PHOSPHODIESTERASE 5 INHIBITION ON DOPAMINERGIC AND

GLUTAMATERGIC NEUROTRANSMISSION: IMPLICATIONS

FOR MEMORY ENHANCEMENT

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PHOSPHODIESTERASE 5 INHIBITION ON DOPAMINERGIC AND GLUTAMATERGIC NEUROTRANSMISSION: IMPLICATIONS FOR MEMORY ENHANCEMENT

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VITA

Subramaniam Uthayathas, son of Appakkuddy Subramaniam and late Sivapakkiam Subramaniam was born on September 25, 1964. He graduated from The Faculty of Agriculture, University of Peradeniya, Sri Lanka in 1991, with his Bachelor's of Science (Agricultutre). Shortly after graduation, he was recruited to the Department of Animal Science of the same college where he earned his degree. Subsequently, he got a tenure track Lecturer position in the Department of Animal Science of the University of Jaffna, Sri Lanka and worked there as a lecturer for about twelve years until he joined the doctoral degree program in pharmacology, at Auburn University in August 2005. He earned his Master's degree from the same place where he had his Bachelor's degree. Uthayathas was born with other six siblings, four sisters and two brothers. He was married to Thayalini Uthayathas and has two daughters Luckshikha Uthayathas and Hamshika Uthayathas. During his stay in Auburn University he obtained several awards and prizes. He received the outstanding graduate student award from Auburn University International Education in 2007 and outstanding doctoral student award from Auburn University in 2008.

DISSERTATION ABSTRACT

PHOSPHODIESTERASE 5 INHIBITION ON DOPAMINERGIC AND GLUTAMATERGIC NEUROTRANSMISSION: IMPLICATIONS FOR MEMORY ENHANCEMENT

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Phosphodiesterases belongs to a family of proteins that metabolize cyclic nucleotides. There are at present eleven types of Phosphodiesterases been isolated and shown to be expressed in various tissues. Phosphodiesterase 5, cyclic guanosine 3',5' monophosphate (cGMP) metabolizing enzyme and influence numerous pharmacological processes including ion channel function and learning. Sildenafil, active substance in the Viagra, is a specific and potent inhibitor of Phosphodiesterase 5. However, the physiological mechanisms underlying the learning and memory enhancement have poorly been studied. In this dissertation the behavioral and molecular modifications due to sildenafil administration in various animal models of Parkinson's and Alzheimer's disease have been investigated.

Effect of sildenafil on fatigue was evaluated using forced swim test in mice. Sildenafil had no effect on fatigue as seen by the swim time. Neuroprotective effect of sildenafil was investigated using two animal models of Parkinson's disease. 6-

hydroxydopamine-lesioned rats were used to determine the effect of sildenafil on rotational behavior. Ipsilateral or contralateral rotational behavior can indicate the amphetamine-like activity or apomorphine-like activity of sildenafil. Sildenafil did not induce contralateral or ipsilateral rotations in 6-hydroxydopamine-lesioned rats. Sildenafil did not protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopamine depletion in the striatum. The results of this study are significant as sildenafil that cross the blood brain barrier, has neither beneficial nor deleterious effect on nigrostriatal dopaminergic system.

Memory enhancing properties of sildenafil were investigated using mice. Single administration of sildenafil enhances hippocampal dependent behavioral tasks. To elucidate the underlying mechanism in the memory enhancement, effects of sildenafil on long term potentiation (LTP) was measured. The level of LTP was significantly elevated in mice treated with sildenafil (1 mg/kg/day) for 15 day compared to control. Thus, these results suggest that the neural mechanisms of memory enhancement through PDE5 inhibition could be due to glutamatergic synaptic modification.

The finding that sildenafil exert glutamatergic synaptic modification in the normal animal motivated the investigation on a disease model. Alzheimer's disease (AD) is a fatal, progressive neurodegenerative disease that occurs in the elderly of the general population. Amyloid plaques, consisting of extracellular deposits of Aβ peptide are found in many AD patients' brains and considered one of the hallmarks of Alzheimer's disease. Intracerebroventricular (icv) infusion of Aβ in rat caused severe memory dysfunction. Consistent with the previous observation in mice, sildenafil significantly attenuated the memory deterioration in the Abeta (1-42) infused AD rat model. Thus, these results suggest that neural disturbances caused by abeta infusion may be rescued by treatment with sildenafil.

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1. INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs) controls the hydrolysis of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) and they influence numerous pharmacological processes including ion channel function and learning. Inhibition of phosphodiesterase enhanced long-term potentiation of synaptic transmission without altering basal synaptic transmission in rats (Boess et al., 2004). Among eleven types of phosphodiesterases, only three; 5, 6 and 9 are cGMP specific (Reneerkens et al., 2008), where as 1, 10 and 11 are dual specificity enzymes that hydrolyse both cAMP and cGMP while the others are specific to cAMP (Francis et al., 2001). Phosphodiesterase 5 hydrolyzes cGMP and found in several parts of the body such as the lungs, platelets, smooth muscle and brain (Reyes-Irisarri et al., 2007). Sildenafil structurally resembles the guanosine base of cGMP and the 3-substituent extension fills a space in the enzyme active site occupied by ribose. Sildenafil selectively inhibit phosphodiesterase-5 and increase the level of cGMP leading to beneficial effects in targeting organs.

Parkinson's disease is a chronic and disabling disorder that affects the motor function and likely to impair a patient's quality of life. There has been wide variation in the management of Parkinson's disease due to a lack of consensus on the best approach. Degeneration of nigral neurons result in the dopamine depletion in striatum. Currently

there are no perfect drugs available for therapeutic use in PD and it is customary to test a substance for its potential protection against neurodegeneration. Results our published study reveal that sildenafil did not prevent neurotoxicity produced by MPTP exposure (Uthayathas et al., 2007a). Recently, Janis et al. (2008) draw the same conclusion and found that sildenafil did not produce any deleterious effect on nigro-striatal dopaminergic neuron function nor did it potentiate the neurotoxic effects of MPTP.

Learning and memory performance in human is determined by the strength of synaptic connections and the ability of neurons to reestablish the communication (Costa-Mattioli et al., 2007). Communication among neurons affected by several factors and to some extent can be manipulated by memory enhancers. The discovery of drugs that act on the memory processes is the main challenge today in the field of neuropharmacology, particularly as regards dementia (Wilcock and Harrold, 1996). In recent times, cholinergic and glutamatergic system have been targeted for development of nootropic agents. Acetylcholine esterase inhibitors like donepezil and allosteric modulator of AMPA receptors such as piracetam, pramiracetam and aniracetam are being primarily used to improve memory, mood and behavior (Wijayawardhane et al., 2007). However, the resulting adverse effects associated with these agents have limited their use. Therefore, it is worthwhile to explore therapeutically safe medicines for the treatment of various cognitive disorders. Regulation of cyclic guanosine monophosphate (cGMP)

pathway is a vital pharmacological response of the AMPA receptor potentiators and contributes to the efficacy of AMPA modulators in rodent models of cognition (Ryder et al., 2006). It has been shown that amyloid beta, a peptide considered as a hallmark in Alzheimer's disease, stimulates cGMP degradation (Paris et al., 1999). Sildenafil has been shown to increase cGMP levels in the prefrontal cortex, hippocampus, and cerebellum (Marte et al., 2008).

Diverge results have been reported targeting phosphodiesterase 5, in account to dopaminergic system. Sildenafil, specific inhibitor of phosphodiesterase 5, did not show protection against Parkinsonian toxins (Uthayathas et al., 2007a; Janis et al., 2008). However, in middle cerebral artery occlusion stroke model, inhibition of phosphodiesterase 5 using sildenafil has been shown to enhance neurogenesis and functional recovery (Zhang et al., 2002). Interestingly, sildenafil has been shown to enhance memory in a range of Alzheimer's disease models (Erceg et al., 2006; Devan et al., 2006). Sildenafil administration improved the cognitive performance in diabetic conditions and electroconvulsive shock-induced animal models (Patil et al., 2006). Inhibition of phosphodiesterase5 by sildenafil has been shown to enhance object memory in mice (Rutten et al., 2005, 2006, 2007a, 2007b). Although there is some evidence for enhancement of memory by phosphodiesterase5 inhibition, studies exploring the action of phosphodiesterase inhibitors in memory enhancement *in vitro* or *in vivo* are still limited and in-depth investigation of effect on cellular signaling is scanty. Retrograde signaling by nitric oxide (NO) signal transduction has been well characterized and shown to play a major role in the neurotransmitter release machinery (Arancio et al., 1996). On

the other part NO activate guanylyl cyclase resulting in the formation of cGMP, with cGMP-dependent protein kinase being one of the downstream mechanisms contribute to synaptic plasticity (Feil et al., 2005). Recent studies indicate that the amyloid-beta peptide induced impairment of synaptic plasticity involves inhibition of the nitric oxide/cGMP/cAMP-responsive element-binding protein pathway activation (Puzzo et al., 2005).

A promising field in memory research focuses on the involvement of second messenger systems. Inhibition of phosphodiesterase (PDE) enzymes is a way to enhance second messenger signaling and consequently influence the pathways involved in learning and memory. Phosphodiesterases can be selective for either cAMP or cGMP or both and these nucleotides play an important role in intracellular signaling and in processes of neuroplasticity, such as long term potentiation (LTP) (Frey et al.,. 1993b; Son et al., 1998). The difference between phosphodiesterase inhibitors as cognition enhancers and more conventional drugs is that most other drugs act on specific neurotransmitter systems whereas phosphodiesterase inhibitors can modulate multiple biochemical systems through second messenger signaling. Phosphodiesterase inhibition elevates concentrations of second messengers (cAMP and/or cGMP) which in turn trigger various intracellular mechanisms. As such, these second messengers are thought to be involved in processes of synaptic plasticity through the mechanism of LTP (Frey et al., 1993b; Impey et al., 1996b).

Long term potentiation (LTP) is a process in the brain that can explain certain aspects of synaptic plasticity. Experimentally, a series of short, high frequency electric

stimulation to a nerve cell synapse can strengthen, or potentiate, that synapse for minutes to hours. Hippocampal LTP has been proposed to be a neuro-physiological correlate of memory (Bliss and Collingridge 1993). Studies that investigated the underlying molecular mechanisms of LTP have provided molecular tools for improving LTP and consequently memory. Among the various molecules involved in LTP, cAMP and cGMP play a prominent role (Bailey et al., 1996a; Chien et al., 2003; Son et al., 1998). Cyclic nucleotides modify LTP processes, because they play an important role in intracellular signaling (Bailey et al., 1996a; Chien et al., 2003; Son et al., 1998). Therefore the hypothesis that phosphodiesterase inhibition could enhance memory through the process of LTP arose and several studies have been performed to investigate this possibility. A couple of very important studies provided the base for some of the behavioral experiments described in this dissertation. These previous studies showed that intra-hippocampal infusion of 8Br-cAMP improved memory performance when injected 3 or 6 h after one-trial training, but not when injected directly after the training trial. In contrast, intra-hippocampal injections with 8Br-cGMP only improved memory performance when injected directly after the learning trial and not when injected 3 h or 6 h later (Bernabeu et al., 1996; Prickaerts et al., 2002a). The time dependent effects of cAMP and cGMP in memory performance may underlie different cellular mechanisms and we speculated that these mechanisms might be related to early and late consolidation processes.

Sildenafil treatment also ameliorated the deficits induced by two different models for diabetes and electro convulsive shocks (Patil et al., 2006; Patil et al., 2004b; Rutten et al., 2005). Previous studies showed no effects of phosphodiesterase5 inhibition on spatial

tasks, i.e. the water escape task or the Y maze (Prickaerts et al., 2004). However, in hyperammonemia or portacaval shunt deficit models for liver failure, both sildenafil and zaprinast reversed spatial recognition deficits in rats (Erceg et al., 2006; Erceg et al., 2005a; Erceg et al., 2005b). Previous work showed that zaprinast reversed the deficits induced by the NOS inhibitor 7-nitroindazole in rats in the object recognition task (Prickaerts et al., 1997). Recent work adds to this in that sildenafil reversed the effects the NOS inhibitor L-NAME, in a complex maze learning paradigm (Devan et al., 2006). Furthermore, various studies investigated the effects of phosphodiesterase5 inhibition on active and passive avoidance learning in rats, mice and neonatal chicks. Memory impairments caused by scopolamine, diabetes model and electro convulsive shocks in rats were reversed by sildenafil treatment (Devan et al., 2004; Patil et al., 2006; Patil et al., 2004b). Although one study failed to show improvement in learning performance after sildenafil treatment in unimpaired and aged rats (Shafiei et al., 2006), others show improvement of learning and memory after treatment with phosphodiesterase5 inhibition in unimpaired and aged mice and in neonatal chicks (Patil et al., 2004a). Taken together, a growing amount of evidence shows cognitive effects of phosphodiesterase5 inhibition in several behavioral memory models. Most research so far has focused on rodent models and it would be of great importance to extend these findings into mechanism of action on the molecular level and the pathways that leads to the neurological benefits. Since phosphodiesterase5 inhibitors are clinically accepted for the treatment of male erectile dysfunction, these drugs can be easily tested in a clinical experimental setup.

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2. REVIEW OF LITERATURE

Phosphodiesterases

Phosphodiesterases (PDEs) are enzymes that break down cyclic nucleotides, i.e. cAMP or cGMP. The family of phosphodiesterase enzymes has eleven classes based on their substrate. Phosphodiesterases can be selective for either cAMP or cGMP or both and these nucleotides play an important role in intracellular signaling and in processes of neuronal plasticity, such as long term potentiation (LTP) (Frey et al., 1993; Son et al., 1998). There are three phosphodiesterases namely; PDE5, PDE6 and PDE9 are specific to cGMP (Reneerkens et al., 2008). Three phosphodiesterases; PDE1, PDE10 and PDE11 are known for hydrolyzing both cAMP and cGMP while the others are specific to cAMP (Francis et al., 2001). Cyclic nucleotide phosphodiesterases controls the hydrolysis of cGMP and cAMP and they influence numerous pharmacological processes including ion channel function and learning. Inhibition of phosphodiesterase2 enhanced long-term potentiation of synaptic transmission without altering basal synaptic transmission in rats (Boess et al., 2004). Therefore, modulating second messenger system through inhibition of phosphodiesterase may be a potential target for central nervous system drug development (Boess et al., 2004; Uthayathas et al., 2007a). Inhibition of several phosphodiesterases has been shown to enhance memory (Prickerts et al., 1997; Rutten et al., 2005; Puzzo et al., 2008). Phosphodiesterase inhibitors can modulate multiple

biochemical systems through second messenger signaling whereas more conventional drugs act on specific neurotransmitter systems. Elevating concentrations of second messengers (cAMP and/or cGMP) through phosphodiesterase inhibition in turn trigger various intracellular mechanisms. These second messengers are thought to be involved in processes of synaptic plasticity through the mechanism of LTP (Frey et al., 1993; Impey et al., 1996).

Memory enhancing effects of phosphodiesterase5 inhibition using zaprinast was first reported in the literature (Prickaerts et al., 1997). Zaprinast is not selective for phosphodiesterase5 it also inhibits phosphodiesterase1, 9, 10, and 11. Currently, highly selective phosphodiesterase5 inhibitors are used for the pharmacological treatment of erectile dysfunction. There are three approved phosphodiesterase5 inhibitors in several countries; sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis) (Blokland et al., 2006). One review is available for a detailed discussion on the behavioral effects of phosphodiesterase5 inhibition (Prickaerts et al., 2004). Thus far several studies have shown effects of selective phosphodiesterase5 inhibitors on memory performance in the object recognition task in rats (Prickaerts et al., 2005; Prickaerts et al., 1997; Prickaerts et al., 2002b).

Interestingly, sildenafil has been shown to enhance memory in a range of Alzheimer's disease models (Erceg et al., 2006; Devan et al., 2006). Sildenafil administration improved the cognitive performance in diabetic conditions and electroconvulsive shock-induced animal models (Patil et al., 2006). Inhibition of phosphodiesterase5 by sildenafil has been shown to enhance object memory in normal

mice (Rutten et al., 2005; Rutten et al., 2007a). In middle cerebral artery occlusion stroke model, inhibition of phosphodiesterase5 using sildenafil has been shown to enhance neurogenesis and functional recovery (Zhang et al., 2002). Although there is some evidence for enhancement of memory by phosphodiesterase5 inhibition, studies exploring the action of phosphodiesterase inhibitors in memory enhancement *in vitro* or *in vivo* are still limited and in-depth investigation of effect on cellular signaling is scanty. Retrograde signaling by nitric oxide (NO) signal transduction has been well characterized and shown to play a major role in the neurotransmitter release machinery (Arancio et al., 1996). On the other part NO activate guanylyl cyclase resulting in the formation of cGMP, with cGMP-dependent protein kinase being one of the downstream mechanisms contribute to synaptic plasticity (Feil et al., 2005; Rutten et al., 2006, 2008). Recent studies indicate that the amyloid-beta peptide induced impairment of synaptic plasticity involves inhibition of the nitric oxide/cGMP/cAMP-responsive element-binding protein pathway activation (Puzzo et al., 2005).

Learning and memory

The memory system represents one of the most fundamental but also one of the most complex systems in the brain. Nearly all living organisms, from Drosophila to primates, exhibit some kind of learning and memory. Evolution has favored those organisms that learn from previous experience and found some way to store previous information for later use. The primitive mechanism of memory formation is believed to be comparable between species with regard to the underlying cellular mechanisms. Therefore, fundamental research using animal models of cognition *in vivo* or *in vitro* can

realize better comprehension of the human memory system. Furthermore, pathological situations in which memory functioning is compromised provide valuable information about the memory system and its underlying mechanisms.

