SYNAPTIC GLUTAMATE RECEPTOR DYSFUNCTION IN TISSUE AND ANIMAL MODELS OF ALZHEIMER'S DISEASE

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SYNAPTIC GLUTAMATE RECEPTOR DYSFUNCTION IN TISSUE AND ANIMAL MODELS OF ALZHEIMER'S DISEASE

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SYNAPTIC GLUTAMATE RECEPTOR DYSFUNCTION IN TISSUE AND ANIMAL

MODELS OF ALZHEIMER'S DISEASE

Patrick M. Kanju

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DISSERTATION ABSTRACT

SYNAPTIC GLUTAMATE RECEPTOR DYSFUNCTION IN TISSUE AND ANIMAL MODELS OF ALZHEIMER'S DISEASE

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The complexity of cognitive decline involved in Alzheimer's disease (AD) warrants a thorough investigation into the molecular mechanism that may hold the basis for the impaired learning and memory. Indeed, certain molecules have been suggested to play significant roles in AD progression. For example it is well known that the two major hallmarks of AD, amyloid beta fragments and hyperphosphorylated tau contribute to the neuropathogenesis of the disease. Another candidate that may play a significant role in AD is the accelerated lysosomal enzymes arising from a dysfunction of these organelles. Additionally, some of the neurotransmitter systems involved in learning and memory are implicated in AD pathogenesis. The most common and well studied neurotransmitter systems are the cholinergic and glutamatergic systems. Indeed, appropriate expression and function of glutamate receptors subtypes N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) are important in cognitive processes.

It is possible that all these molecules and systems may work synergistically to execute the cognitive decline observed in AD. However, it is important to unravel the early events that lead to the cascade of neurodegeneration prior to the overt signs of cognitive decline. To study these events, tissue and animal models have been used by several investigators. The model of choice depends on the intended target point in the neuropathological cascade. In the current we utilized hippocampal organotypic slice cultures exhibiting lysosomal dysfunction to study the early events of neurodegeneration. Progressive changes were achieved by treating the slices with lysosomotropic agent chloroquine for 3, 6 and 9 days. We studied the functional properties of glutamate receptors subtypes AMPA and NMDA in hippocampal slices and in isolated synaptosomes at each stage of lysosomal dysfunction. Our results indicate that there is correlation between lysosomal dysfunction and glutamate receptor function. This study also shows that the altered AMPA channel properties after 9 days of chloroquine treatment can be reversed by the nootropic compound ampakine CX516. To complement the *in vitro* slice model we investigated the early changes in hippocampal glutamatergic function associated with cholinergic denervation in live animals. The functional properties of synaptic AMPA and NMDA receptors, 4 to 6 days after selective medial septum lesioning with immunotoxin 192 IgG-saporin were studied. To correlate this with slice model we examined whether cholinergic denervation is accompanied by lysosomal dysfunction. It is interesting to note that after 4-7 days of medial septal lesions there was suppression of lysosomal function and modifications in AMPA and NMDA receptor mediated synaptic responses. The data

suggest that in both in vitro and in vivo models of AD, the early neuropathogenesis is associated with synaptic glutamatergic dysfunction.

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1. INTRODUCTION

Over the last decade there has been significant progress in understanding the mechanistic basis underlying neurodegeneration. Several neurodegenerative diseases are known to exhibit many common features including neuronal and synaptic loss that lead to memory impairment (Hirono et al., 1999; Ramaekers et al., 2004; Ghosh et al., 2004). However, many questions remain unanswered regarding what takes place during the early events of neurodegeneration and indeed many factors, both intra- and extracellular may interact in a complex but rather poorly understood way to cause cognitive decline. It is not well known which receptor systems play the major role in executing memory loss but there are speculations that it might involve receptors required for fast synaptic transmission such as glutamate receptors. In order to understand the molecular mechanism involved in neurodegenerative disorders such as Alzheimer's disease (AD), it is necessary to unravel the early events that cause intercellular and intracellular interaction between normal proteins and aberant molecules to influence neuronal function prior to neuronal damage.

