nicolet tubal tip inserts into the ear of each subject. One repetition of the paradigm consists of 300 tones. It is presented twice, before and after drug

application. Evaluation of the rare responses consists of defining and comparing the P300 in terms of integrated area before and after drug. P300 area is calculated by integration of area under the curve.

Evaluation: Values are expressed as means and standard error per group and percent changes from control. Statistical evaluation consists of paired t-tests to demonstrate significant differences.

Transgenic animal model[14]

***Principle*:** Mutation responsible for the rare cases of familial Alzheimer disease (FAD)—only to realize that not one but numerous mutations were actually able to cause the disease. Mutations are indeed located not only in the APP gene from which the Aβ peptide is cleaved, but also in the genes of presenilin 1 or 2 that are directly involved in Aβ production from APP. All of the mutations that have been tested; when transfected in cellular models, induce an increase in the Aβ42/Aβ40 ratio with the noticeable exception of the Arctic mutation directly involving the A_ sequence itself. All mutations induce an overproduction of Aβ except the mutation V715M, in which, however, the ratio Aβ42/ Aβ40 is increased. Transfecting the APP gene induces an overproduction of the protein, and APP overproduction may be sufficient to increase Aβ peptide secretion: both the β and the γ enzymatic activities do not appear to be rate limiting. There are two transgenic models: Amyloid-β Transgenic Mouse Models and Tau Transgenic Mouse Models[17].

***Procedure*[15]:** Twenty-seven APP-PS1 (15 males and 12 females) mice and 30 female B6_SJL wild-type mice spanning in age from 66 to 904 days were used in this study. Some of the mice were scanned repeatedly. Eight mice were scanned twice, and two were scanned three times. Additionally, three 624-day-old APP mice were scanned. Mice were anesthetized by using 1.0–1.5% isoflurane and O_2/NO_2 and positioned in a custom built device to immobilize the head during experiments. Body temperature was maintained at 37°C by warm water circulation, and physiological monitoring was used for temperature, respiration, and ECG.

Evaluation: Statistical analysis was conducted by using SAS software for Windows. The ratios of concentration of NAA, Glu, and mIns to tCr and NAA to mIns were compared between wild-type and APP-PS1 mice over time by using analysis of variance. Only the first observation in the case of the repeatedly scanned animals was used in the analysis. A Kruskal–Wallis one-way analysis of variance by ranks test (23) was used to compare the median mIns and taurine levels among the wild-type, APP, and APP-PS1 mice. Subsequent Mann–Whitney *U* tests (23) were used to make pair wise comparisons between different mice groups.

CONCLUSION

Although drug discovery is based upon many factors, animal models provide a crucial part in identifying chemical compounds with potential for clinical efficacy. It is challenging task to develop appropriate animal model for dementia in the absence of truly affective therapeutic agent. For an ideal animal model it should exhibit some or all the behavioral and neurological dysfunction known to be associated with disorder. In the mean time many models are developed successfully which are currently used and there is huge scope for the development of new advanced screening models for learning and memory enhancer drugs.

Table 1: Research works done on Plant and Synthetic Drugs having Nootropic activity

Sr No	Plant drug/ synthetic drug	Chemical constitute	Uses	Animal model used
1	Wormwood extracts (*Artemisia absinthium L.*)[18]	Flavonoids, Cinnamic acid, Coumarin	Antioxidant	Inhibition of acetylcholine-esterase activity
2	Metoclopramide[19]	Benzamide derivative	5-HT_3 antagonist, D_2antagonist, weak 5-HT_4 agonist.	Scopolamine induced, runway apparatus, radial arm maze.
3	Sildenafil[20]		Correcting erectile dysfunction	Discrimination learning.
4	Combination of insulin		Lowering blood	Discrimination learning.

	with dextrose, D(-)fructose & diet[21]		glucose level.	
5	Clitoria ternatea root extract[22] (Shanka pushpi)	Oleo resin	Nervine tonic, nervous debility.	Two compartment passive avoidance and spatial learning.
6	5-HT_3 Antagonist Ondensetron[23]		Anti-emetic agent	Passive avoidance test, holeboard test and Conditioned avoidance response.
7	Gly^{14}]-Humanin[24]	Linear Polypeptide	Protect cell death	Scopolamine induced
8	Ferulic acid[25]	Phenol	Antioxidant and anti inflammatory	Pasiive avoidance test and Discrimination learning.
9	Nicotine[26]		Anxiolytic activity	Runway avoidance, open field test.
10	Lipopolysaccharide[27]			Pasiive avoidance test and Discrimination learning.
11	*Ocimum sanctum Linn (Labiateae)*[28]	Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)	Expectorant, antiseptic. Antioxidant, anti-stress.	Inhibition of acetylcholin esterase activity and Discrimination learning.
12	*Vitex Negunda (Verbenaceae)*[29]	Volatile oil, flavonoid, glycoside.	Anti-amensic.	Scopolamine induced
13	Tacrine, Bis-tacrine, Donepezil, Rivastigmine, Galantamine, heptyl-Physostigmine, TAK-147 and Metrifonate[30]		Acetylcholineste rase inhibitor.	Molecular forms of acetylcholine esterase from rat frontal cortex and striatum.
14	Soman[31]		Acetylcholineste rase inhibitor.	Inhibition of acetylcholine-esterase activity
15	Nitric oxide[32]		Gaseous chemical messenger.	Pole jumping method
16	BDNF and Carnitine[33]		Neuronal plasticity,enhanc e neuronal survival and differentiation.	Spatial learning
17	Regular exercise[34]			Passive avoidance test and jumping avoidance.
18	(R)-α-methylhistamine [35]		H_3 selective agonist.	Release of [3H] Ach.
19	Latrepirdine (Dimebon™)[36]		Antihistamine.	AF64A model of Ach deficiency.
20	*Nardostachy jatamansi, Withania somnifera, etc*[37]	Jatamansone, Anaferine,	Antispasmodic, diuretic, sedative hypnotic.	Inhibition of acetylcholine-esterase activity
21	Tetrahydro-N, N-dimethyl-5, 5-diphenyl-		Potent muscarinic and	Passive avoidance test , water maze and [H]-N-

	3-furanmethanamine hydrochloride[38]		sigma$_1$ receptor ligand.	methylscopolamine binding
22	Mentat [39]		Improves memory quotient.	Active avoidance and conditioned response
23	Donepezil[40]		Acetylcholinesterase inhibitor.	Passive avoidance test and Inhibition of acetylcholine-esterase activity.
24	Memantine[41]	1-amino adamantane	NMDA-receptor antagonist.	Long term potentiation
25	Benzylidene ketal, Anthranilamide [42]		Muscarinic receptor antagonist,	Passive avoidance test
26	4-fluronaphthamide, SCH 57790(piperazine)[42]	Napthamides, piperazine.	M_2 receptor antagonist	[3H] Oxotremorine-m binding to muscarinic cholinergic receptors in rat forebrain
27	OM99-1 and OM99-2[43]		β-secretase inhibitor	Tg2576 transgenic AD mice model
28	BMS 289948 (benzene sulfonamide hydrochloride), BMS 299897 (flurobenzenepropanoic acid)[43]		γ-secretase inhibitor.	transgenic AD mice model

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