Memory impairment can occur as a result of normal aging or pathological conditions. Due to increase in life expectancy and the middle-aged cohort is greater than previous decades, the absolute number of old people will increase substantially. As a consequence of this double aging, the age-associated problems will increase. One of the more serious problems older people are facing is a decline in cognitive functions (Mattson et al., 2002). Memory impairments have a strong negative impact on the daily activities and quality of life of aged people. The loss of cognitive functioning is even more serious in pathological conditions such as Alzheimer's disease or other dementia. But also in depressed and schizophrenic patients, prominent memory deficits are present (Blaney, 1986; Frith, 1996).

So far, several preventive strategies have been described which could ameliorate or slow down the cognitive decline resulting from brain aging. Research has focused on avoiding genetic and environmental factors that cause neuronal dysfunction and death or by enhancement of the ability of neurons to adapt to the aging process (Mattson et al., 2002). Examples of avoiding genetic factors are genetic counseling or germ line gene therapy and examples of avoiding environmental factors are dietary restrictions or behavioral modification. These strategies to some extent reduce the risk of cognitive decline and dementia (for a review see Mattson et al., 2002) however may not eliminate

the problem. Therefore, there is a great need for drugs that counteract the processes involved in ageing and more specifically the decline of cognitive functions and memory.

Various drug targets have been suggested based on neurotransmitter systems for the enhancement of memory or reversal of cognitive deficits. Serotonergic, cholinergic and dopaminergic neurotransmitter systems have been shown to be involved in memory. Furthermore, cognitive performance, including memory, can be facilitated by numerous biological factors. Memory can be improved or impaired for example by neurotransmitters, neuromodulators, intracellular molecules, hormones, plant extracts and nutritional ingredients (Cahill et al., 1994; Davis and Squire, 1984; DeZazzo and Tully, 1995; Izquierdo et al., 1998; McGaugh, 1989; Messier, 2004).

Targeting second messenger systems may be a promising field in memory research. Enhancing second messenger signaling through inhibition of phosphodiesterase enzymes may be a practical venue to control the pathways involved in learning and memory. Studies were conducted to asses the effects of phosphodiesterase inhibition on intact memory as well as deficit memory models. In general, the effects of cyclic guanosine monophosphate (cGMP) manipulations were studied on learning and memory by means of inhibition of specific phosphodiesterases.

Long term potentiation

Long term potentiation (LTP), an activity-dependent synaptic plasticity, plays a key part in the forms of memory mediated by hippocampus. LTP is the increase in the chemical strength of a synapse after a tetanus stimulation that lasts for over an hour. Experimentally, a series of short, high frequency electric stimulation to a nerve cell

synapse can strengthen, or potentiate, that synapse for minutes to hours. Hippocampal LTP has been proposed to be a neurophysiological correlate of memory (Bliss and Collingridge, 1993). Studies that investigated the underlying molecular mechanisms of LTP have provided molecular tools for improving LTP and consequently memory. Among the various molecules involved in LTP, cAMP and cGMP play a prominent role (Bailey et al., 1996; Chien et al., 2003; Son et al., 1998).

In general, LTP is often divided into two phases, an early phase (E-LTP), which is short lasting and a late phase (L-LTP) that lasts longer and depends on gene transcription and protein synthesis (Pang and Lu, 2004; Voronin et al., 1995). LTP can be experimentally induced in the CA1 area of the hippocampus by applying a couple trains of tetanic stimulation to the synaptic connection between two neurons. Induction of LTP involves the activation of NMDA receptors, a subclass of glutamate receptors. Post-synaptic depolarization after short tetanus stimulation removes the voltage-dependent magnesium block of NMDA receptors. This results in an influx of Ca^{2+} through the NMDA receptor in the post-synaptic neuron (Pang and Lu, 2004). Elevated intracellular Ca^{2+} levels trigger a cascade of reactions including the production of Nitric Oxide (NO) and activation of the Ca2+-calmodulin-dependent protein kinase II (CaMKII). Initially, cGMP was thought to act mainly pre-synaptically in the LTP as part of a NO/sGC/cGMP pathway. In this respect NO is regarded as a retrograde messenger. Elevation of cGMP could result in either more glutamate release via activation of pre-synaptic voltage-gated cGMP-dependent ion channels, or more glutamate synthesis via cGMP-dependent protein kinase (PKG). However, post-synaptic mechanisms of action are also suggested, via the

insertion of post-synaptic AMPA receptors by activation of CaMKII (Lu and Hawkins, 2002).

Maintenance of LTP for hours after induction requires activation of cAMP-dependent protein kinase (PKA) and results in synthesis of RNA and proteins (Lu and Hawkins, 2002). Several pathways might be involved in maintenance of LTP. Cyclic AMP response element binding protein (CREB) is thought to be the primary transcription factor in the cascade of gene expression that leads to permanent structural changes at the level of the synapse (Impey et al., 1996). Recently, it has been shown that a cGMP/PKG/CREB pathway is also involved in L-LTP (Lu and Hawkins, 2002; Lu et al., 1999). Thus, cAMP and cGMP play an important role in intracellular signaling and in processes of neuroplasticity such as long term potentiation (LTP).

Phosphodiesterase inhibition and LTP

Two key players in LTP processes are the cyclic nucleotides cAMP and cGMP, because they play an important role in intracellular signaling (Bailey et al., 1996; Chien et al., 2003; Son et al., 1998). Therefore the hypothesis that phosphodiesterase inhibition could enhance memory through the process of LTP arose and several studies have been performed to investigate this possibility. A couple of very important studies provided the base for some of the behavioral experiments described in this thesis. These previous studies showed that intra-hippocampal infusion of 8Br-cAMP improved memory performance when injected 3 or 6 h after one-trial training, but not when injected directly after the training trial. In contrast, intra-hippocampal injections with 8Br-cGMP only improved memory performance when injected directly after the learning trial and not

when injected 3 h or 6 h later (Bernabeu et al., 1996; Prickaerts et al., 2002a). The time dependent effects of cAMP and cGMP in memory performance may underlie different cellular mechanisms and we speculated that these mechanisms might be related to early and late consolidation processes.

Sildenafil treatment ameliorated the memory deficits in different animal models (Patil et al., 2006; Patil et al., 2004b) where as few studies showed no effects of phosphodiesterase5 inhibition on spatial tasks, i.e. the water escape task or the Y maze (Prickaerts et al., 2004). In another study, hyperammonemia or portacaval shunt deficit models for liver failure, both sildenafil and zaprinast reversed spatial recognition deficits in rats (Erceg et al., 2006; Erceg et al., 2005a; Erceg et al., 2005b). Recent work adds to this in that sildenafil reversed the effects the nitric oxide synthase inhibitor L-NAME, in a complex maze learning paradigm (Devan et al., 2006). Furthermore, various studies investigated the effects of phosphodiesterase5 inhibition on active and passive avoidance learning in rats, mice and neonatal chicks (Devan et al., 2004; Patil et al., 2006; Patil et al., 2004b). Interestingly, phosphodiesterase5 inhibition in unimpaired and aged mice and in neonatal chicks has improved learning and memory (Campbell and Edwards, 2006; Patil et al., 2004a). Taken together, a growing amount of evidence shows pro-cognitive effects of phosphodiesterase5 inhibition in several behavioral memory models. Most research so far has focused on rodent models of behaviors and it would be of great importance to extend these findings into molecular mechanisms by employing multiple techniques.

Since phosphodiesterase5 inhibitors are clinically accepted for the treatment of male erectile dysfunction, these drugs can be easily tested in a clinical experimental setup. The effects of sildenafil (100 mg) on early information processing and memory processes in human have only sporadically been studied. Thus far, no clear effects on the behavioral measures of attention and verbal recognition memory have been reported (Schultheiss et al., 2001). However, there are some methodological considerations since only short term memory was measured at an already optimal performance allowing a minimal window for treatment effects.

Sildenafil and Memory enhancement

Alzheimer's disease is a progressive neurodegenerative disorder that is mainly characterized by cognitive impairment. An estimated 4 million people, most of them elderly, have Alzheimer's disease in United States, affecting 30-50% of individuals aged 85 and older (Evans et al., 1989). The specific cause of Alzheimer's disease is unknown, but genetic abnormalities appear to play a role and neuroinflammation is now recognized as a prominent feature in Alzheimer's pathology. Therapeutic treatment consists of alleviating symptoms, providing long term care at a minimal cost with fewer adverse effects. Progressive neurodegeneration results in chronic cognitive decline culminating in memory loss and motoneuronal dysfunctions. Alzheimer’s disease patients find difficulty reasoning, making judgments, communicating and carrying out daily activities. With the progression of Alzheimer’s disease, patients may also experience changes in personality, behavior and life style, such as anxiety, suspiciousness or agitation, as well as delusions or hallucinations (Heese and Akatsu, 2006).

Prevalence of Alzheimer's disease is increasing in trend, particularly in economically well developed countries due to many reasons including increase in life expectancy and change in food habit (Uthayathas et al., 2007b). Huge investments are currently made for the therapeutic intervention and cure of Alzheimer's disease. However there is no perfect drug currently available for the therapeutic treatment. In recent times research on memory focuses on the involvement of second messenger systems. Inhibition of phosphodiesterase enzymes is one possible intervention to enhance second messenger signaling and consequently influence the pathways involved in learning and memory. Studies have shown that phosphodiesterase5 inhibitors can counteract deficits in long-term memory caused by pharmacological agents or aging (Devan et al., 2004; Devan et al., 2006; Erceg et al., 2005a; Erceg et al., 2006). Therefore, targeting phosphodiesterase5 with selective inhibitor sildenafil may offer a novel therapy aimed at slowing progression, prevention and, eventually, therapy of Alzheimer's disease. Event related brain potentials recorded following sildenafil administration suggest an enhanced ability in young men to focus attention on auditory stimuli (Schultheiss et al., 2001). This finding is significant as a first attempt to study the cognitive effects of sildenafil in humans using electrophysiological techniques. Animal studies have shown sildenafil to enhance memory (Baratti and Boccia, 1999; Domek-Lopacinska and Strosznajder, 2005; Erceg et al., 2005b; Prickaerts et al., 2005; Rutten et al., 2005; Singh and Parle, 2003).

Administration of sildenafil after the first trail in object recognition task has been shown to improve memory in mice (Rutten et al., 2005; Uthayathas et al., 2007c).

Similarly, sildenafil has been shown to attenuate memory impairment induced by nitric oxide synthase inhibition (Domek-Lopacinska and Strosznajder, 2005), hyperammonemia (Erceg et al., 2005b) and blockage of muscarinic cholinergic receptors (Deven et al., 2006). Sildenafil administration improved the cognitive performance in diabetic conditions and electroconvulsive shock induced animal models (Patil et al., 2006).

There are several theories proposed to explain the memory enhancement by phosphodiesterase inhibition. Phosphodiesterase5 inhibition causes vasodilatation, probably through cGMP, in rats (Dundore et al., 1992, 1993). Thus one of the suggested mechanisms is memory improvement through increased blood flow and consequent glucose metabolism in the brain (Prickaerts et al., 2005). A unifying hypothesis intended to explain the sildenafil mediated memory enhancement proposes that accumulation of cGMP initiates a complex cascade. Presynaptic phosphodiesterase5 inhibition increases the cGMP level and triggers the release of glutamate and subsequent NMDA receptor activation. Postsynaptic inhibition of phosphodiesterase5 increases protein synthesis and synaptogenesis. Increased activity of cGMP mediated ion channels may lead to early consolidation of information into memory. Cascade of events related to memory enhancement is presented in Figure-2.1. Electrophysiological experiments with long term potentiation revealed that cGMP must be kept high but below a certain threshold to attain maximum learning performance (Erceg et al., 2006). Bernabeu et al., (1996) found administration of cAMP into hippocampus enhanced passive avoidance learning, suggesting cAMP is involved in later stages of memory consolidation processes. Direct

administration of cGMP into the hippocampus improved object memory in rats and there was no improvement by cAMP. Cyclic GMP-regulated processes in the hippocampus play a significant role in the early stages of memory consolidation and cAMP signaling pathways are occupied in the late post-training memory processing of inhibitory avoidance learning (Baratti and Boccia, 1996).

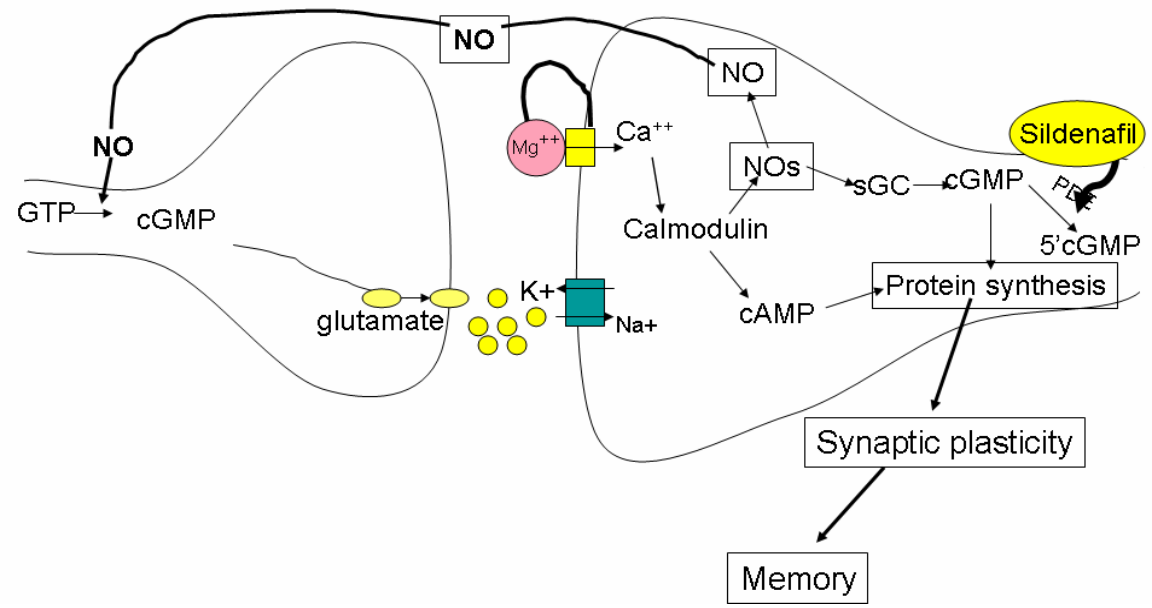

Figure 2.1.: NO-Glu- cGMP pathway and memory

Long term potentiation is mainly a postsynaptic event. However, long term potentiation is expressed by increasing the presynaptic release of glutamate through the guanylate cyclase/cGMP/PKG pathway (Monfort et al., 2001; Domek-Lopacinska and Strosznajder, 2005). Glutamate-Nitric oxide-cGMP pathway modulates important cerebral processes such as intercellular communication, the circadian rhythms and long-term potentiation (Blokland et al., 2006; Boulton et al., 1995). Sildenafil may reduce the

cognitive deficits associated with aging and has the potential for treating age-related neurodegeneration. Production of nitric oxide and cGMP is reduced with age and to some extent contributes to age related memory decline (Taddei et al., 2001). Sildenafil, a specific inhibitor of cGMP degrading enzyme, therefore offers a new strategy for memory improvement and a novel therapy in the future.

Role of amyloid beta in the development of Alzheimer's disease.

Pathophysiological hallmarks in Alzheimer's disease (AD) include severe atrophy in cortex, hippocampus and amygdale (Chan et al., 1999) and senile plaques composed of deposits of amyloid beta (Aβ) peptides and neurofibillary tangles of hyperphosphorylated tau protein (Lee et al., 2001; Selkoe, 2003). The Aβ peptides are produced as a result of two step proteolytic cleavage of transmembrane amyloid precursor protein (APP) by β- and γ-secretases. When γ-secretase cleavage occurs in endoplasmic reticulum it produces $A\beta_{1-42}$ and produces $A\beta_{1-40}$ in trans-Golgi network. The APP cleavage by α-secretase is nonamyloidogenic as this enzyme cleaves within Aβ sequence of APP. The modulation of APP processing is discussed in detail elsewhere (Tang and Liou, 2007). The $A\beta_{1-40}$ and $A\beta_{1-42}$ are the most predominant species found in AD brains (Hsieh et al., 2006). Though other sequences like $A\beta_{25-25}$ have been used extensively in research, the effects of this peptide may be different from the ones found in the brain (Giovannelli et al., 1995). According to amyloid hypothesis (Hardy and Selkoe, 2002), Aβ peptides are the etiological agents of AD pathology. In particular, among the various assembly forms, oligomeric Aβ has been shown to be very potent in disrupting plasticity mechanisms and causing memory impairment (Barghorn et al., 2005; Cleary et al., 2005; Lesne et al.,

2005; Townsend et al., 2006). The levels and ratio of different Aβ fragments determine the toxicity. Among the several Aβ fragments tested, $A\beta_{1-42}$ has been shown to be severe than other forms (Parameshwaran et al., 2007). Amyloid hypothesis is continued to be supported by several reports which utilized animal models of AD over expressing or excessive build up of Aβ, and *in vitro* nerve cells/tissues subjected to Aβ insult. Increasing evidences suggest that AD is due to synaptic pathological processes in which synaptic loss and synaptic dysfunction begins several years prior to severe neuronal loss (Parameshwaran et al., 2008). In such cases synaptic loss in living AD brain neurons could be as high as 38% and that disturbances in APP processing initiate pathologic changes, probably involving synapses (Yao et al., 2003). Synaptic pathology, especially diminished synaptic plasticity in hippocampal Shaffer collateral synapses has been identified as the earliest manifestations of neurodegenerative tauopathies like Alzheimer's disease. There are several strategies proposed for the clearance and or prevention of Aβ deposit, however, this remains a challenge in AD research.