In a mammalian brain, ionotropic glutamate receptors mediate a significant proportion of excitatory synaptic transmission in the central nervous system (Cotman *et al.*, 1987; Monaghan *et al.*, 1989; Boulter *et al.*, 1990). Members of glutamate receptors have been divided into three subtypes according to their

preferred pharmacological agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainite. The two major glutamate receptor subtypes, NMDA and AMPA receptors are involved in the induction (Bliss and Collingridge 1993) and expression (Muller and Lynch 1988; Isaac et al 1995; Durand *et al*., 1996; Ben-Ari *et al*., 1997) of long term potentiation (LTP), respectively. The LTP phenomenon is a form of synaptic plasticity thought to underlie learning and memory and may be impaired during the process of neurodegeneration (Bliss and Lomo 1973). Thus, the role played by AMPA and NMDA receptors in memory acquisition is of particular interest in studying the events of neurodegeneration in cognitive disorders. Understanding the functional properties of these two receptors in the early events of cognitive decline in AD is a valuable tool in designing future drug interventions.

A common feature of most cognitive disorders is the accelerated production of lysosomal hydrolases indicating a dysfunction of this cellular organelle in age-related disorders (Bahr et al., 1994; Hajimohammadreza et al 1994; Nixon et al., 2000). Lysosomal dysfunctions may thus be among the earliest events of neurodegeneration (Cataldo et al., 1990. Cataldo et al., 1991) and hence an important candidate for studying disease progression. Indeed, studies show that induction of lysosomal dysfunction in organotypic slice cultures results in accumulation of abnormal proteins including abeta and tau isoforms similar to those observed in AD (Bahr et al., 1994). Studies also show that lysosomal changes occur in AD vulnerable neurons before the onset of pathology (Bi et al.,

1999). In keeping with such findings are studies that show neurodegeneration prior to any overt sign of cognitive decline. Hence, it is of particular interest to investigate the link between lysosomal dysfunction and the receptors that are required for synaptic communication that consequently enhance the cognitive function. As mentioned above one of the candidate receptors are the glutamate receptors, particularly the AMPA and NMDA subtypes whose kinetics may be altered during the early events of AD. Indeed, our results indicate that there could be an indirect link between lysosomal dysfunction and the functional modifications of glutamate receptors.

In order to investigate the events that take place during neurodegeneration, several animal and slice models have been suggested. Although none of the animal and slice models completely mimic the cognitive disorders in question, such models equip researchers with valuable information at least for a particular stage in the cascade of events. However, selecting the model that particularly addresses the problem at hand with limited shortcomings has always been a challenge to many researchers. In this study we use both in vitro and in vivo models to study the early progression of synaptic glutamatergic dysfunction in AD. Specifically, we induce lysosomal dysfunction in hippocampal slice cultures by treating the slices with the lysosomotropic drug chloroquine. The AMPA and NMDA receptor mediated synaptic currents were studied in whole cell configuration utilizing organotypic hippocampal slices. The single channel properties of synaptic AMPA and NMDA receptors were also

investigated using isolated synaptoneurosomes reconstituted in lipid bilayers. To complement the in vitro data, we utilized an in vivo animal model exhibiting cholinergic denervation.

Although most current drugs for AD target the cholinergic system, recently the glutamatergic system has received much attention. Drugs like mementine and ampakine are being used in clinical trials for cognitive decline. Mementine targets NMDA receptors to antagonize their function while ampakines potentiate the AMPA receptor function. Here we utilized ampakines to determine their effects on the altered AMPA channel properties. Since our focus is on the early events of neurodegeneration we only studied ampakine effects and not mementine which may perhaps be useful at a later stage of neurodegeneration when NMDA receptors get involved in excitotoxicity of the neuron. Our study may thus give an insight as to when AMPA receptor modulation by ampakines may be useful as a therapeutic intervention of cognitive decline.