Interpreting genotype-to-phenotype relationships in Alzheimer's disease has advanced during the last two decades and it became apparent that the key challenge for understanding and ultimately treating Alzheimer's disease was to focus not on what was degenerating neurons over the course of the disease but rather on what was interfering subtly and intermittently with episodic declarative memory well before widespread neurodegeneration had occurred (Selkoe, 2002). In other words, one wishes to recognize the factors underlying early synaptic dysfunction in the hippocampus and then mange to neutralize, perhaps even before a definitive diagnosis of Alzheimer's disease can be

made. This steady movement of the field towards earlier stages of the disorder is exemplified by the recognition and intensive study of mild cognitive impairment–amnestic type (Petersen et al., 1999). Patients who die with a diagnosis of mild cognitive impairment have been found to already have a histopathology essentially indistinguishable from classical Alzheimer's disease (Price and Morris, 1999). Therefore, even earlier phases of this continuum are likely to become recognized, and these might show milder histopathology and might have biochemically, but not yet microscopically, detectable Aβ species that mediate synaptic dysfunction.

A wealth of evidence from many published data now supports the once controversial hypothesis that the accumulation and aggregation of Aβ initiates a complex cascade of molecular and cellular changes that gradually leads to the clinical features of mild cognitive impairment (MCI) –amnestic type and then frank Alzheimer's disease (Hardy and Selkoe, 2002; Hardy and Higgins, 1992; Selkoe, 1991). As a result, understanding precisely how Aβ accumulation and assembly compromise synaptic structure and function has become the centerpiece of therapeutically oriented research on the disease.

Several studies have been conducted using synthetic Aβ peptides of either 40 or 42 amino acids, representing the two most common lengths of Aβ found in normal human brain and in the cortical and vascular amyloid deposits of Alzheimer's disease patients. But naturally generated Aβ peptides in brain, cerebrospinal fluid (CSF) or the media of cultured cells are considerably more heterogeneous in length (Busciglio et al., 1993; Haass et al., 1992; Iwatsubo et al., 1994; Podlisny et al., 1995, Saido et al., 1995;

Seubert et al., 1992; Shoji et al., 1992; Vigo-Pelfrey et al., 1993; Wang et al., 1996). Studies of synthetic Aβ peptides have applied concentrations upwards of 1μM, often 10–40μM, because the critical concentration allowing relatively rapid assembly of synthetic $A\beta_{1\text{-}40}$ (the most commonly used peptide) into amyloid fibrils is in the high nanomolar range or greater. Also, synthetic Aβ peptides are often solublized, at least initially, in highly non-physiological solvents (acetonitrile, trifluoroacetic acid, sodium hydroxide, etc.), since these allow the hydrophobic, relatively insoluble peptide to be dissolved and used experimentally.

Different types of synthetic Aβ, including amyloid fibrils, protofibrils (PFs), annular structures, paranuclei, Aβ-derived diffusible ligands and globulomers, have been described over the last two decades (Caughey et al., 2003; Teplow, 1998). Even some of the smaller synthetic aggregates do not fulfill the definition of soluble oligomers: Aβ assemblies that are not pelleted from physiological fluids by high-speed centrifugation. For example, protofibrils are intermediates that were observed in the course of studying the fibrillization of synthetic Aβ (Harper et al., 1997; Hartley et al., 1999; Walsh et al., 1999). They are flexible structures that can continue to polymerize *in vitro* to form amyloid fibrils or can de-polymerize to lower order species. Protofibrils are narrower than bona-fide amyloid fibrils (~4–5nm versus 8–10nm). Ultrastructural analyses of synthetic protofibril preparations by electron microscopy (EM) and atomic force microscopy (AFM) have revealed both straight and curved assemblies up to 150 nm in length. Synthetic Aβ protofibrils have been shown to contain substantial β-sheet structure, as they are able to bind Congo red or Thioflavin T in ordered fashion.

Annular assemblies of synthetic Aβ are donut-like structures with an outer diameter of 8–12 nm and an inner diameter of 2.0–2.5 nm that are distinct from protofibrils by AFM and EM (Bitan and Teplow, 2005; Lashuel et al., 2002). Smaller oligomeric species of synthetic Aβ than protofibrils and annuli have been observed, depending on how synthetic Aβ is prepared and incubated, and some have been designated as Aβ-derived diffusible ligands (ADDL) (Lambert et al., 1998). Apparent ADDL-like oligomeric assemblies have been isolated from postmortem AD brains and their presence correlated with memory loss (Gong et al., 2003). In separate work, chemical stabilization of synthetic $A\beta_{42}$ assembly intermediates has revealed an apparent hexamer periodicity, with hexamer, dodecamer, and octadecamer structures observed (Bitan et al., 2003).

In striking contrast to the properties of these various synthetic assemblies, Aβ peptides that are generated *in vivo* by humans and lower mammals or by cultured cells are diverse with regard to their N- and C-termini, occur naturally in extracellular fluids at low to sub-nanomolar concentrations (Gravina et al., 1995; Näslund et al., 2000; Scheuner et al., 1996; Walsh et al., 2002) and can begin to assemble into metastable dimers, trimers and higher oligomers while still at low nanomolar levels (Podlisny et al., 1995; Walsh et al., 1999). Dimeric, trimeric and apparently tetrameric soluble oligomers have been described in cultured cells (Podlisny et al., 1995; Walsh et al., 1999), and sodium dodecyl sulfate (SDS) stable oligomers of varying sizes have also been detected by Western blotting in amyloid precursor protein (APP) transgenic mouse brain and human brain (Enya et al., 1999; Funato et al., 1999; Kawarabayashi et al., 2004; Lesne et

al., 2006; McLean et al., 1999; Roher et al., 1996). Such natural Aβ oligomers can be resistant not only to SDS but also to chaotropic salts like guanidine hydrochloride and to the Aβ-degrading protease insulin-degrading enzyme which can only efficiently digest monomeric Aβ (Walsh et al., 2002). Aβ oligomers produced by cultured cells could be related to the recently described Aβ*56 species, which represents a soluble, SDS-stable dodecamer found in the brains of at least some APP transgenic mouse lines (Lesne et al., 2006). Like the Aβ oligomers produced from cultured cells (Walsh et al., 2002), Aβ*56 can disrupt synaptic function and thus affect memory (Lesne et al., 2006). Whether Aβ*56, the cell-derived dimers and trimers (Podlisny et al., 1995) and other soluble oligomers observed in biological systems represent stable assemblies of solely Aβ under native conditions or whether such small oligomeric assemblies stably associate with one or more "carrier" proteins *in vivo* is currently unclear. Upon further study, Aβ*56 and Aβ trimers secreted by cultured cells could turn out to share common synaptotoxic properties.

Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway (Townsend et al., 2008). There is considerable evidence that Aβ can bind to several different molecules on the neuronal surface. It has been reported that a brief treatment of cultured neurons with trypsin reduces synthetic Aβ binding (Lambert et al., 1998). Moreover, Klein and co-workers (Gong et al., 2003) have used a far Western technique to demonstrate the binding of synthetic Aβ oligomers called Aβ-derived diffusible ligands to discrete proteins in cortical brain homogenates. Various laboratories have identified candidate receptors that may play a role in Aβ neurotoxicity, including

the α7-type nicotinic acetylcholine receptor (Dineley et al., 2001; Snyder et al., 2005; Wang et al., 2000), the metabotropic glutamate receptor 5 (mGluR5) (Wang et al., 2004), the β-adrenergic receptor (Huang and Gibson, 1993), and the IR (Xie et al., 2002). It is unclear whether some or all of these cell surface proteins contribute to the rapid effects of Aβ on long term potentiation (LTP).

Significant progress has occurred over the last decade in elucidating the molecular basis of memory in the hippocampus (Lynch, 2004). In particular, the synapses in the CA1 region undergo an N-methyl –D- Aspartic acid (NMDA) receptor-mediated LTP that has been well studied from the molecular to the behavioral levels. Among five kinases (Calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), protein kinase C (PKC), Mitogen-activated protein (MAP) kinases (MAPK/Erk), and protein kinase B (Akt/PKB) (which is activated via phosphatidylinositol 3-kinase)) that are known to be necessary for early steps in the LTP cascade, naturally secreted Aβ inhibited the activation of only a subset of these kinases in 14-day-old hippocampal cultures (Townsend et al., 2008).

Sildenafil and Parkinson Disease.

James Parkinson, the English physician, deserves credit for providing the first clinical description of the disease that now bears his name (Parkinson, 1817). However, it was Jean-Martin Charcot, the French neurologist, who recognized all the cardinal signs of the disease (Goetz, 1986). The four cardinal signs of Parkinson's disease (PD) (tremor, bradykinesia, rigidity and postural instability) were all described by Charcot in series of lectures given at the Salpetriére in 1887, 70 years after Parkinson's publication. In addition to cardinal signs, there are other severe and often disabling signs in patients with

Parkinson's disease (Adams et al., 1998). Personality changes are among the earliest and most striking neurobehavioral abnormalities seen in patients with Parkinson's disease (Stocchi and Brusa 2000). Depression is a common disturbance reported in patients with Parkinson's disease and its role is relevant because it can worsen Parkinsonian disability (Hauser and Zesiewicz 1997; Gotham et al., 1986). The frequency of depression in Parkinson's disease is reported to be about 40% (Cummings, 1992). Patients with idiopathic Parkinson's disease can show depressive symptoms associated to erectile dysfunction (ED). ED has been defined by the National Institutes of Health (NIH) as the inability to achieve or maintain an erection sufficient for satisfactory sexual performance (NIH 1993). Sildenafil is an orally active agent for the treatment of ED (Shah, 1998; Goldenberg 1998; Saldana et al., 1998; Goldstein, 1998; Booleil, 1996). Treatment of Parkinson's disease is primarily aimed at improving motor function. However, especially in advanced stages, Parkinson's disease is often complicated by additional problems such as treatment related complications, falls, depression, which may have much greater impact on the patient's quality of life than the cardinal features of Parkinson's disease. Sexual dysfunction has been observed in men with Parkinson's disease, and ED seems to be correlated with disease severity and depressive symptoms (Brown et al., 1990; Cleeves and Findley, 1987; Helfried et al., 2000). In these patients, the higher prevalence of ED can be related to motor symptoms (Longstreth and Linde 1984) and the use of anti-Parkinsonian drugs (Lipe et al., 1990), or, often, can be a consequence of reduced dopaminergic tone and high incidence of depression (often prior to the onset of motor symptoms) (Commings, 1985; Mayeux, 1990), such as in patients treated with anti-dopaminergic drugs (such as reserpine or α-methyldopa) that reduce the functional

efficiency of dopaminergic terminals (McKinney and Kane, 1967; Willner, 1983) or anti-psychotic drugs which antagonize dopamine receptors in the central nervous system. Several observations suggest the involvement of the central dopaminergic system in the overall aetiology of ED (Brown et al., 1990; Cleeves and Findley, 1987). In the last 10 years, however, attention has increasingly been focused on the mesolimbic dopaminergic system, which is now recognized as the major reward pathway in the central nervous system. The last pathway coincides with the mesolimbic dopaminergic system, which projects from the ventral segmental area (a mesencephalic region located in the vicinity of the pars compacta of the substantia nigra) to the nucleus accumbens and other limbic areas (Koob, 1996). The mesolimbic dopaminergic pathway responds to natural or artificial rewards by generating the sensation of pleasure, by encoding the memory of pleasurable cues and by promoting motor and behavioral responses that direct the individual towards the source of pleasure.

For example, this pathway is activated during breast feeding in neonates, during sexual activity or in individuals performing other pleasant tasks. Most of the common drugs of abuse (including psychostimulants, nicotine, diamorphine or heroin, and alcohol or ethanol) activate the mesolimbic system to produce an intense sensation of pleasure and induce drug-seeking behavior (Koob, 1996). Sildenafil has no central action on dopaminergic tone, but an improvement in ED could counter balance the dysfunction of pleasure sensation such as mood disorders. Sildenafil's mechanism of action involves the competitive and selective inhibition of cGMP-specific Phosphodiesterase 5, the primary phosphodiesterase in cavernosal tissue. Inhibition of Phosphodiesterase 5 results in an increase in cGMP levels, with a corresponding increase in cavernosal tumescence and

rigidity (Goldstein et al., 1998). Sildenafil appears to be a safe and effective oral drug for the treatment of ED in patients with Parkinson's disease stage Hoehn-Yahr stages 1–3 and moderate depressive symptoms, only receiving L-dopa (Raffaele et al., 2002). Sildenafil improves, significantly, ability to achieve and maintain erection in PD with depression. Sildenafil has been shown to improve orgasmic function, sexual desire, satisfaction with sexual intercourse and overall satisfaction in Parkinson's patients (Brown et al., 1990; Cleeves and Findley, 1987; Helfried et al., 2000). Several studies suggest that in men with idiopathic Parkinson's disease, moderate depression can be either a consequence or a cause, of ED. It is well known that quality of life in idiopathic PD is primarily influenced by disease severity, disability and depression, but sexual problems, and particularly ED, should therefore, gain greater importance in the treatment of the disease.

Parkinson's disease is associated with increased oxidative stress in the substantia nigra (Przedborski et al., 1995; Thrash et al., 2007; Uthayathas et al., 2007b) and drugs showing antioxidant activity became accepted agents to manage disease severity (Dhanasekaran et al., 2008). It has been shown that sildenafil inhibits the formation of superoxide and the expression of gp47 (phox) (the active sub unit of NADPH oxidase) induced by the thromboxane A2 mimetic, U46619, in corpus cavernosal smooth muscle cells (Koupparis et al., 2005)

Erectile dysfunction is common among individuals with Parkinson's disease, but it is unknown whether it precedes the onset of the classic features of Parkinson's disease. Nitric oxide (NO) is a short lived diatomic free radical species synthesized by nitric oxide synthases (NOS). The physiological roles of NO depend on its local concentrations as

well as availability and the nature of downstream target molecules. At low nanomolar concentrations, activation of soluble guanylyl cyclase (sGC) is the major event initiated by NO. The resulting elevation in the intracellular cyclic GMP (cGMP) levels serves as signals for regulating diverse cellular and physiological processes. The participation of NO and cGMP in diverse physiological processes is made possible through cell type specific spatio-temporal regulation of NO and cGMP synthesis and signal diversity downstream of cGMP achieved through specific target selection. Thus cyclic GMP directly regulates the activities of its downstream effectors such as Protein Kinase G (PKG), Cyclic Nucleotide Gated channels (CNG) and Cyclic nucleotide phosphodiesterases, which in turn regulate the activities of a number of proteins that are involved in regulating diverse cellular and physiological processes. Localization and activity of the NO-cGMP signaling pathway components are regulated by G-protein coupled receptors, receptor and non receptor tyrosine kinases, phosphatases and other signaling molecules. NO also serves as a powerful paracrine factor. At micromolar concentrations, NO reacts with superoxide anion to form reactive peroxinitrite, thereby leading to the oxidation of important cellular proteins.

In summary this section provides literature support for the link between Parkinson's disease erectile dysfunction also harmful effects of beta amyloid on learning and memory processes, and the beneficial effects of sildenafil in the central nervous system.

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3. EVALUATION OF NEUROPROTECTIVE AND ANTI-FATIGUE EFFECTS OF SILDENAFIL

Abstract

Sildenafil, a phosphodiesterase5 inhibitor is widely used for the treatment of erectile dysfunction. Recently FDA approved the use of sildenafil in the therapeutic treatment of pulmonary arterial hypertension. Sildenafil crosses the blood brain barrier and has been shown to enhance memory. Tremor, rigidity and akinesia are the most common symptoms seen in Parkinson's disease. Fatigue and sexual dysfunction are the other prominent features seen in Parkinson's disease. Interestingly, sildenafil is used therapeutically to treat sexual dysfunction in Parkinson's disease patients. Currently research on Parkinson's disease focuses on developing novel drug therapies for retarding the nigral dopaminergic neurodegeneration. Hence, we investigated the anti-fatigue and neuroprotective effects of sildenafil. In this study, the effect of sildenafil on fatigue was evaluated using forced swim test in mice. Sildenafil had no effect on fatigue as seen by the swim time. With regard to neuroprotective effects, we investigated the effects of sildenafil using two animal models of Parkinson's disease. In this study, 6-hydroxydopamine-lesioned (unilateral) rats and MPTP treated mice were used as the animal models of Parkinson's disease. 6-hydroxydopamine-lesioned rats were used to determine the effect of sildenafil on rotational behavior. Ipsilateral or contralateral

rotational behavior can indicate the amphetamine-like activity or apomorphine-like activity of sildenafil. Sildenafil did not induce contralateral or ipsilateral rotations in 6-hydroxydopamine-lesioned rats. Sildenafil did not protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopamine depletion in the striatum.

Introduction

Parkinson's disease occurs due to the progressive degeneration of dopaminergic neurons in the substantia nigra compacta region. Degeneration of nigral neurons result in the dopamine depletion in striatum. Certain dopaminergic agonists (bromocriptine, apomorphine) increase the sexual function and also protect the nigrostriatal dopaminergic system. MPTP and 6-hydroxydopamine (6-OHDA) are potent dopaminergic neurotoxins that can severely damage the nigral dopaminergic neurons resulting in striatal dopamine depletion (Dhanasekaran et al., 2006; Heikkila et al., 1984, Manyam et al 2004; Muralikrishnan and Ebadi, 2001; Ungerstedt et al., 1974). Hence MPTP-treated mice and 6-OHDA lesioned (unilateral) rats have been used to study the etiopathology of Parkinson's disease. These animal models of Parkinson's disease have also been used to evaluate the neuroprotective properties of various drugs (Beal et al., 1998; Dhanasekaran et al., 2006; Manyam et al., 2004; Muralikrishnan and Ebadi, 2001; Schober, 2004; Thongsaard and Marsden, 2002). 6-OHDA-lesioned rat model has a measurable motor deficit, as seen by the stereotypic rotation (Ungerstedt et al., 1974). Hence 6-OHDA-lesioned rat model of Parkinson's disease has proved useful in the pharmacological characterization of numerous drugs for their effects on dopamine receptor or dopamine

release (Dunnett and Bjorklund, 1999; Manyam et al., 2004; Shimohama et al., 2003; Tharakan et al., 2006).

Fatigue is one of the most frequent and most disabling non-motor problems and results in negative impact on cognitive and physical function, and quality of life in patients with the Parkinson's disease. Morning tiredness increases the risk for erectile dysfunction due to fatigue and a decrease in libido. Chronic fatigue occurs with aging, depression, diabetes, and Parkinson's disease and is one of the most common symptoms in primary care (Kroenke et al., 1988; Margel et al., 2004; Tharakan et al., 2006). In spite of this, there are very few pharmacological drugs or therapies in the treatment of fatigue.

Sildenafil, a potent inhibitor of phosphodiesterase 5, has been therapeutically used for men with erectile dysfunction in Parkinson's disease (Raffaele et al., 2002). Sildenafil can cross the blood-brain barrier and significantly improve learning/memory by modulating glutamate-NO-cGMP signal transduction pathway. Sildenafil has been shown to increase neurogenesis, functional recovery and decrease the neurological deficits in rats after stroke (Wang et al., 2005, Zhang et al., 2002, 2006). Theophylline and sildenafil (phosphodiesterase inhibitors) have shown to prevent oxidative stress by increasing intracellular cAMP and cGMP (Milani et al., 2005). In addition to its cognitive enhancing ability recent studies have shown that sildenafil possess neuroprotective properties (Uthayathas et al., 2006, 2007; Wang et al., 2005; Zhang et al., 2002). Sildenafil has been approved by FDA for use in erectile dysfunction and pulmonary hypertension. Recently sildenafil has been shown to enhance memory in Alzheimer's disease animal models (Devan et al., 2006). However, the anti-fatigue and

neuroprotective effect of sildenafil are unknown. In the present study, we evaluated the neuroprotective effect of sildenafil against MPTP-induced dopaminergic neurotoxicity in mice. Dopamine releasing action or agonistic activity of sildenafil was investigated using 6-OHDA lesioned rat.

Materials and methods

Chemicals: Coomassie plus protein assay reagent kit was purchased from Pierce Biotechnology Inc. (Rockford, IL). Sildenafil was kindly donated by Eczacibasi Drug Company (İstanbul, Turkey). All other chemical were purchased from Sigma chemicals, St. Louis, MO, USA.

Animals: Male C57/BL6 mice purchased from Charles River laboratories (Wilmington, MA) weighing 20–25g were used for both MPTP experiment and swim test. Commercially available 6-OHDA lesioned (unilateral) rats weighing 250-300g purchased from Charles River laboratories (Wilmington, MA) were used for 6-OHDA model. All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the Auburn University.

MPTP treated mice: Mice were divided into 4 groups (A–D). Mice in group A were used as normal controls, and injected with saline intraperitoneally. Mice in group B received two intraperitonial injections of MPTP (30mg/kg, 16 hours apart). Mice in group C were injected with MPTP similar to group-B mice and in addition received sildenafil (10mg/kg, i.p.) 30 minute prior to MPTP injection. Similar regime of administration of D-deprenyl protected nigrostriatal neurons against MPTP induced

dopaminergic neurotoxicity previously (Muralikrishnan et al., 2003). Mice in group D received intraperitonial injection of sildenafil only (10mg/kg).

Behavioral analysis: For a period of 3 hours after MPTP/sildenafil treatment, mice were continuously observed by two examiners (trained in evaluating different animal behaviors and blind to the drug treatment). Akinesia, hind limb abduction, jumping and tremor were monitored during this period following the previously established procedure (Mitra et al., 1992; Sedelis et al., 2001, Dhanasekaran et al., 2006). Tremor was scored for intensity on a scale of 0–4. Akinesia was measured by noting the latency of the animals to move all four limbs in a unit of time (seconds). Mice exhibited tremor 2–4 minutes and akinesia 90 minutes after receiving MPTP.

Neurochemical analysis: Saline and drug treated mice were sacrificed six days after the last MPTP injection. Brain nuclei, substantia nigra and striatum were micropunched (Muralikrishnan and Ebadi, 2001). Micropunched brain nuclei were sonicated in 0.4N perchloric acid. These samples were then centrifuged and the supernatant was used for the analysis of neurotransmitters and their metabolites using HPLC-electrochemical detector. The results were expressed as Pico moles per milligram of tissue (Dhanasekaran et al., 2006). The mitochondrial P_2 fractions were prepared from mice brains. For monoamine oxidase assay, 100µl of the mitochondrial preparation was incubated with 10 mM potassium phosphate buffer, pH 7.2, and the reaction was started by the addition of 3.07 mM kynuramine. The reaction was terminated by addition of 300µl ice-cold 0.4N perchloric acid. The product spun at 10,000g for 10 minute and the supernatant was added to twice the volume of 1N sodium hydroxide. Fluorescence intensity was measured at excitation 315 nm and emission 380 nm. Quantity of formed,

4-hydroxyquinoline, was determined from a standard curve. The enzyme activity is expressed as nmol of 4-hydroxyquinoline formed/mg protein/hour (Muralikrishnan and Ebadi, 2001). Protein was assayed employing the coomassie plus protein assay reagent.

6-Hydroxydopamine lesioned rats: The efficacy of 6-OHDA lessoning was validated using amphetamine (5mg/kg, i.p.) and tested in the rotometer for rotational behavior. Rats exhibiting more than seven ipsilateral rotations per minute to amphetamine were considered as a valid parkinsonian animal model and used in the present study. This test allows us to select rats with the lesion of niagro-striatal tract of at least 90 percent in the lesioned site. After allowing sufficient wash out period (10 days from amphetamine experiment), the validated 6-OHDA lesioned rats were injected intraperitoneally with 1, 2.5, 5 and 10 mg/kg sildenafil and their rotational behavior was monitored in automated rotometer bowls for 60 minutes (Manyam et al., 2004; Tharakan et al., 2006).

Forced swim test: Mice were subjected to forced swim test immediately after the administration of sildenafil (2.5, 5 and 10mg/kg, i.p.). Swim test was performed by adapting the previously described techniques (Porsolt et al., 1977; Tharakan et al., 2006). Forced swim until complete exhaustion is considered as a reliable method for evaluating fatigue in animals (Kim et al., 2002). The total swim time of mice was calculated from the time they entered the water until complete exhaustion as evidenced by sinking (Tharakan et al., 2005, 2006). Once the animal sunk, they were sacrificed with an overdose of pentobarbital.

Statistical analysis: All the data were expressed as the means ± SEM. The statistical significance was evaluated by the one-way analysis of variance (ANOVA) using Sigma Stat version 2.03. Values of p less than or equal to 0.05 were considered significant.

Results

The present study evaluated the neuroprotective action of sildenafil in two different animal models of Parkinson's disease. The first experiment was conducted to assess the neuroprotective potential of sildenafil against MPTP-induced dopamine depletion. Effects of sildenafil and MPTP on serotonin, norepinephrine, dopamine and its metabolites in the nigrostriatal tract is presented in Table 1. Administration of MPTP caused a significant reduction in the levels of dopamine in the striatum (Table 1, [a]$p<0.001$). Sildenafil did not prevent the dopamine depletion induced by MPTP in the striatum (Fig.2). Sildenafil (10mg/kg) alone did not affect the content of dopamine, serotonin or norepinephrine in the nigrostriatal tract (Table 1). Intraperitoneal administration of MPTP in mice induced significant behavioral abnormalities including tremor, straub tail, hind limb abduction, jumping and akinesia. MPTP caused a long-lasting motor impairment in C57/BL6 mice (Table 2). Sildenafil (10mg/kg) alone did not induce any significant behavioral changes, however there was licking of the genitals. Sildenafil or MPTP had no effect on the total mitochondrial monoamine oxidase activity in the mice brain (Fig. 1). Administration of sildenafil (1, 2.5, 5 and 10 mg/kg) to 6-OHDA-lesioned rats did not induce any stereotypic rotations. Sildenafil (2.5, 5 and 10 mg/kg) did not show any significant difference on the total swim time as compared to the controls (Fig. 3).

Discussion

Sildenafil has been shown to have modulatory activity on central dopaminergic pathways (Ferrari et al., 2002). However, in the present study sildenafil had no protective effect against MPTP-induced behavioral changes and dopamine depletion. Sildenafil did not induce ipsilateral or contralateral rotations in 6-OHDA lesioned rat. In the forced swim test, sildenafil had no anti-fatigue activity. MPTP has to be converted to MPP^+ and specifically taken up by the striatal dopamine to exert is toxicity. Hence monoamine oxidase plays an important role in MPTP-induced toxicity. However, sildenafil had no effect on monoamine oxidase activity.

Amantadine, alone or in combination with levodopa, reduces fatigue, tremor and bradykinesia in Parkinson disease (Moser and Besler-Panos, 2006). Sildenafil exert its action through phosphodiesterase5 inhibition and has shown to combat erectile dysfunction. Fatigue is an important component of sexual dysfunction. Hence, we evaluated the anti-fatigue effect of sildenafil using forced swim test. However, sildenafil (10mg/kg) did not possess any anti-fatigue effect.

Akinesia, jumping, tremor and straub tail were observed immediately after MPTP administration. These acute behavioral changes induced by MPTP may be due to the excessive release of monoaminergic neurotransmitters (dopamine and serotonin) in the nigrostriatal tract (Mitra et al., 1992). Deprenyl, salicylic acid and bromocriptine have different pharmacological effects but they all possessed significant antioxidant activity as seen by their ability to scavenge the hydroxyl radicals. The above drugs due to their antioxidant activity protected against MPTP-induced behavioral changes and dopamine depletion (Muralikrishnan et al., 2003). Our unpublished results also show that sildenafil

had no antioxidant activity. Interestingly, the results seen in MPTP clearly correlate with the findings on the 6-hydroxydopamine lesioned rats. Bromocriptine, SKF-38393 and apomorphine (dopamine agonist) induces contralateral rotation in 6-OHDA lesioned (unilateral) rats and also exhibited neuroprotective effects in mice treated with MPTP. Sildenafil had no effect on the 6-OHDA lesioned (unilateral) rats, indicating the lack of agonistic or dopamine releasing effect.

Recent advances in our understanding of the pathophysiological and molecular mechanisms involved in Parkinson's disease have led to the development of novel and rational pharmacological therapies. Phosphodiesterases are widely expressed in the body. Phosphodiesterase 5 inhibitors are widely used for the treatment of erectile dysfunction and currently tested for therapeutic use in many disorders. It has been shown that the major phosphodiesterase expressed in nigrostriatal tract is phosphodiesterase-10A (Xie et al., 2006). Higher levels of cGMP in the substantia nigra pars compacta was found in the MPTP induced parkinsonian animals (Chalimoniuk et al., 2006). Chronic treatment of sildenafil has been shown to have neuroprotection by enhancing neurogenesis and functional recovery in a middle cerebral artery occlusion model of stroke in rats (Zhang et al., 2002). The experimental protocols used in our study differ considerably from this work as the drug was administered acutely and prior to the toxin in MPTP model. Therefore, a more chronic administration of sildenafil along with appropriate dose timing might acquiesce beneficial results in 6-OHDA and MPTP models of Parkinson's disease.

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Legends

Table 3.1: Effects of sildenafil &/or MPTP on norepinephrine and serotonin in the nigrostriatal tract: C57/BL 6 mice were treated with MPTP (30 mg/kg, i.p.) and/or sildenafil (10 mg/kg, i.p.). Effects of Sildenafil and MPTP on the nigrostriatal dopamine, serotonin, DOPAC and norepinephrine content (pmoles/mg of tissue) were determined using HPLC-ECD.

Table 3.2: Effects of sildenafil on MPTP-induced behavioral changes in mice: C57/Bl 6 mice were treated with MPTP (30 mg/kg, i.p.) and/or sildenafil (10 mg/kg, i.p.). Five animals were treated in each group. MPTP-treated animals differed significantly. Long-lasting motor impairment in mice caused by MPTP is not reversed by administration of sildenafil 30 minutes prior to treatment. (*p>0.05, as compared to the control, n=5)

Fig. 3.1: Effects of sildenafil and/or MPTP on MAO activity in the mitochondria of mice brain. The mitochondrial P_2 fractions were prepared from mice brains. Sildenafil did not have any significant effect on MAO activity. Data represents total MAO activity measured in 4-hydroxy-quinoline formed (nmole /hr/mg protein, n=5). Data are mean ± SEM, n=5.

Fig. 3.2: Effects of sildenafil and/or MPTP on MPTP induced significant depletion of dopamine ($^a p$<0.001, as compared to the control, n=5). Sildenafil did not block MPTP-induced toxicity. Values represent Mean ± SEM.

Fig. 3.3: Effect of Sildenafil on fatigue. Sildenafil (2.5, 5 & 10 mg/kg) did not exhibit anti-fatigue effect (as seen in the swim time) in mice. Data represent mean swim time ± SEM (n=5).

Table 3.1: Effects of sildenafil &/or MPTP on norepinephrine and serotonin in the nigrostriatal tract:

Treatment	NE (pmol/mg tissue)	5 HT (pmol/mg tissue)
Control	17.39±2.26	9.52±1.56
MPTP	16.90±1.53	9.52±0.92
Sildenafil	15.37±0.91	7.71±0.89
MPTP + Sildenafil	15.95±0.96	7.90±0.72

Table 3.2: Effects of sildenafil on MPTP-induced behavioral changes in mice:

	Control	MPTP	Sildenafil	Sildenafil +MPTP
Tremor	ND	3.14 ±.16	ND	3.23 ±.18
Straub tail	ND	Detected	ND	ND
Akinesia	2.15 ±.35	21.23±.14	2.46 ±.27	23.42±.51
Jumping	ND	Observed	ND	Observed
Hind limb abduction	ND	Observed	ND	Observed