In summary, this project was designed to elucidate the early events of neurodegeneration with a primary focus on glutamatergic transmission. AMPA and NMDA receptors were selected as the candidates for this study. Their electrical properties in both whole cell and single channel recordings in isolated synaptosomes were determined. To investigate the early events of neurodegeneration, both slice (in vitro) and animal (in vivo) models of AD were used in the current study. In the slice model lysosomal dysfunction was induced

in the hippocampus cultured slices by treating the slices with chloroquine to induce the events that take place prior to overt signs of neurodegeneration. In the in vivo model, cholinergic denervation in the medial septum region was performed as this has been observed in early stages of AD. The whole cell electrophysiological recordings of synaptic AMPA and NMDA receptor currents in both models were performed in the CA1 region of hippocampus. Ampakines are drugs currently being investigated for effects on cognitive decline, and the ampikine 1-(quinoxalin-6-ylcarbonyl)-piperidine (CX516) was used to determine its effect on the altered AMPA channel properties in this study. In addition, western blot analysis was performed to determine the level of glutamate receptor subunits (GluR1) and tau protein isoforms. This project therefore gives an insight into the early events of cognitive decline and highlights the future targets for drug intervention.

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2. LITERATURE REVIEW

2.1. Neuropathological features of Alzheimer's disease

Alzheimer's disease is characterized by synapse degeneration and neuronal death in brain regions involved in learning and memory processes. AD begins by a lengthy preclinical phase followed by a malignant stage associated with neuronal degeneration, loss of synaptic connections and progressive cognitive decline. The disease is the most common cause of dementia in the elderly population affecting 30-50% of the adults aged 70 and above. To date the cellular and molecular mechanisms underlying the neurodegenerative process in AD are unclear. However, studies indicate that intracellular neurofibrillary tangles (NFTs) and the extracellular amyloid deposits in the senile plaques (SP) constitute two major pathological hallmarks of AD. Overwhelming evidence also indicates that amyloid beta fragments of between 39 and 43 amino acids long are the major constituents found in extracellular amyloid plaques observed in the brain of AD patients (Masters et al., 1985; Haass et al., 1993; Iversen et al., 1995; Rochet et al., 2000).

The AD pathological process has no direct effects on most functions of the body but the neuronal degeneration that occurs is highly selective for certain brain regions and types of neurons. Abnormal structures of senile plaques and neurofibrillary tangles have been observed in the neocortex as well as amygdala,

hippocampus and parahippocampus. Other parts of the brain such as striatum and cerebellum exhibit a lower degree of neurodegeneration. A subpopulation of pyramidal cells in layers II, III and V that use excitatory amino acids as transmitters appear to be highly vulnerable in AD. Accumulating evidence indicates that neuronal degeneration in AD begins rapidly from the medial temporal lobe and gradually spreads to other areas through cortico-cortical and cortico-subcortical connections (De Lacoste and White, 1993, Vickers *et al* 2000). In all these brain regions beta amyloid aggregation into senile plaques and hyperphosphorylation of tau protein that lead to microtubule destabilization and formation of neurofibrillary tangles, appear to play a significant role in AD pathology. The roles of these two major hallmarks of AD are discussed below.

2.1.1. Role of Amyloid Beta in Alzheimer's Disease

The amyloid cascade hypothesis is perhaps one of the most studied among the known neuropathological cascades of AD. The amyloid beta peptide has thus been proposed as a candidate and in fact the main component of plaques observed in AD (Glenner and Wong, 1984; Selkoe, 1994; Checler, 1995; Mudher and Lovestone, 2002). The aggregates of the amyloid proteins are therefore considered as some of the markers for AD and accompanying neurodegeneration. However, there have been controversies as to whether the cognitive decline observed in AD directly correlates with the level of amyloid fragments (Neve and Robakis, 1998). Hence the specific role of amyloid beta in

the development of the clinical symptoms, the generation of neurofibrillary pathology, neurite formation, and neuronal death is not fully understood and remains a subject for further investigation.

Amyloid beta peptides are generated as normal physiological products of its precursor (Estus et al., 1992; Golde et al., 1992) and are soluble components of the plasma and the cerebrospinal fluid (Seubert et al., 1992). The precursor for amyloid protein is a type I transmembrane glycoprotein (Kang et al., 1987) that belongs to a protein family that also includes APP-like protein (APLP) 1 and 2. APP has a large extracytoplasmic domain, a membrane-spanning domain containing the amyloid beta peptide, and a short intracytoplasmic domain (Selkoe et al., 1996) and is derived by differential splicing of a single gene transript located on the long arm of chromosome 21. APP exists as three alternatively spliced isoforms, ranging from 695 to 770 amino acids in length and is expressed in mammalian neuronal and non-neuronal cells. The APP isoform of 695 amino acids is the most abundant. The newly synthesized APP matures in the secretory pathway by the addition of O-glycosyl and N-glycosyl residues as well as tyrosine sulfation in the *trans*-Golgi network (Sinha, & Lieberburg, 1999). Until recently, many investigators believed that amyloid beta is generated by aberrant metabolism of APP. The aggregation of amylod beta fragments into plaques is considered to be of pathological importance in AD (Dumery et al., 2001). Studies show that APP is expressed on the cell surface in neurons (Storey et al., 1996; Jung et al., 1996) where it plays a role in promoting neurite

outgrowth, may participate in synaptic vesicle recycling (Marquez-Sterling et al., 1997) and may also inhibit proteolytic activity under certain conditions (Gandy and Greengard 1994; Selkoe et al., 1994). Neural stem cell studies indicate that APP performs physiological functions that regulate successful formation and replacement of crucial developing structures and neuronal circuits (see review by Sugaya, 2003)

Proteolytic cleavage of APP is first by the extracellular protease, secretase β to release soluble APP (sAPP) (figure 2.1). The remaining membrane-bound APP is then cleaved by secretase γ that is embedded in the plasma membrane to release Aβ and carboxyl-terminal fragments (CTF) of APP, which have been implicated in the pathogenesis of Alzheimer's disease (Checler, 1995; Selkoe, 1999). APP may be processed via two pathways that identify the products as either amyloidogenic or nonamyloidogenic. The nonamyloidogenic pathway is the major processing route where the APP is cleaved by the α-secretase within the Aβ domain between Lys16 and Leu17 to release soluble fragments of APP (sAPPα) and a membrane-bound fragment p3CT. The p3CT fragment can then undergo γ-secretase cleavage to release the C-terminal p3 peptide preventing the formation of Aβ peptides (Sinha, & Lieberburg, 1999; Kosik, 1999). In the amyloidogenic pathway, cleavage by the β-secretase releases the soluble NH_2-terminal fragment (sAPPβ) and a membrane-bound A4CT fragment which in turn undergo further processing by γ-secretase to generate the Aβ40 or Aβ42 peptides (Selkoe, 1994). The membrane-spanning region of Aβ42 is primarily α-

helix but is released from the membrane as a β-strand which is normally cleared rapidly, either by proteolysis, or by transport across neurovascular endothelium. Failure to clear the abeta fragments results in dimerization of oligomers which in turn aggregates into fibrils which then form the plaques.

Several proteins have been shown to play an important role in amyloid beta generation by either directly or indirectly interacting with the enzymes involved in the amyloid cascade. For example the γ-secretase may be an inefficient protease, and hence its optimal activity requires a proper conformation of the whole complex and the binding of modulatory factors. Among the proteins that interact with the amyloid beta enzyme cascades are the presenelines (PS1 and PS2) family which are ubiquitously expressed in peripheral tissue and in the nervous system where they are thought to regulate neuronal differentiation, development or synaptic function. To expedite these functions, PS1 forms a complex composed of at least three other transmembrane proteins namely nicastrin, Aph-1 and Pen-2 (Kimberly et al., 2003; Kim et al., 2003; Takasugi et al., 2003). PS1-derived fragments in neurons have been found within the synaptic vesicles, synaptic plasma membranes, synaptic adhesion sites, and neurite growth cone. Studies show that nicastrin, Aph-1 and Pen-2 play an important role in PS1-mediated intramembraneous γ–secretase processing of type I membrane proteins. Interestingly, several lines of evidence suggest that presenilin activity is closely related to the activity of γ-secretase (Suzuki et al., 1994; Duff et al., 1996; Iwatsubo et al., 1994)

Degradation of amyloid beta by protease is important in maintaining and regulating its level in neuronal systems. Although several proteases in the brain have been proposed to potentially participate in amyloid beta turnover, the mechanism of catabolism has not been well understood. Among the degrading enzymes proposed to catabolize amyloid beta are cathepsin D and E, aminopeptidase, gelatinase A and B, trypsinor chymotrypsin-like endopeptidase, serine protease and insulin-degrading enzyme (Saido, 2000). Studies show that lysosomal hydrolases such as cathepsin D are accelerated in AD brain in a manner corresponding to amyloid beta accumulation. Perhaps the future drug intervention may be in part found in the abeta degrading enzyme.