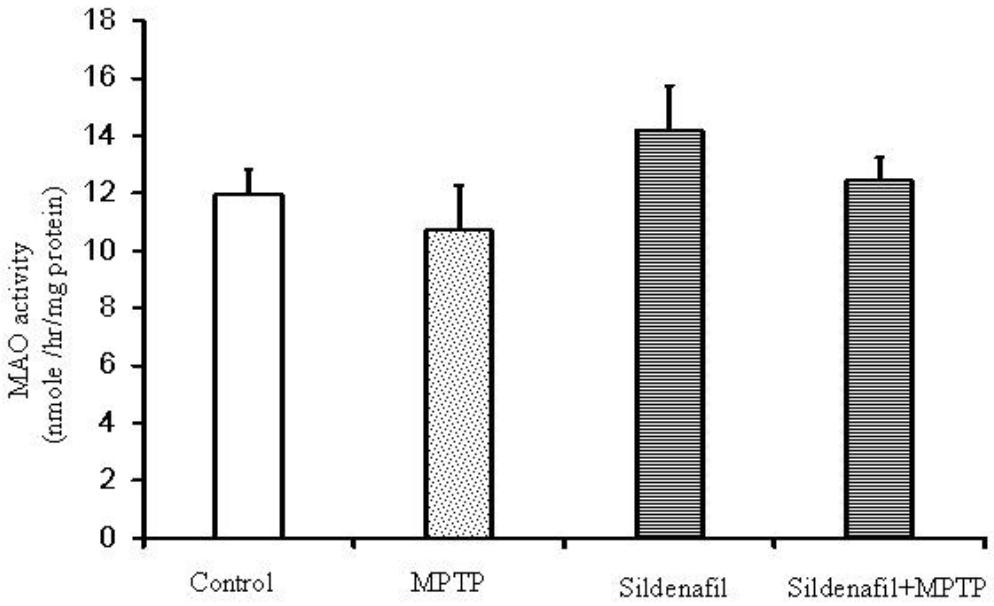

Figure. 3.1

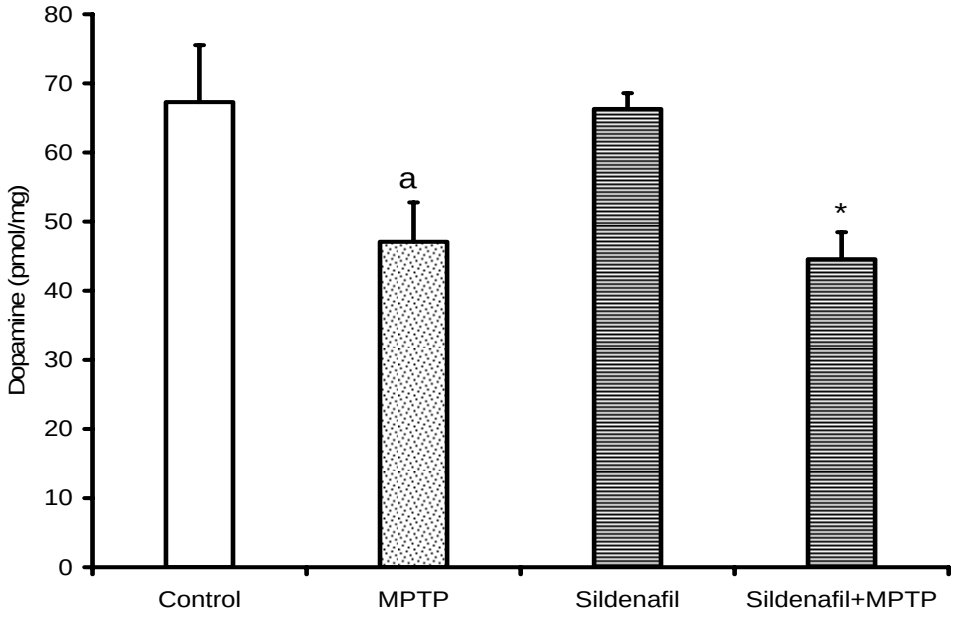

Figure.3.2

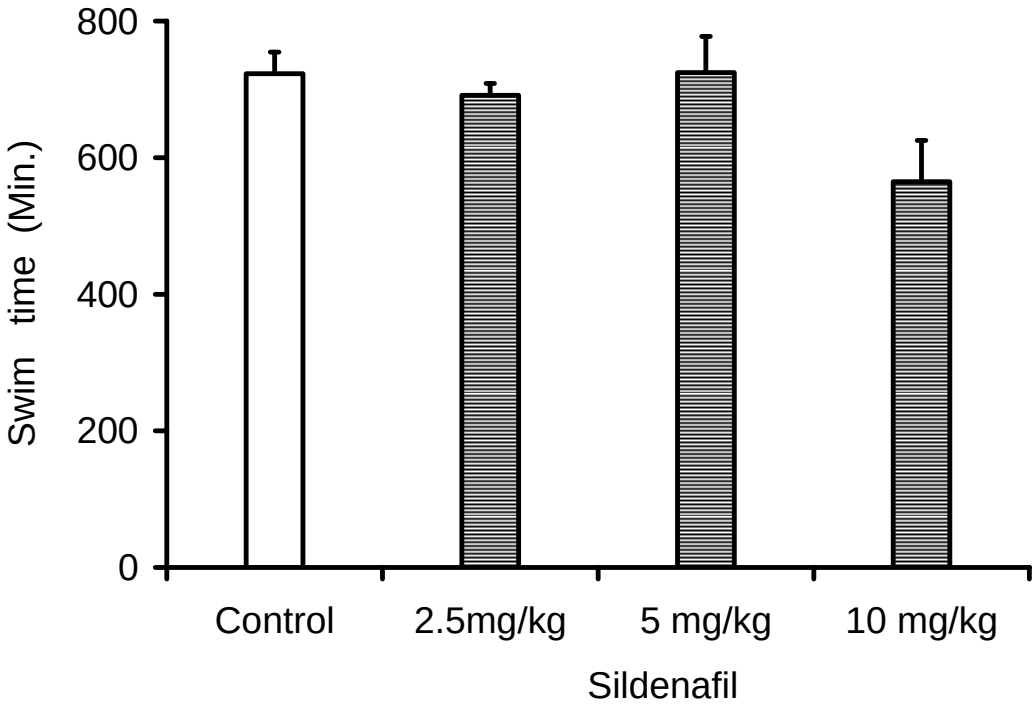
800
600
400
200
0
Swim time (Min.)
Control
2.5mg/kg
5 mg/kg
10 mg/kg
Sildenafil

Figure. 3.3

4. SELECTIVE INHIBITION OF PHOSPHODIESTERASE 5 ENHANCES GLUTAMATERGIC SYNAPTIC PLASTICITY AND WORKING MEMORY IN MICE

Abstract

Phosphodiesterases belongs to a family of proteins that control the metabolism of cyclic nucleotides and phosphodiesterase 5 (PDE5) is expressed in several brain regions. Targeting phosphodiesterase 5 (PDE5) for enhancing cellular function is one of the therapeutic alternatives and widely recognized after the work with sildenafil for male erectile dysfunction. We have investigated in mouse whether phosphodiesterase5 inhibition *in vivo* enhance synaptic transmission in the hippocampus. We have found that single administration of sildenafil, a specific phosphodiesterase5 inhibitor, enhances hippocampal dependent behavioral tasks. To elucidate the underlying mechanism in the memory enhancement, effects of sildenafil on long term potentiation (LTP) was measured. The level of LTP was significantly elevated in mice treated with sildenafil (1 mg/kg/day) for 15 day compared to control. Thus, our results suggest that the neural mechanisms of memory enhancement through phosphodiesterase5 inhibition could be due to glutamatergic synaptic modification.

Introduction

Learning and memory performance in human is determined by the strength of synaptic connections and the ability of neurons to reestablish the communication (Costa-Mattioli et al., 2007). Communication among neurons affected by several factors and to

some extent can be manipulated by memory enhancers. Memory enhancing substances have been used in prehistoric period as part of the tradition of Ayurveda, a system of folk medicine that originated in India several thousand years ago (Misra, 1998). In recent times, cholinergic and glutamatergic system have been targeted for development of nootropic agents. Choline esterase inhibitors like donepezil and allosteric modulator of AMPA receptors such as piracetam, pramiracetam and aniracetam are being primarily used to improve memory, mood and behavior (Wijayawardhane et al., 2007). However, the resulting adverse effects associated with these agents have limited their use. Therefore, it is worthwhile to explore therapeutically safe medicines for the treatment of various cognitive disorders. Regulation of cyclic guanosine monophosphate (cGMP) pathway is a vital pharmacological response of the AMPA receptor potentiators and contributes to the efficacy of AMPA modulators in rodent models of cognition (Ryder et al., 2006). It has been shown that amyloid beta, a peptide considered as a hallmark in AD, stimulates cGMP degradation (Paris et al., 1999).

Cyclic nucleotide phosphodiesterases (PDEs) controls the hydrolysis of cGMP and cAMP and they influence numerous pharmacological processes including ion channel function and learning. Inhibition of phosphodiesterase2 enhanced long-term potentiation of synaptic transmission without altering basal synaptic transmission in rats (Boess et al., 2004). Therefore, modulating second messenger system through phosphodiesterase may be a potential target for central nervous system drug development (Boess et al., 2004; Uthayathas et al., 2007b). In middle cerebral artery occlusion stroke model, inhibition of phosphodiesterase5 using sildenafil has been shown to enhance neurogenesis and functional recovery (Zhang et al., 2002). Sildenafil has been shown to

cross the blood brain barrier and did not produce any beneficial effect on nigro-striatal dopaminergic neuron function nor did it potentiate the neurotoxic effects of MPTP (Uthayathas et al., 2007a; Janis et al., 2008). Interestingly, sildenafil has been shown to enhance memory in a range of Alzheimer's disease models (Erceg et al., 2006; Devan et al., 2006). Sildenafil administration improved the cognitive performance in diabetic conditions and electroconvulsive shock-induced animal models (Patil et al., 2006). Inhibition of phosphodiesterase5 by sildenafil has been shown to enhance object memory in normal mice (Rutten et al., 2005). Although there is some evidence for enhancement of memory by phosphodiesterase5 inhibition, studies exploring the action of phosphodiesterase inhibitors in memory enhancement *in vitro* or *in vivo* are still limited and in-depth investigation of effect on cellular signaling is scanty. Retrograde signaling by nitric oxide (NO) signal transduction has been well characterized and shown to play a major role in the neurotransmitter release machinery (Arancio et al., 1996). On the other part NO activate guanylyl cyclase resulting in the formation of cGMP, with cGMP-dependent protein kinase being one of the downstream mechanisms contribute to synaptic plasticity (Feil et al., 2005).

Present work was undertaken to elucidate the memory enhancing mechanism of phosphodiesterase5 inhibition in mice. We postulate that sildenafil act on cGMP degrading phosphodiesterase 5 in the hippocampus and augment postsynaptic AMPA receptor expression to enhance synaptic plasticity. Here we show that sildenafil administration enhances hippocampal dependent memory and long term potentiation in mice. In addition, we demonstrate that these beneficial effects of sildenafil could be

explained by the changes in the communication ability of neurons and strengthened synaptic connections in the hippocampus. Our data indicate that moderate amounts of sildenafil daily may provide surprising protection against, or delay, the onset of AD.

Materials and Methods

Animals: Two month old male C57/BL6 mice (Charles River, Wilmington, MA) weighing 20–25g were used for both behavioral study and electrophysiological experiments. All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the Auburn University.

Behavioral experiments

Mice were analyzed in two behavioral tasks: first, in the Object Recognition task; and second, in the Two trial Y Maze task, in order to establish an effective dose. Mice were intensively handled (5 d by ~3 min per mouse) and weighed once a week before behavioral testing.

Object Recognition Task (ORT)

Mice were randomly divided into four experimental groups (10 mice per group) as follows: vehicle; sildenafil 0.5mg/kg; sildenafil 1mg/kg; and sildenafil 2mg/kg. Object recognition task was performed as described elsewhere (Rutten et al., 2005). The apparatus consisted of a Plexiglas open-field box (52×52×40cm). Half of the 40cm high wall was covered with blue paper and the other half was left transparent. Two transparent glass bottles (diameter 2.5cm, height 6.5cm) filled with sand were placed symmetrically about 5cm away from the blue wall. During the second trial a gray color

plastic cube (2.5×2.5×6cm) was placed for one of the glass bottle. A testing session comprised of two trials. The duration was 3 and 5min for first (exploration) and the second (retention test) trial respectively. Mice were injected intraperitoneally (i.p.) either vehicle or sildenafil immediately after trial one and put back in its home cage. Subsequently, after a predetermined delay interval (24h), the mouse was put back in the apparatus for the second trial (T2). Before each trial, the surface of the box was sprayed with a diluted ethyl alcohol solution to erase any scent cues. In the present experiment, the inter trial interval of 24 hours was used because pilot data indicated that a 3-min training trial induces significant retention at a 1-hr, but not 24-hr, delay with normal mice. Behavior was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. Times spent exploring each object during T1 and T2 was recorded manually using a personal computer and a timer. In order to avoid the presence of olfactory cues the objects was always thoroughly cleaned using diluted ethyl alcohol before each trial.

Two trial Y Maze test

Two trial Y Maze was used to measure spatial recognition memory as described previously (Ma et al., 2007). The Y-maze test consisted of two trials separated by a time interval. In the first (acquisition) trial, one arm of the maze (subsequently called novel arm) was closed with a guillotine door. Mouse was placed at the distal end of one arm (starting arm), head pointing away from the center of the maze, and was allowed to visit the two accessible arms of the maze for 3 min. At the end of the acquisition trial, the

mice were injected with either vehicle or sildenafil (0.5, 1, 2mg/kg) and placed in their cage for a period, called inter-trial interval (ITI) of 4hrs. During the second (Retention test) trial mouse has free access to the three arms and was allowed to explore the maze for 5 min. Behavior of mice in maze was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. This test is based on the interest of mice for novelty; hence they explore preferentially unknown territories. The number of explorations of each arm was counted every min and the percentages of visits in the novel arm with respect to the total number of visits in the three arms during each min of the test was calculated.

Electrophysiological recordings

Drug treatment and slice preparation

Results of behavioral experiments were analyzed to find out the effective dose and a dose of 1mg/kg was administrated for 15 days daily. At the end of 15 days, *in vitro* brain slice experiments were performed as described previously (Bukalo et al., 2004). After mice were deeply anaesthetized and decapitated, the brain was quickly extracted and immediately placed into ice-cold cutting solution (in mM): 250 sucrose, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgCl_2$) that was oxygenated with 95% O_2 and 5% CO_2. The brain was sliced (350-µm transverse slices) using a Vibratome (Warner instruments, Hamden, CT) and incubated in oxygenated artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgCl_2$) at room temperature for one hour.

Extra-cellular recordings of fEPSPs

Slices were transferred to submerged type recording chamber and continuously perfused (2ml/min) with oxygenated ACSF. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum using glass microelectrodes (2–4 MΩ) filled with ACSF. Evoked EPSPs were elicited by stimulating the Schaeffer collateral fibers with a bipolar Teflon-coated platinum electrode placed ~300μm closer to the CA3 sub field than the recording electrode. Test pulses were set at an intensity that evoked 50% of maximum EPSP amplitude before intrusion of a population spike. Field excitatory postsynaptic potentials (fEPSPs) in CA1-CA3 synapses were recorded on a computerized stimulating and recording unit. Basal synaptic transmission was monitored for 15 minutes before inducing long term potentiation.

Analysis of synaptic transmission and plasticity

Long-term potentiation (LTP) was induced by a theeta burst (TBS) protocol consisting of five TBS delivered at 20 seconds interval. TBS consisted 10 bursts delivered at 5 Hz. Each burst consisted four pulses delivered at 100Hz. Data were acquired and fEPSPs were measured using LTP program software and analyzed offline. Post-tetanic potentiation (PTP) and LTP were defined as the percentage change from baseline for the 7 minutes immediately after TBS and for the last 5 minutes of the 60-min post-TBS recording period, respectively.

Statistical analysis

Experiments were performed with the investigator blinded to the treatment the animals received. Data were expressed as mean ± S.E.M. Electrophysiological results were normalized to the basal values and analyzed by ANOVA with Turkey's post-hoc analysis. The level of significance was set for $P<0.05$. All statistical calculations were performed on a computer using a commercially available statistical package.

Results

Behavioral assessment

We examined the memory enhancing capabilities of sildenafil using a battery of behavioral tests in order to establish the effective dose since there is disparity in dose among the literature. Further more, there is a lack of reports on memory enhancing ability of sildenafil in this age of mice.

Effect of PDE5 inhibition on object recognition memory

We first studied the effect of sildenafil on object recognition memory by first allowing them to explore two identical objects and after a delay of 24 hours memory retention was tested with a novel object. Post-hoc analysis shows that the level of exploration as measured by object discrimination index (DI) was high in groups treated with 1 and 2 mg/kg sildenafil compared to .5 mg/kg and control (Figure 4.1.A, $P<.05$). Control mice had sufficient time to loose the memory and were unable to discriminate between the novel object and familiar object and the DI was of chance level.

Effect of PDE5 inhibition on spatial recognition memory

We then studied the effect of sildenafil on spatial memory by first restricting them to explore only in two arms of Y maze and after a delay of 4 hours memory retention was tested by allowing to explore in all arms. Effect of sildenafil on the number of entry in novel arm, the arm opened only during second trial, in Y maze is presented in Figure 4.1.B. The spatial memory was significantly enhanced in the group treated with 1 mg/kg sildenafil compared to the groups control, .5 mg/kg and 2 mg/kg. Control mice in Y maze test had sufficient time to loose the memory and were unable to discriminate between the novel arm and familiar arms. During the second trial, control group showed equal alterations to all three arms, no arm differences were found, that validate the delay time. Similar results of no arm differences after 4 hours delay time in normal mice were reported previously (Ma et al., 2007).

Electrophysiological assessment

Basal synaptic transmission in PDE5 inhibition

To characterize whether phosphodiesterase5 inhibition altered the basal synaptic function to enhance the memory performance, presynaptic fiber volley (FV) and field excitatory postsynaptic potential (fEPSP) from CA1 synapses in hippocampal Schaffer collateral projections were recorded for different stimulations given at CA3 neurons. In order to elucidate the possible presynaptic modifications, change in amplitude of FV with stimulation intensity was analyzed. Size of FV is considered as a measure of number of recruited presynaptic neurons (Xiao et al. 2007). The amplitude of FV was significantly increased in the group treated with sildenafil compared to control (Fig. 4.2.A), indicating

the enhanced ability to transfer the presynaptic stimulus into an axonal depolarization by the phosphodiesterase5 inhibition. Similarly, analysis of input out put curve (Fig. 4.2.B) revealed that the slope of fEPSP's for a given stimulus intensity was significantly elevated by phosphodiesterase5 inhibition. Combination of FV amplitude versus slope of fEPSP suggest that phosphodiesterase5 inhibition results in a significant increase in basal synaptic transmission in hippocampal slices across a broad range of stimulus intensities. Examination of both FV and fEPSP input output data reveal a possible modification of pre and post basal synaptic function by phosphodiesterase5 inhibition. Surprisingly phosphodiesterase5 inhibition did not alter the paired pulse facilitation (PPF) (Fig. 4.2.C). Presynaptic dependent short term plasticity is measured by PPF that believed to correlate the release probability of glutamate (Chan et al., 2006). Overall enhancement of basal synaptic transmission through phosphodiesterase 5 inhibition however failed to produce a difference in short term plasticity in our experiment.

Post tetanic potentiation and long term potentiaton in PDE5 inhibition

To examine how phosphodiesterase 5 inhibition can affect different forms of plasticity, we analyzed the magnitude of PTP and LTP. A robust theta burst tetanization paradigm consisting of five trains with an inter-train interval of 20 s was used to induce LTP. In agreement with the results of basal synaptic transmission, PTP another form of short term plasticity was unaffected by the inhibition of phosphodiesterase5 (Fig. 4.3.A). Interestingly, LTP was significantly elevated by phosphodiesterase 5 inhibition (Fig. 4.3.A); sixty minutes after the induction of LTP the slope of fEPSP were145±3.3 and 124±4.7 percent of base line in sildenafil treated group and control group respectively

(Fig 4.3.B). Collectively these results shows that phosphodiesterase5 may play a role in the synaptic plasticity, predominantly on the protein synthesis dependent LTP.

Discussion

Results in this study clearly demonstrate that *in vivo* administration of selective phosphodiesterase5 inhibitor positively modulate the hippacampal function by enhancing basal synaptic transmission, synaptic plasticity and working memory. Learning and memory function is chiefly determined by hippocampal synaptic plasticity (Milner et al., 1998); therefore we examined the excitatory synaptic transmission in synapses between the glutamatergic fibers of hippocampal CA3 and CA1 pyramidal cells. Interestingly, daily application of phosphodiesterase5 inhibitor for a brief 15 days (1 mg/kg) was capable of producing enhanced hippocampal function. Phosphodiesterase 5 is expressed in hippocampus, cortex and cerebellum of mice and rat (Blokland et al., 2006; Shimizu-Albergine et al., 2003; Van Staveren et al., 2003), therefore, a selective phosphodiesterase5 inhibitor can act on these brain regions to enhance memory. Synaptic plasticity is a critical component of the neural mechanisms underlying learning and memory. Long-term potentiation, an activity-dependent synaptic plasticity, plays a key part in the forms of memory mediated by hippocampus. Hippocampus is one of the brain structures more directly related with learning and memory and widely used as a model to study synaptic plasticity. LTP in general regarded as postsynaptic event (Zamanillo et al., 1999) and supported by the evidences that LTP could occur due to an increase in single channel conductance or an increase in the number of AMPARs expressed at synapses (Benke et al., 1999; Luthi et al., 2004). LTP expression also involves additional

AMPARs insertion into the postsynaptic membrane and stimulation of their biosynthesis. Phosphorylation of AMPRs is one means by which the biophysical properties of these channels are modified.

Paired pulse facilitation (PPF) in consistent with post titanic potentiation (PTP) propose a minimum contribution of presynaptic mechanism compared to the postsynaptic mechanism in the enhanced synaptic plasticity in our study. Normal Schaffer collateral LTP has been linked to post synaptic mechanisms (Zamanillo et al., 1999) however the possibility of involvement of presynaptic mechanism should not be over looked (Choi et al., 2003, Lisman, 2003, Luscher et al., 2000, Sanes and Lichtman, 1999; Zakharenko et al., 2001). Enhanced LTP and unaffected PTP in our study suggest that the memory enhancing mechanism of chronic sildenafil treatment might be chiefly at the postsynaptic glutamatergic fibers.

Also consistent with elctrophysiological findings behavioral tests revealed that administration of sildenafil enhance the memory retention. These results are in agreement with the findings reported elsewhere (Rutten et al., 2005). The delay between first trial and retention test was preset to 4 hours and 24 hours in Y maze and ORT respectively since we expected the drug treatments to improve memory performance. This delay was sufficient to produce a loss of memory retention in mice of this age group. As expected the control mice did not discriminate between the objects above chance level and in Y maze they had equal alterations in all 3 arms.

Learning and memory capabilities have been directly linked to hippocampal synaptic plasticity and phosphodiesterase5 inhibition may play a role in determining

related cognitive/ behavioral functions in mice, although modulation in other forebrain regions may also contribute to these enhancements. Since the behavioral battery used in this study has been widely recognized for the hippocampal dependant memory the results discussed herein give strong evidence for the involvement of glutamatergic alterations in the memory enhancing mechanism of phosphodiesterase5 inhibition. To our knowledge this is the first report giving evidence for the glutamatergic synaptic modification through phosphodiesterase5 inhibition explaining memory enhancing capability of sildenafil. Application of sildenafil for a brief period of 15 days in our study might have extended the action of cGMP to effect de novo protein synthesis in the postsynaptic element. These findings raise the interesting possibility that regulators of cGMP coupled ion channels could serve as therapeutic targets for the enhancement of memory in neurodegenerative disorders such as Alzheimer's disease.

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Legends

Fig 4.1. Sildenafil treated mice exhibited enhanced object memory (A) and spatial learning memory (B). Mice receiving sildenafil 1mg/kg immediately after first trial had significantly higher object memory ($P<.01$). Discrimination index (DI) was calculated as a ratio of additional time mice spent with novel object to the total time spent on exploring objects. Mice received sildenafil 1mg/kg immediately after first trial remembered the spatial orientation than other groups ($P<.05$). Number of entries in each arm during the second trial was analyzed against the number of entries into novel arm. Values are mean ± SEM.

Fig. 4.2. Basal synaptic transmission in phosphodiesterase5 inhibited mice. Increase in FV amplitude was seen in the group treated with sildenafil compared to control (A), slope of fEPSP's was significantly elevated by *in vivo* phosphodiesterase5 inhibition in input out put data (B). Paired pulse facilitation (PPF) (C) remained unaltered by phosphodiesterase5 inhibition.

Fig. 4.3. Enhanced LTP in PDE5 inhibited mice. LTP and PTP in sildenafil treated and control mice measured in hippocampal slices at Schaffer collateral/CA1 synapses (A). LTP was induced with a tetanic stimulus at 100 Hz and an intensity that evoked 50% of a maximal fEPSP. The fEPSPs slopes show significant differences between PDE5 inhibited (●) and control mice (○). Representative traces are shown for PDE5 inhibited and control mice at baseline (Trace 1) and 60 min (Trace 2) after tetanic stimulation. Scale bars equal 1mV and 10ms. (B) PTP and LTP in PDE5 inhibited and control mice (open bar) measured at 1–7min and 55–60min, respectively. At 1–7min there was no significant

difference on PTP in PDE5 inhibited mice (160±6.6%) compared to control mice (145±7.3; p=0.1797). At 55–60min LTP was significantly lower in control mice (124±5%; n=7 slices; 4 mice) compared to sildenafil treated mice (145±3.3%, p=0.005).

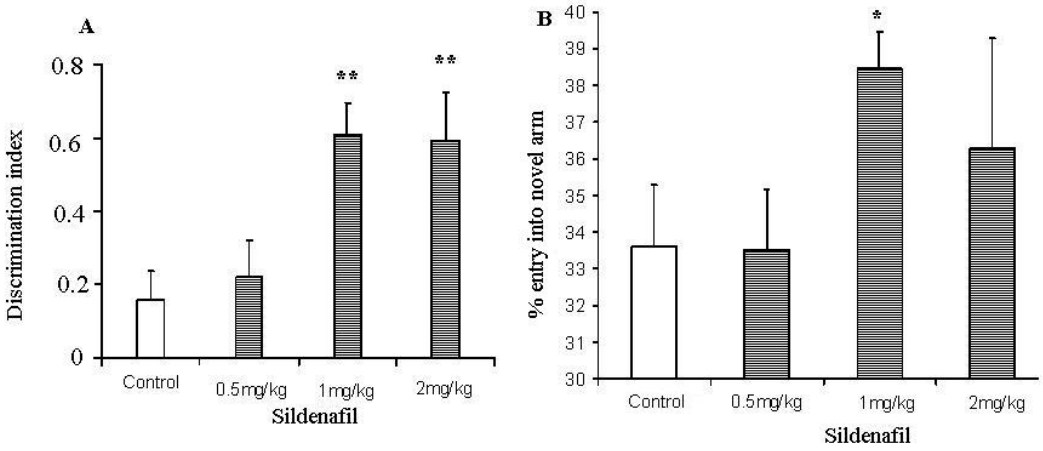

A
Discrimination index
0.8
0.6
0.4
0.2
0
**
**
Control
0.5mg/kg
1mg/kg
2mg/kg
Sildenafil
B
% entry into novel arm
40
39
38
37
36
35
34
33
32
31
30
*
Control
0.5mg/kg
1mg/kg
2mg/kg
Sildenafil

Figure. 4.1.

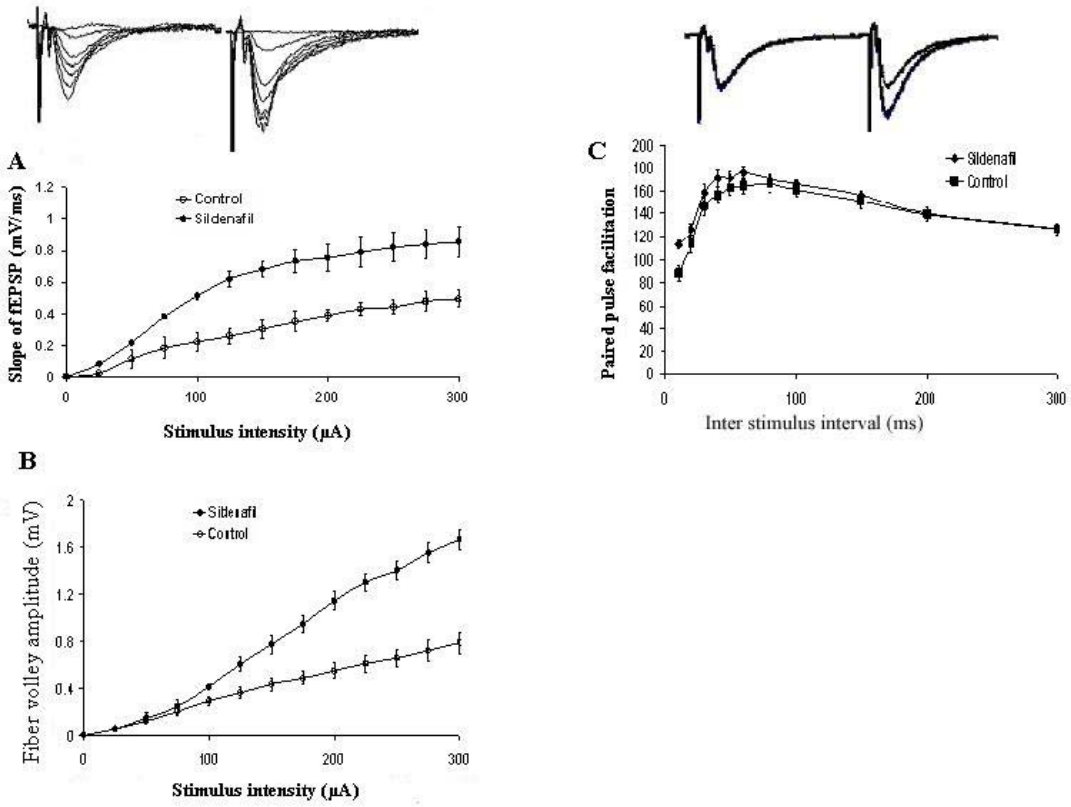
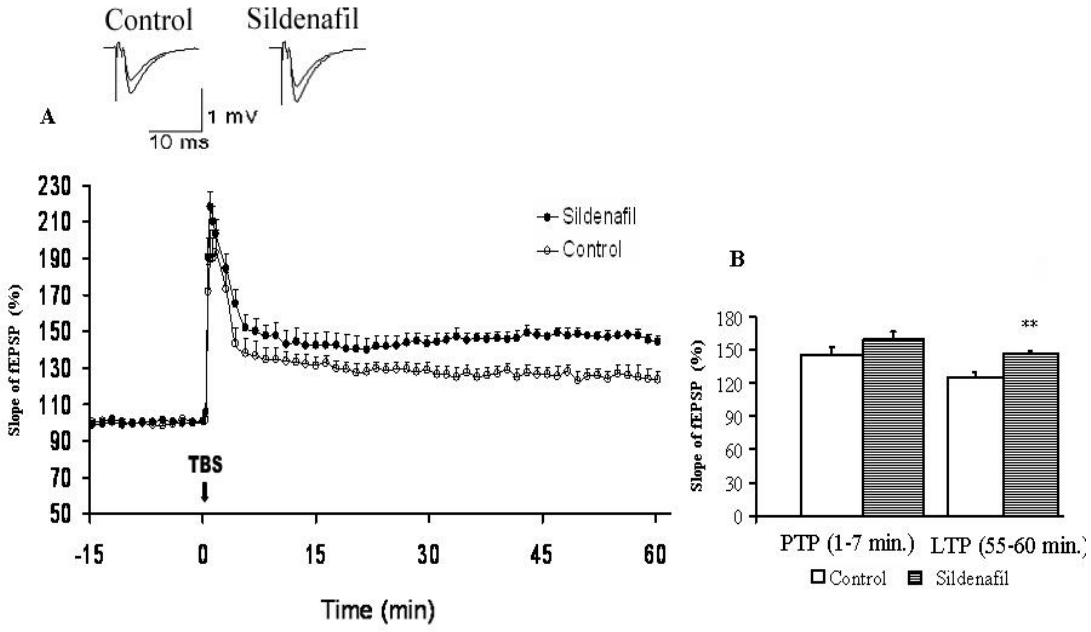

A
Slope of fEPSP (mV/ms)
Control
Sildenafil
Stimulus intensity (µA)
B
Fiber volley amplitude (mV)
Sildenafil
Control
Stimulus intensity (µA)
C
Paired pulse facilitation
Sildenafil
Control
Inter stimulus interval (ms)

Figure. 4.2.


Control
Sildenafil
A
1 mV
10 ms
230
210
190
170
150
130
110
90
70
50
Slope of fEPSP (%)
Sildenafil
Control
TBS
-15
0
15
30
45
60
Time (min)
B
180
150
120
90
60
30
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Slope of fEPSP (%)
**
PTP (1-7 min.)
LTP (55-60 min.)
Control
Sildenafil

Figure. 4.3.

5. SILDENAFIL IMPROVES MEMORY DEFICITS CAUSED BY INTRACEREBROVENTRICULAR INFUSION OF AMYLOID BETA PEPTIDE

Abstract

Accumulating experimental evidence indicated that involvement of cyclic guanosine monophosphate (cGMP) and its downstream regulation on the synaptic plasticity. Pharmacological manipulations of cGMP may reduce the relative risk for AD or may be a useful therapeutical intervention. Alzheimer's disease (AD) is a fatal, progressive neurodegenerative disease that occurs in the elderly of the general population. Amyloid plaques, consisting of extracellular deposits of Aβ42 peptide are found in many AD patients' brains and considered one of the hallmarks of Alzheimer's disease. Intracerebroventricular (icv) infusion of abeta (1-42) oligomers in rats caused severe memory dysfunction and impaired long-term potentiation. Consistent with our previous observation in mice, we recently found that moderate intake of sildenafil, a potent and highly selective phosphodiesterase 5 inhibitor, significantly attenuated the memory deterioration in the Abeta (1-42) infused AD rat model. We have found that administration of sildenafil enhances hippocampal dependent behavioral tasks such as object recognition and Y maze. The level of LTP was significantly elevated in abeta infused rats treated with sildenafil (1 mg/kg/day) for 10 days. Thus, our results suggest that neural disturbances caused by abeta infusion may be rescued by treatment with sildenafil.

Introduction

Alzheimer's disease (AD) is the most common cause of progressive cognitive impairment in the elderly (Hendrie 1997; Lobo et al., 2000; Koo et al., 1999; Woo et al., 1998). The characteristic neuropathologies of AD are the formation of senile plaques and neurofibrillary tangles in vulnerable brain regions (Terry, 1963; Braak and Braak, 1997). Senile plaques are the more complex extracellular lesion and are composed of amyloid β (Aβ) peptide fragments. The senile plaques are primarily composed of Aβ peptide, which is a 40–42 amino acid peptide fragment of the amyloid protein precursor that plays an important role in the development of AD. However, the mechanism by which Aβ causes neuronal injury and cognitive impairment is not yet clearly understood.

Aβ peptide has been shown to have the potential to induce oxidative stress and inflammation in the brain, which have been postulated to play important roles in the pathogenesis of Alzheimer's disease (Behl, 1999; McGeer and McGeer, 1999). Oxidative stress is a cause of neurodegeneration during aging (Leutner et al., 2001; Floyd and Hensley, 2002), as it causes cell death and consequent cognitive decline. Indeed, the intracerebroventricular (i.c.v.) administration of $A\beta_{25–35}$ peptide into rodent brain induced histological and biochemical changes, memory deficits, and oxidative stress (Stepanichev et al., 1998) within 1 or 2 weeks of administration. These findings suggest that antioxidants could be candidate treatments for various neurodegenerative diseases. Moreover, in terms of the pathology of AD, because many active substances produced by Aβ peptides stimulate inflammatory responses (Griffin et al., 1998), anti-inflammatory agents might also be effective treatment candidates.

Nabeshima and his colleagues demonstrated that a continuous infusion of Aβ $_{(1–40)}$ into the cerebral ventricle in rats results in learning and memory deficits that were accompanied by a reduction of choline acetyl transferase activity, suggesting that accumulation of Aβ is related to cognitive impairments in AD (Nitta et al., 1994, 1998). Memory impairment induced by Aβ $_{(1–42)}$ is potentiated by the long-term deprivation of estrogens in female rats (Yamada et al., 1999). In rats treated with Aβ (1–40), dysfunction of cholinergic and dopaminergic neuronal systems are observed, as evidenced by the decrease in the nicotine- and KCl-induced stimulation of acetylcholine and dopamine release *in vivo* (Itoh et al., 1996). Furthermore, long-term potentiation is impaired in the CA1 field of the hippocampal slices prepared from the rat brain after continuous i.c.v. infusion of Aβ $_{(1–40)}$ (Itoh et al., 1999).