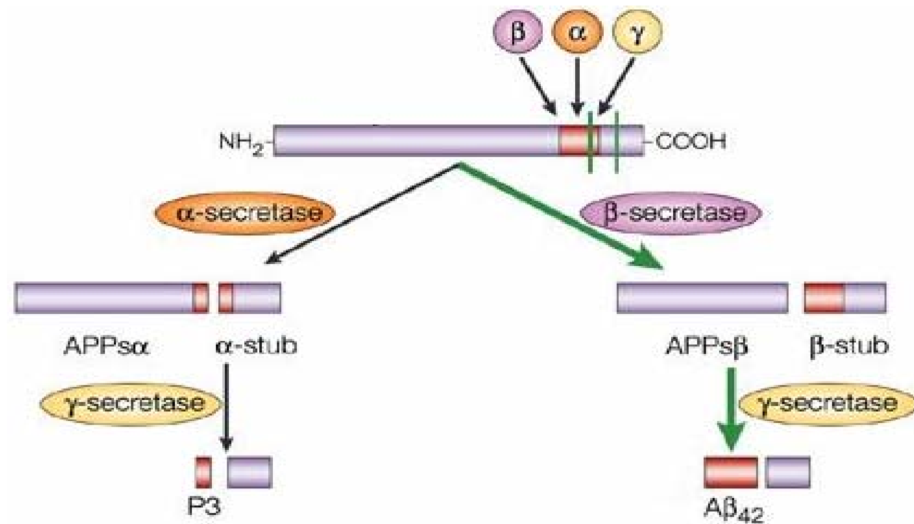

Figure 2.1: Schematic diagram of APP processing. APP is sequentially cleaved, first by β-secretase to form the soluble ectodomain APPsβ and β-stub and then by γ–secretase amyloid beta 1-42 (Ab42). APP can also be alternatively cleaved by α-secretase to form soluble APPsα and the α-stub. The α-stub can be cleaved by γ–secretase to give P3.

The mechanism of amyloid beta-mediated neurotoxicity in AD is still unclear. Several in vitro (Yankner et al., 1990) and in vivo (Games et al., 1995, Johnson-Wood et al., 1997) studies have attempted to characterize the neurotoxicity mechanisms. Some studies indicate that the amyloid beta toxicity is via excitotoxicity of the neurons mainly through abeta-induced alterations in Ca^{2+} homeostasis (Koh et al., 1990, Mattson et al. 1992) while others implicate oxidative stress, induction of apoptosis and mitochondrial dysfunction (Behl et al., 1994; Mark et al., 1997a & b; Mattson, 1997). Although there is apparent evidence for amyloid beta-induced oxidative stress, attempts to block amyloid beta neurotoxicity with antioxidants or free radical scavengers to offer neuroprotection have failed (Lockart et al., 1994; Pike and Cotman, 1996; Zhang et al., 1996]. In addition, cannula infusion of amyloid beta into specific brain region failed to induce abeta related toxicity (Games et al., 1995; Clemens and Stephenson, 1992) suggesting that a precise conformation state of intermolecular beta sheet structures is required for abeta to induce neurotoxicity. In agreement with this idea is the finding that direct interaction of abeta with membrane receptors is required to elicit neurotoxicity (Yankner et al., 1990; Boland et al., 1995; Yan et al., 1996). A summary of different mechanisms of amyloid beta toxicity are shown in table 2.1 below.

Table 2.1: Mechanisms of Amyloid beta Toxicity

Toxicity	Enzymes/receptors involved	Effects	References
Generation of c-terminus peptide (C31)	Caspases	Apoptotic cell death	Lu et al., 2003
Lipid peroxidation	Peroxidase	Synaptic degeneration	(Mark et al., 1997a,b; Keller et al., 1997).
Impaired Na^+/Ca^{2+} exchange homeostasis	Ca^{2+} and Na channels	Increased Ca^{2+}	Wu et al., 1997
Enhancement of glutamate-mediated excitotoxicity	Glutamate receptors	Increased Ca^{2+} influx and excitotoxicity	Harkany et al., 2000
Impairment of Na^+/K^+ ATPase	ATPase	Increased Ca^{2+}	Mark et al., 1995