In addition, they demonstrated that the continuous infusion of Aβ $_{(1–40)}$ into the cerebral ventricle induced a time-dependent expression of inducible nitric oxide synthase and an overproduction of nitric oxide in the hippocampus although Aβ $_{(40–1)}$ had no effect (Tran et al., 2001). Amyloid beta form senile plague leading to cognitive dysfunction by impairing the signal cascade of the phosphatidylinositol-3-OH kinase (PI-3K) pathway (Kubo et al., 2002). The Aβ-induced overproduction of nitric oxide which reacts rapidly with superoxide radical to yield highly reactive peroxynitrite caused an increase in tyrosine nitration of a synaptic protein synaptophysin in the hippocampus (Tran et al., 2003). We have also demonstrated that the prolonged infusion of Aβ $_{(1–42)}$ results in a significant reduction of the immunoreactivity of antioxidant enzymes in the rat brain areas, although the same treatment with Aβ $_{(40–1)}$ had little effect (Kim et al., 2003).

Evidence suggests that oxidative stresses are involved in the mechanism of Aβ-induced neurotoxicity (Butterfield et al., 1994; Butterfield and Stadtman, 1997; Butterfield and Lauderback, 2002), and AD pathogenesis (Markesbery, 1997; Yankner, 1996). For example, exposure to Aβ increases lipid peroxidation, protein oxidation, and the formation of hydrogen peroxide in cultured cells (Behl et al., 1994). Similarly, increases in lipid peroxidation, protein carbonyl and oxidation of mitochondrial DNA have been observed in the brains of AD patients (Lyras et al., 1997).Yamada et al. (1999) demonstrated that treatment with antioxidants, such as idebenone and α-tocopherol prevents the learning and memory deficits induced by $A\beta_{(1–42)}$. However, they did not find increased lipid peroxidation in the brains of the $A\beta_{(1–42)}$-infused rats.

Sildenafil has been shown to enhance memory in animal models basically its cGMP retaining ability in the target tissue (Erceg et al., 2006; Devan et al., 2006 van Donkelaar et al., 2008; Reneerkens et al., 2008; Puzzo et al., 2008; Rutten et al., 2007a). Behavioral studies in non human primates also provide evidence for memory enhancing properties of sildenafil (Rutten et al., 2008c). Sildenafil participate in the regulation of signal transduction by means of regulation of cyclic nucleotides so that the response to cell stimuli is both specific and activates the correct third messengers. Several phosphodiesterase inhibitors as well as knock out animals have been used to study the second messenger systems involved in the synaptic plasticity (van der Staay et al., 2008; Rutten et al., 2008b; Rutten et al., 2007a). Although sildenafil and several other phosphodiesterase inhibitors have been studied in behavioral pharmacology, reports on studies employing electrophysiology and molecular biology are scanty.

The purpose of this study was to investigate whether rat administered i.c.v. $A\beta_{1-42}$ show learning and memory impairments, and if so, whether these impairments can be attenuated by sildenafil. In the present study, cognitive function was evaluated using object recognition and Y-maze tasks. We also investigated the alterations in long term potentiation (LTP) a process in the brain that can explain certain aspects of synaptic plasticity.

Materials and methods

Animals Two month old male rats (Charles River, Wilmington, MA) weighing 200–225g were used for both behavioral study and electrophysiological experiments. Aβ (1-42, AnaSpec, CA) was infused intracerebroventricularly (icv), 4 nmol, to Sprague Dawley rats. Sildenafil (1 or 2 mg/kg, i.p.,) was administered for 10 days starting 24 hours after the Aβ lesioning. All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the Auburn University.

Behavioral experiments

Rats were randomly divided into four experimental groups (10 rat per group) as follows: Control (infused with vehicle); control (infused with Abeta); Abeta infused rats treated intraperitoneally (i.p.) sildenafil 1mg/kg or 2mg/kg for 10 days. Changes in behavioral memory were tested by employing Object Recognition Task (ORT) and Two trial Y Maze task. Rats were intensively handled (5 d by ~3 min per rat) and weighed once a week before behavioral testing.

Object Recognition Task (ORT)

The apparatus consisted of a Plexiglas open-field box (52×52×40cm). Half of the 40cm high wall was covered with blue paper and the other half was left transparent. Two transparent glass bottles (diameter 2.5cm, height 6.5cm) filled with sand were placed symmetrically about 5cm away from the blue wall. During the second trial a gray color plastic cube (2.5×2.5×6cm) was placed for one of the glass bottle. The objects could not be displaced by a rat. A testing session comprised of two trials (Rutten et al., 2005, 2008b). The duration was 3 and 5min for first (exploration) and the second (retention test) trial respectively. Inter trial interval used in this experiment was 24 hours. Rat was put back in the apparatus for the second trial and one of two identical objects was replaced by a novel object. Before each trial, the surface of the box was sprayed with a diluted ethyl alcohol solution to erase any scent cues. Behavior was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. Times spent exploring each object during T1 and T2 was recorded manually using a personal computer and a timer. In order to avoid the presence of olfactory cues the objects was always thoroughly cleaned using diluted ethyl alcohol before each trial. Discrimination index was calculated for each animal using the time spent exploring each object.

Two trial Y Maze test

Two trial Y Maze was employed to analyze spatial recognition memory using the previously described procedure (Ma et al., 2007). The Y-maze test consisted of two trials separated by a time interval. In the first (acquisition) trial, one arm of the maze

(subsequently called novel arm) was closed with a guillotine door. Rat was placed at the distal end of one arm (starting arm), head pointing away from the center of the maze, and was allowed to visit the two accessible arms of the maze for 3 min. During the second (Retention test) trial rat has free access to the three arms and was allowed to explore the maze for 5 min. Behavior of rat in maze was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. This test is based on the interest of rat for novelty; hence they explore preferentially unknown territories. The number of explorations of each arm was counted every min and the percentages of visits in the novel arm with respect to the total number of visits in the three arms during each min of the test was calculated.

Electrophysiological recordings

Drug treatment and slice preparation

Control rats, abeta infused rats and abeta infused sildenafil treated rats were analyzed in field electrophysiology to determine the mechanism behind the behavioral changes. Since 1mg/kg sildenafil produced significant memory reversal in behavioral study, only that effective drug treatment was analyzed for electrophysiology. At the end of 10 days, *in vitro* brain slice experiments were performed following the methods described previously (Bukalo et al., 2004). Rats were deeply anaesthetized and decapitated, the brain was quickly extracted and immediately placed into ice-cold cutting solution (in mM): 250 sucrose, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgCl_2$) that was oxygenated with 95% O_2 and 5% CO_2. The brain was sliced (350-

μm transverse slices) using a Vibratome (Warner instruments, Hamden, CT) and incubated in oxygenated artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgCl_2$) at room temperature for one hour.

Extra-cellular recordings of fEPSPs

Brain slices were transferred to submerged type recording chamber and continuously perfused (2ml/min) with oxygenated ACSF. Evoked EPSPs were elicited by stimulating the hippocampal Schaeffer collateral fibers with a bipolar Teflon-coated platinum electrode placed in the CA3 sub field. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum using glass microelectrodes (2–4 MΩ) filled with ACSF placed ~300μm apart from recording electrode. Test pulses were set at an intensity that evoked 50% of maximum EPSP amplitude before intrusion of a population spike. Field excitatory postsynaptic potentials (fEPSPs) in CA1-CA3 synapses were recorded on a computerized stimulating and recording unit. Response to different stimulation intensities was recorded, the slope of fEPSPs and the amplitude of fiber volley were analyzed. In order to study the changes in presynaptic function, paired pulse facilitation was analyzed. Ratio of the slope of first response and the subsequent response against different inter-stimulus interval was analyzed. Basal synaptic transmission was monitored for 15 minutes before inducing long term potentiation.

Analysis of synaptic transmission and plasticity

Long-term potentiation (LTP) was induced by a theeta burst (TBS) protocol consisting of five TBS delivered at 20 seconds interval. TBS consisted 10 bursts

delivered at 5 Hz. Each burst consisted four pulses delivered at 100Hz. Data were acquired and fEPSPs were measured using LTP program software and analyzed offline. Post-tetanic potentiation (PTP) and LTP were defined as the percentage change from baseline for the 7 minutes immediately after TBS and for the last 5 minutes of the 60-min post-TBS recording period, respectively.

Biochemical analysis

The effect of sildenafil and abeta infusion on oxidative stress and mitochondrial function as seen in superoxide dismutase activity, glutathione, and lipid peroxides was analyzed. Glutathione (GSH) content was estimated by employing OPT-condensation reaction with the tripeptide to yield a fluorescent product. Readings were taken at the activation/emission wavelengths of 337/423 nm spectrofluorimeter. Catalase activity (CAT) was assayed by rate of decrease in absorbance of H_2O_2 and measured by spectrophotometrically at 240nm. Acetylcholine esterase (AChE) activity was measured spectrophotometrically at 412nm. Choline acetyl transferase (ChAT) activity was measured spectrophotometrically by reading the absorbance of acetyl choline yield at 324nm. Super oxide dismutase (SOD) activity was measured spectrophotometrically using pyrogallol as substrate at 420nm. Protein was assayed using the coomassie plus protein assay reagent kit. Bovine serum albumin was used as standards.

Statistical analysis

Experiments were performed with the investigator blinded to the treatment the animals received. Data were expressed as mean ± S.E.M. Electrophysiological results were normalized to the basal values and analyzed by ANOVA with Turkey's post-hoc

analysis. The level of significance was set for $P<0.05$. All statistical calculations were performed on a computer using a commercially available statistical package.

Results

Results of the current study indicates that intracerebroventricular (icv) infusion of $A\beta_{(1-42)}$ to Sprague Dawley rats caused severe memory impairments sufficient to mimic Alzheimer's type of neural function. The brief treatment of Aβ infused rats with sildenafil rescued the neuronal deficits. We first studied the effect of infusion of Aβ on object recognition memory. Post-hoc analysis shows that the level of exploration as measured by object discrimination index (DI) was high in groups treated with 1 mg/kg sildenafil and the Control rats compared to Aβ infused group however, there was no significant difference between Control and Aβ sildenafil (Figure 5.1.A, $P<.05$). Treating Aβ animals with higher dose of sildenafil didn't show significant changes on the memory performances.

We then studied the effect of infusion of Aβ on spatial memory by first restricting them to explore only in two arms of Y maze and after a delay of 3 hours, memory retention was tested by allowing access to all three arms. Effect of infusion of Aβ and or sildenafil on the number of entry in novel arm, the arm opened only during second trial, in Y maze is presented in Figure 5.1.B. The spatial memory was significantly impaired by infusion of Aβ and rescued in the group treated with 1 mg/kg sildenafil. Similar to the results obtained in the object memory test the higher dose of sildenafil didn't cause a significant change.

To characterize whether infusion of Aβ altered the basal synaptic function to impair the memory performance, presynaptic fiber volley (FV) and field excitatory postsynaptic potential (fEPSP) from CA1 synapses in hippocampal Schaffer collateral projections were recorded for different stimulations given at CA3 neurons. In order to elucidate the possible presynaptic modifications, change in amplitude of FV with stimulation intensity was analyzed. Size of FV is considered as a measure of number of recruited presynaptic neurons (Xiao et al. 2007). The amplitude of FV was significantly impaired by infusion of Aβ compared to Control (Fig. 5.2.A), indicating the impaired ability to transfer the presynaptic stimulus into an axonal depolarization. Similarly, analysis of input out put curve (Fig. 5.2.B) revealed that the slope of fEPSP's for a given stimulus intensity was significantly lowered by infusion of Aβ. Examination of both FV and fEPSP input output data reveal a possible modification of post basal synaptic function by of infusion of Aβ. Treating the Aβ infused rats with sildenafil rescued those impairments. Surprisingly neither infusion of Aβ nor treatment of sildenafil altered the paired pulse facilitation (PPF) (Fig. 5.2.C). Presynaptic dependent short term plasticity is measured by PPF that believed to correlate the release probability of glutamate (Chan et al., 2006). Overall enhancement of basal synaptic transmission through sildenafil treatment however failed to produce a difference in short term plasticity in our experiment.

To examine how infusion of Aβ and treatment of sildenafil can affect different forms of plasticity, we analyzed the magnitude of PTP and LTP. A robust theta burst

tetanization paradigm consisting of five trains with an inter-train interval of 20 s was used to induce LTP. In agreement with the results of basal synaptic transmission, PTP another form of short term plasticity was unaffected all three treatment groups (Fig. 5.3.A). Interestingly, LTP was significantly impaired by infusion of Aβ and treatment of sildenafil rescued to normal level (Fig. 5.3.B).

We also analyzed the effect of sildenafil and abeta infusion on oxidative stress and mitochondrial function using known markers. Glutathione content, activity of catalase, superoxide dismutase and glutathione peroxidase (Fig. 5.4) were not significantly altered by abeta infusion or sildenafil treatment. Yamada et al. (1999) demonstrated that treatment with antioxidants, such as idebenone and α-tocopherol prevents the learning and memory deficits induced by $A\beta_{(1–42)}$. However, they did not find increased lipid peroxidation in the brains of the $A\beta_{(1–42)}$-infused rats (Yamada et al., 1999). There was no significant difference among treatment groups in the activities of Choline acetyl transferase and Acetylcholine esterase (Fig.5.4). Activities of enzymes involved in cholinergic system have been used to analyze the effect of drug or toxin on the cholinergic neurotransmission, a very early cause/ marker of Alzheimer's disease.

Collectively these results shows that intracerebroventricular (icv) infusion of $A\beta_{(1-42)}$ to Sprague Dawley rats produce a valid animal model to study the Alzheimer's pathology. The beneficial effect of sildenafil in memory enhancement is also evidenced from the current results.

Discussion

We found that a single intracerebroventricular injection of betaamyloid (1–42) peptide leads to sustained memory impairment and impaired glutamatergic synaptic transmission. Although variable in terms of the injected peptide, injection procedure, and behavioral test employed, different studies have consistently shown the occurrence of behavioral deficits related to memory impairment after intracerebral injections of amyloid peptides (Delobette et al., 1997; O'Hare et al., 1999; Harkany et al., 2001; Malin et al., 2001; Nakamura et al., 2001; Ammassari-Teule et al., 2002; Christensen et al., 2008). These studies also demonstrated that the behavioral effects are specific to the sequence of the peptide, as injection of amyloid fragments with scrambled or inverse sequences are without any effects (Ammassari-Teule et al., 2002; Malin et al., 2001). The levels and ratio of different Aβ fragments determine the toxicity and most importantly $A\beta_{1-42}$ has been shown to be severe than other forms (Parameshwaran et al., 2007). Amyloid hypothesis is continued to be supported by several reports which utilized animal models of AD over expressing or excessive build up of Aβ, and *in vitro* nerve cells/tissues subjected to Aβ insult. Increasing evidences suggest that AD is due to synaptic pathological processes in which synaptic loss and synaptic dysfunction begins several years prior to severe neuronal loss (Parameshwaran et al., 2008). Based on these observations, we believe that the behavioral effects we observed are due to a specific effect of the Aβ(1–42) peptide.

Administration of selective PDE5 inhibitor, sildenafil, positively modulates the hippacampal function by enhancing basal synaptic transmission, synaptic plasticity and working memory. Learning and memory function is chiefly determined by hippocampal synaptic plasticity (Milner et al., 1998); therefore we examined the excitatory synaptic transmission in synapses between the glutamatergic fibers of hippocampal CA3 and CA1 pyramidal cells. Interestingly, daily application of sildenafil for a brief 10 days (1 mg/kg) was capable of producing enhanced hippocampal function. Phosphodiesterase 5 is expressed in hippocampus, cortex and cerebellum of mice and rat (Shimizu-Albergine et al., 2003; Van Staveren et al., 2003; Blokland et al., 2006), therefore, a selective PDE5 inhibitor can act on these brain regions to enhance memory. Nitric oxide acts in the presynaptic neuron to produce long-term potentiation in cultured hippocampal neurons (Arancio et al., 1996). This study and several other findings suggest the involvement of NO-cGMP signaling pathway in the memory function. Synaptic plasticity is a critical component of the neural mechanisms underlying learning and memory. Long-term potentiation, an activity-dependent synaptic plasticity, plays a key part in the forms of memory mediated by hippocampus. Taken together, these observations indicated that sildenafil treatment can functionally ameliorate the Aβ -induced memory loss possibly by minimizing the inhibitory effect of Aβ on hippocampal glutamatergic transmission.

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Legends

Fig 5.1. Effect of Intracerebroventricular (icv) infusion of Aβ (1-42) and treating with sildenafil on object memory and spatial learning memory: Intracerebroventricular (icv) infusion of Aβ (1-42) to Sprague Dawley rats caused severe memory impairments and treating with sildenafil to the Aβ infused rat exhibited enhanced object memory (A) and spatial learning memory (B). Aβ infused rat receiving sildenafil 1mg/kg had significantly higher object memory ($P<.01$). Discrimination index (DI) was calculated as a ratio of additional time rat spent with novel object to the total time spent on exploring objects. Aβ infused rat received sildenafil 1mg/kg remembered the spatial orientation than other groups ($P<.05$). Number of entries in each arm during the second trial was analyzed against the number of entries into novel arm. Values are mean ± SEM.

Fig. 5.2. Basal synaptic transmission in Aβ infused and sildenafil treated rat. Increase in FV amplitude was seen in the control and sildenafil treated Aβ animals compared to Aβ infused rat (A), Aβ ifusion significantly lowered the slope of fEPSP's compared to control and sildenafil treatment rescued the defect in input out put data (B). Paired pulse facilitation (PPF) (C) remained unaltered in all three groups.

Fig. 5.3. LTP in Aβ infused and sildenafil treated rat. LTP and PTP in Aβ infused, sildenafil treated and control rat measured in hippocampal slices at Schaffer collateral/CA1 synapses (A). LTP was induced with a tetanic stimulus at 100 Hz and an intensity that evoked 50% of a maximal fEPSP. The fEPSPs slopes show significant differences between Aβ infused rat (●) and sildenafil treated rat (▪). There was no significant difference between sildenafil treated rat and control rat (○). Representative traces are shown for all three groups at baseline (Trace 1) and 60 min (Trace 2) after tetanic stimulation. Scale bars equal 1mV and 10ms. (B) PTP and LTP in Aβ infused, sildenafil treated and control rat (open bar) measured at 1–7min and 55–60min, respectively. At 1–7min there was no significant difference on PTP among treatment groups. At 55–60min LTP was significantly lower in Aβ infused rat (n=6 slices; 4 rat) compared to sildenafil treated rat and control (p<0.01).

Fig. 5.4. Neurochemical changes in sildenafil treated and Abeta infused rats: Superoxide dismutase activity in Aβ infused and sildenafil treated rat (A) was not altered. Catalase activity (B), Glutathione content (C), Glutathione peroxidase activity (D) and Acetylcholine esterase activity (E) were not significantly altered in Aβ infused and sildenafil treated rat. Abeta infusion had no effect on the choline acetyl transferase activity (F).

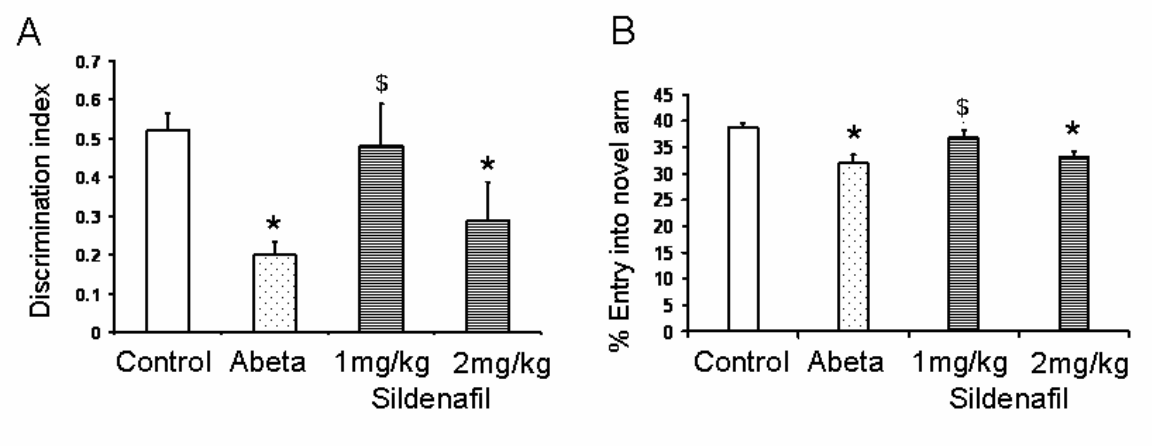
A
Discrimination index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$
*
*
Control
Abeta
1mg/kg
2mg/kg
Sildenafil
B
% Entry into novel arm
45
40
35
30
25
20
15
10
5
0
*
$
*
Control
Abeta
1mg/kg
2mg/kg
Sildenafil

Figure. 5.1.

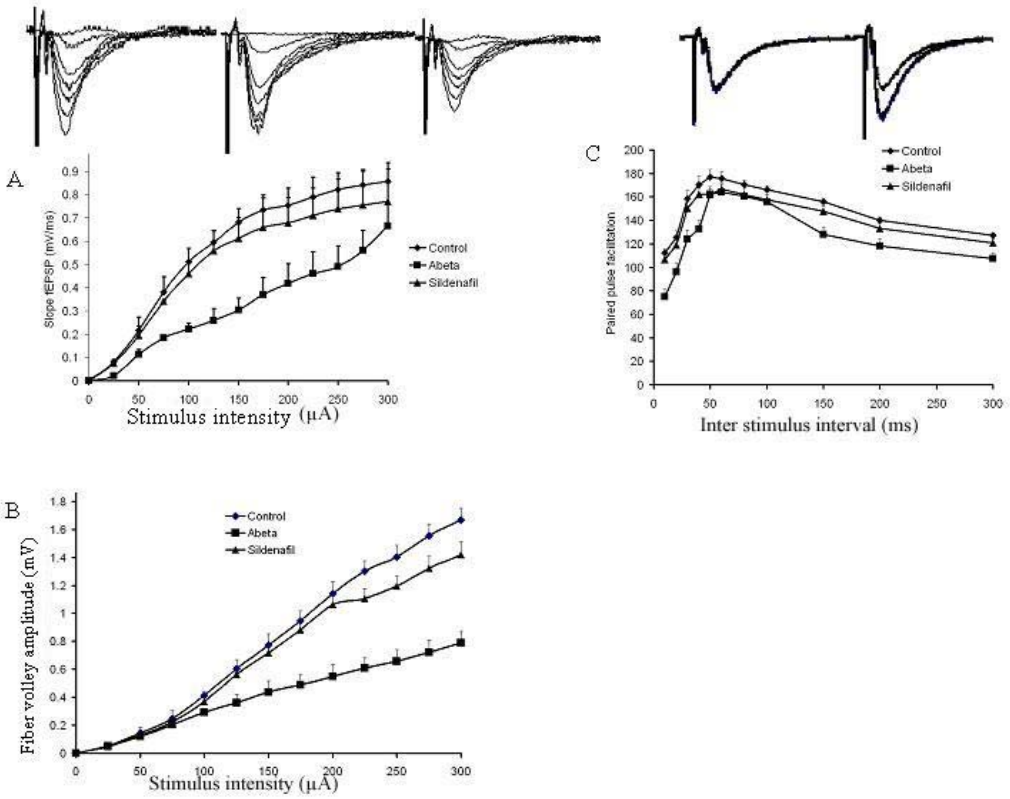
A
Slope fEPSP (mV/ms)
Stimulus intensity (µA)
Control
Abeta
Sildenafil
C
Paired pulse facilitation
Inter stimulus interval (ms)
Control
Abeta
Sildenafil
B
Fiber volley amplitude (mV)
Stimulus intensity (µA)
Control
Abeta
Sildenafil

Figure. 5.2.

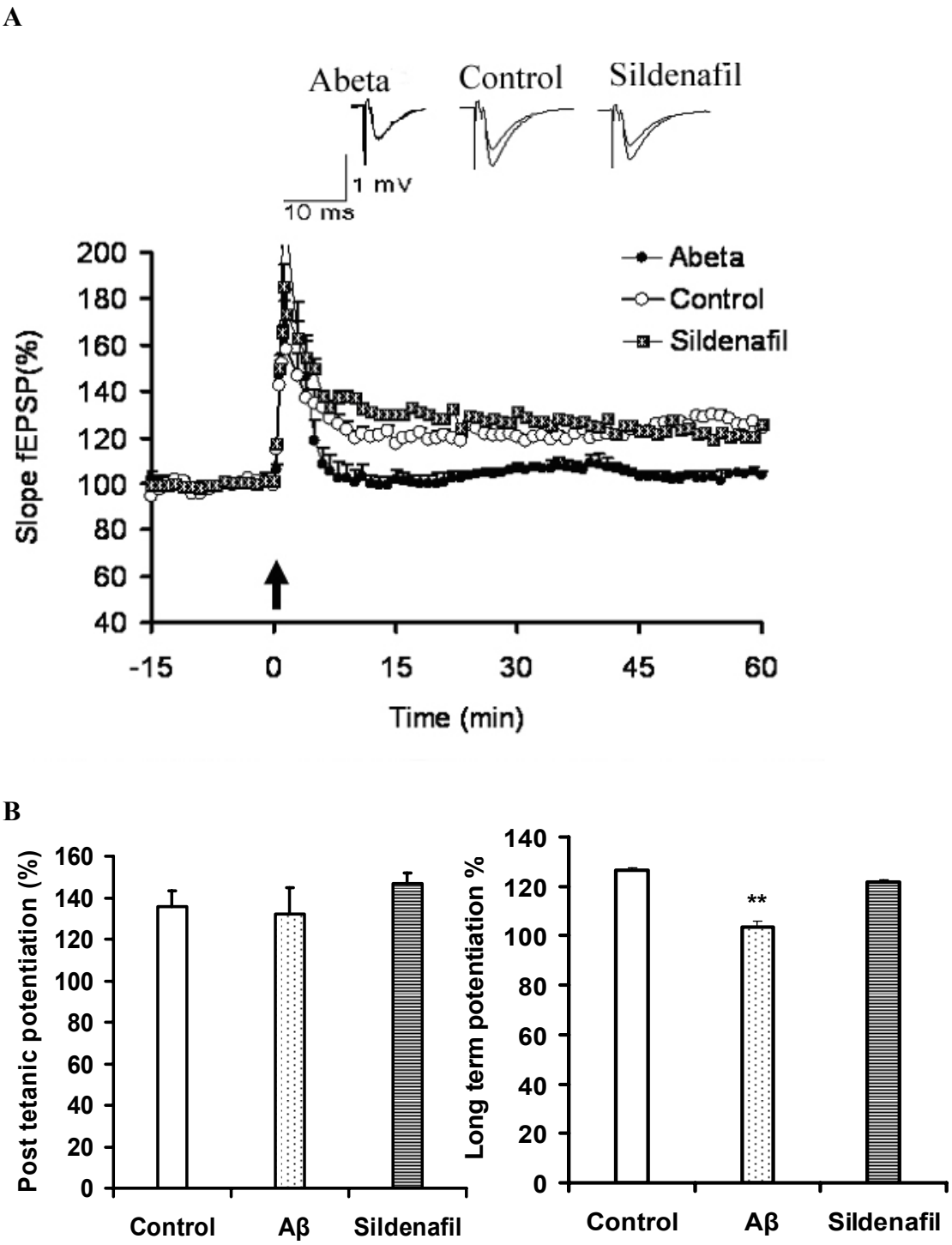
A
Abeta
Control
Sildenafil
1 mv
10 ms
Slope fEPSP(%)
200
180
160
140
120
100
80
60
40
-15
0
15
30
45
60
Time (min)
Abeta
Control
Sildenafil
B
Post tetanic potentiation (%)
160
140
120
100
80
60
40
20
0
Control
Aβ
Sildenafil
Long term potentiation %
140
120
100
80
60
40
20
0
**
Control
Aβ
Sildenafil

Figure. 5.3.

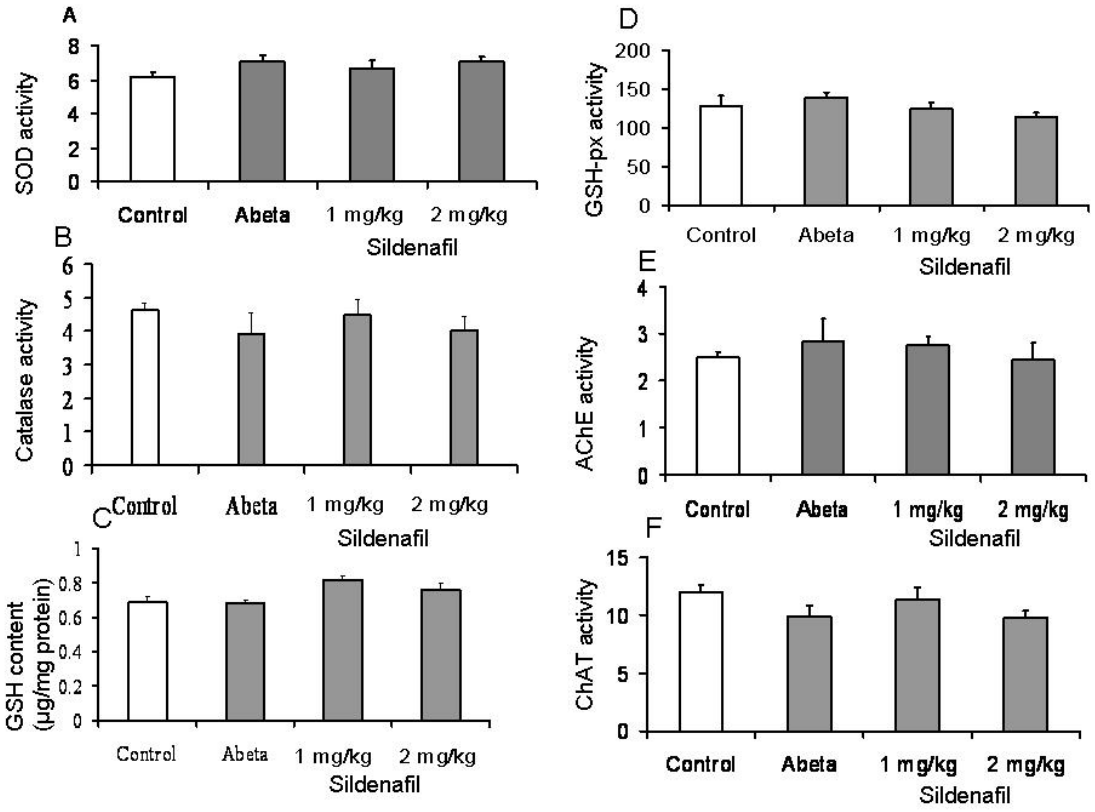
A
SOD activity
Control
Abeta
1 mg/kg
2 mg/kg
Sildenafil
B
Catalase activity
C
GSH content (µg/mg protein)
D
GSH-px activity
E
AChE activity
F
ChAT activity

Figure. 5.4.

6. SUMMARY AND CONCLUSIONS

Parkinson's disease, a movement disorder, frequently affects the erectile function and as the results considerable proportion of patients are treated with sildenafil. Sildenafil has been shown to cross the blood brain barier; it was therefore interesting to investigate the neurological effects of this substance. The experiments presented in third chapter of this dissertation is an in depth investigation employing several animal models for Parkinson disease and fatigue. The results revealed that sildenafil would not protect the dopaminergic cell loss against a specific nigro-striatal toxin nor potentiate the severity of the toxic insult. Recently, one year after we publish this finding, Janis et al. (2008) draw the same conclusion and found that sildenafil did not produce any beneficial effect on nigro-striatal dopaminergic neuron function.

Although the underlying mechanisms of learning and memory are not yet explained or fully understood, experimental results from morphology, electrophysiology, biochemistry, and behavioral experiments point towards critical roles for cAMP and cGMP in this process. These second messengers play an important role in intracellular signaling and in processes of neuroplasticity such as long term potentiation (LTP). Hippocampal LTP has been proposed to be a neurophysiological correlate of memory. The studies presented in this dissertation investigated the effects of phosphodiesterase5 inhibitor on memory performance in several test models (object recognition and spatial

recognition) and species (mice and rats). The results from studies presented in the fourth chapter of this dissertation support the importance of the role of the second messengers such as cGMP in learning and memory processes. A large part of this dissertation investigated the effects of specific phosphodiesterase5 inhibitor on long-term memory performance employing behavioral, biochemical and electrophysiological techniques.

Experiments presented in the chapter 5, focused on two aspects; firstly identifying and characterizing an animal model to be used in drug screening for Alzheimer's disease. Secondly, employ the animal model for testing sildenafil on the possible therapeutic benefit. Intracerebroventricular (icv) infusion of abeta (1-42) oligomer in rat caused severe memory dysfunction and impaired electrophysiological correlates in the hippocampus such as longterm potentiation. Interestingly, a brief application of sildenafil restored the memory deficits and elctrophysiological alterations to the level comparable to the normal animal.

Taken together, the results presented in the present dissertation show convincing cognitive enhancing properties of phosphodiesterase5 inhibition. The enhancing effects of phosphodiesterase5 inhibition on memory performance have been shown in rats and mice. It appears that cGMP is mainly involved in early consolidation processes and cAMP in late consolidation processes. The underlying pathways are yet to be fully explained, although cellular processes that are related to LTP seem to be a possible mechanism. The cGMP/PKG/CREB pathway for early consolidation processes and the cAMP/PKA/CREB pathway for late consolidation processes might be the most probable mechanisms underlying the observed behavioral effects. In addition, the pre-synaptic

NO/sGC/cGMP pathway may also exert effects on short-term memory, but this has not yet been investigated at present. Finally, the understanding of the underlying mechanisms of phosphodiesterase5 inhibition and cognition can be further complicated, since evidence has shown that early LTP can be converted to late LTP. Isoform specific studies are promising new ways to further investigate the underlying mechanisms of cognition enhancement in animal models. Genetic knock-out models for phosphodiesterase4 show behavioral differences in cognition and depression, but developmental deficits in these animals might confound the outcome of these studies. Although sildenafil shows some promise as a therapeutic agent in Alzheimer's disease, well-designed clinical trials are needed before the agent can be recommended for use in this disorder. Novel techniques, such as gene silencing, could further increase our understanding of the mechanisms of phosphodiesterase5 inhibitors in cognition and might provide more selective targets for cognitive enhancement.