2.1.2 Role of Tau Protein in Alzheimer's Disease

Tau protein is a microtubule-associated protein that undergoes several post-translational modifications and aggregates into paired helical filaments (PHFs) in AD and other neuropathological conditions. Although tau is not restricted to the brain, it is found in this region in high concentrations, where it was originally isolated (Cleveland et al., 1977a,b). Further studies indicate that tau mRNA and proteins have been detetected in several peripheral tissues such as heart, kidney, lung, muscle, pancreas and in fibroblasts (Grundke-Iqbal et al., 1986; Ingelson et al., 1996; Vanier et al., 1998). In the brain, in addition to neurons tau protein expression has been reported in glial cells, mainly during pathological conditions (Chin et al., 1996). In neurons, tau is normally found in axons (Binder et al., 1985),

but becomes redistributed to the cell body and dendrites in the tauopathies. Axonal tau is colocalized with microtubule, a member of a neurofilament family that is involved in axonal transport from the cell body to pre-synaptic sites. The phosphorylation of microtubule tau may act as a mechanism for regulating microtubule assembly. The filamentous deposits of abnormally modified tau are considered as some of the hallmarks of AD and other neurodegenerative diseases that are collectively known as tauopathies (Table 2.2).

The functions of tau protein in promoting and stabilizing the microtubule involve the assembly of different tubulin subunits (Cleveland et al., 1977a; Weingarten et al., 1975). Stabilized microtubules are important in ensuring efficient axonal transportation of proteins from the cell body to the synaptic site (Stamer et al., 2002). Tau protein may also have other physiological functions in addition to stimulating microtubule assembly. For example tau have been shown to inhibit kinesin-dependent trafficking of vesicles and may interact with the mitochondria and nucleic acids (Ebneth et al., 1998; Kampers et al., 1996; Rendon et al.,1990; Hua et al., 2003). Tau proteins have also been suggested to play a significant role in modifying the cell shape by acting on the submembranous actin cytoskeleton through the src-family tyrosine kinase signalling pathway (Lee et al., 1998).

In adult brain, tau exist in six isoforms that range from 352 to 441 amino acids and about 45 to 69 kDa which include; 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R (Goedert et al., 1989) each of which possibly has its particular

physiological role and differential biological activity (Utton et al., 2001; Stanford et al., 2003). All six isoforms of tau protein are abnormally phosphorylated (Buee and Delacourte, 1999). However, 4R-tau isoforms are more efficient at promoting microtubule (MT) assembly and have a greater MT binding affinity than do 3R-tau isoforms (Goedert & Jakes 1990, Butner & Kirschner 1991). Following the post-translational modifications normal and functional tau may fail to perform its physiological role and undergoes functional transition into a toxic molecule and aggregation into paired helical filaments. Conversely, these abnormal forms of tau are found in high concentration in PHFs and neurofibrillary tangles. It is still not clear whether polymerization of tau into filaments and consequently into a PHF is a protective mechanism against the unsequestered hyperphosphorylated tau. Moreover the role of paired helical filaments in inhibiting the axonal transport is still not well known.

Tau modifications include hyperphosphorylation, glycosylation, ubiquitination, glycation, polyamination, nitration, and proteolysis. However, the hyperphosphorylation processes are of primary interest to the molecular pathogenesis of neurofibrillary degeneration of AD. Although the effect of glycosylation of tau is not well known, the nonenzymatic glycosylation (glycation) of advanced glycation end products (AGEs) may be responsible for PHF insolubility, since a cross-linking reaction leading to the formation of insoluble aggregates of proteins is attributed to proteins glycation (Kent et al., 1985; Ko et al., 1999; Smith et al., 1996). In addition, immunohistochemical

studies show that AGEs are colocalized with both senile plaques and NFT in AD (Sasaki et al., 1998; Smith et al., 1995). Interestingly, although uncontrolled phosphorylation (hyperphosphorylation) of tau protein play a pathogenic role in AD, phosphorylation of tau is required for its normal physiological functions. Indeed, studies show that increasing tau phosphorylation negatively regulates microtubule binding (Drechsel et al 1992, Bramblett et al 1993, Yoshida & Ihara 1993, Biernat et al 1993). Hence, although microtubule assembly depends partially upon the degree of phosphorylation, hyperphosphorylated tau proteins are less effective than hypophosphorylated Tau on microtubule polymerization (Buee et al., 2000). In addition, phosphorylation of Ser262, located in the first microtubule-binding domain, dramatically reduces the affinity of tau for microtubules in vitro. However, this site alone is insufficient to abolish Tau binding to microtubules. The microtubules have four binding motifs of which the binding domains of tau are localized to the carboxy-terminal end. Studies show that the motifs are composed of highly conserved 18–amino acid long sequences that are separated by flexible, but less conserved, inter-repeat sequences of 13–14 amino acids (Himmler et al 1989, Lee et al 1989, Butner & Kirschner 1991). An important fact to note is that tau phosphorylation is developmentally regulated such that tau from immature brain is phosphorylated at more sites than tau from adult brain (Goedert et al., 1993)

Of particular interest is the question yet to be resolved as to which phosphorylation site distinguishes AD from other tauopathies. There are

suggestions that this site may be the threonine 181 phosphorylation site (Nagga et al., 2002). The hyperphosphorylated tau protein is quite resistant to proteolytic degradation (Wang et al., 1995) and its diminished turnover may be responsible for the formation of neurofibrillary tangles (NFT). The formation of neurofibrillary tangles therefore corresponds to the aggregation of abnormally phosphorylated tau proteins into filaments referred to as paired helical filaments (Terry 1963), within certain vulnerable neuronal populations. There are reports that the *in vitro* polymerization of tau is facilitated by sulfated glycosaminoglycans (sGAG) (Arrasate et al., 1997; Goedert et al., 1996; Perez et al., 1996). Such findings are supported by previous studies that reported colocalization of NFT with sGAG-bearing proteoglycans in AD patients (De Witt et al., 1993; Perry et al 1991). More recently, there are suggestions that GAGs may enhance tau aggregation and disturb microtubule assembly since GAGs were shown to bind to the microtubule-binding domains of tau proteins (Spillantini et al., 1999). Indeed, assembly of individual 3R-tau isoforms gives a typical paired-helical-like filament when incubated with heparin or heparan sulfate, whereas assembly of individual 4R-tau isoform gives straight filaments (Goedert et al 1996, Perez et al 1996). Although sGAG-like chondroitin or heparan sulfate are potent enhancers of tau aggregation, which perhaps contribute to cognitive decline, the findings are intriguing since heparin sulfate was shown to play a significant role in synaptic plasticity (Sinnarajah et al., 1999). However, if the formation of NFT is a neuroprotective mechanism as it has been suggested, then

the physiological role of sGAGs in synaptic function is consistant with these findings.

Table 2.2: Tau Isoforms in Different Neuropathological Conditions

Disease	Tau isoforms	Filamentous inclusions	Region	Reference
Progressive supranuclear palsy (PSP)	Tau 64 & tau 69	PHF & SF	basal ganglia, brainstem, & cerebellum	Flament et al., 1991; Vermersch et al., 1994
Pick disease	Tau 55 & Tau 64	Pick Bodies	frontotemporal	Delacourte et al., 1996; Hof et al., 1994; Buee-Scherrer et al., 1996
AD	Tau 55 Tau 64 & Tau 69	PHF & SF	entorhinal cortex & hippocampus	Vermersch, et al., 1992,1995; Delacourte et al., 1998, 1999
PHF= Paired helical filament, SF straight filaments.				

Abnormal phosphorylation of tau protein and its aggregation into intracellular filaments that may consequently lead to neuronal death have been reported by several investigators (Lee et al., 2001; Buee et al., 2000). Hyperphosphorylation is also believed to be an early event in the pathway that procedes from soluble to insoluble and filamentous tau protein (Braak et al 1994). However, it is not known what causes hyperphosphorylation but there are suggestions that it might involve an increase in kinase activity and/or a decrease in phosphotase activity (Brion et al., 2001; Buee et al., 2000). Several protein kinases have been implicated in tau phosphorylation among them are; glycogen synthase kinase-3b (GSK-3b) (Ishiguro et al., 1993), mitogen-activated protein kinase (MAPK) (Drews et al., 1992), Ca^{2+}/calmodulin-dependent kinase II (Sironi et al., 1998), casein kinase I (Singh et al., 1995), MARK kinase and protein kinase

A (PKA) (Schneider et al., 1999). The neuronal Cyclin-dependent kinase 5 (Cdk5) has also been shown to play a specific role in the tau phosphorylation process (Patrick et al., 1999; Matsushita et al., 1995; Michel et al., 1998). Surprisingly, cdk5 is required for normal development of the mammalian central nervous system. To carry out this role and for its activity, Cdk5 has to associate with its regulatory subunit, p35. However, a truncated form of p35, p25, accumulates in neurons in the brains of patients with AD, whose accumulation correlates with an increase in Cdk5 activity. Abnormal tau accumulation has been observed in more than 20 neurological disorders with dementia (Buee et al., 2000). There is a close relationship between the extension of tau pathology and cognitive deficits observed in AD and other dementia disorders (Delacourte A, Sergeant N and Buee L., 2002; Delacourte et al., 2002; Fewster et al., 1991).

Although there is speculation about the link between neurofibrillary tangle formation and β-amyloid deposition the precise relation between the two is still not clear. Studies show that whereas Aβ fibrils can induce tau-containing neurofibrillary lesions, the toxicity is species specific which may be pronounced in some and less significant in others (Geula, et al., 1998). In addition, the amyloid precursor protein is a phosphoprotein whose metabolic regulation requires the PKC and casein kinases (Walter et al., 1997; Walter et al., 2000). Despite these links between abeta and tau protein it is still not clear which one plays a major role in producing the dementia. However, studies show that the degree of dementia correlates with the amount of tau rather than Aβ deposits

(Arriagada *et al.,* 1992). In addition, amyloid beta deposits are sometimes found in cognitively normal individuals in the absence of tau deposits.

2.2. Alzheimer's Disease Vs Normal Aging

Although progressive cognitive decline is characteristic of AD, it is not unique to this disease. Interestingly, the process of normal aging may exhibit similar features as those observed in AD. Indeed, changes during normal aging of the human brain show quantitative overlap with the phenomena characterizing AD including low levels of abeta deposits and tau aggregates (Morris et al., 1996). These findings are supported by the fact that the percentage of the population affected by a brain disease increases dramatically after the age of 70 years and 40% of the population aged 90 years and over are demented. Surprisingly, a huge number of brain lesions may be observed in some non-demented patients. However, neuroplasticity and neuronal compensation may overcome the pathological process giving the patient a non-dementia status. Despite the non-dementia state, all lesions found in a brain from a non-demented patient, even with excellent and proven cognitive functions, could be a signal of a pathological process. Brain aging is a regionally differentiated process at least in humans with meganeurites occuring in the superficial but not deep layers of the frontal cortex. On the other hand, severe effects of Alzheimer's disease, are found in the superficial entorhinal cortex and subiculum compared other parts of the forebrain (Braak and Braak 1994; Honer, et al., 1992; Morrison and Hof 1997). The meganeurites are more pronounced in field CA1 but not field CA3 of the

hippocampus (Braak 1984). Similarly the neurofibrillary tangles and amyloid plaques occur with very different frequencies in different zones of the cortical telencephalon (Braak and Braak 1991).

Studies show that a subtle decline of memory function commonly occurs in normal aging but may not progress to AD (Petersen et al., 1992; Small et al., 1999). Although cognitive decline during normal aging and in AD may be similar, many investigators consider the two mechanisms to be very different. It was initially thought that both conditions exhibit neuronal loss but experiments have now demonstrated that while there is some cell loss in the aging brain, neuronal loss in the areas involved with memory storage such as the entorhinal cortex is normally not significant (Price et al., 2001) or very minimal (Insausti et al., 1998). Thus, during normal aging memory loss may occur as a result of alteration in brain chemistry that may include neurotransmitters and their receptors that in turn result in changes in the way the neurons communicate. On the other hand the memory loss and other cognitive changes in AD are thought to be a result of profound neuronal loss in the parts of the brain critical for memory.

As will be discussed later both Alzheimer's and aged brain exhibit overactivation of the lysosomal system. However, Alzheimer-related alterations of the lysosomal system far exceed those accompanying normal aging. This can be depicted by increased levels of Cathepsin D and constant or even decreased

levels of several other cathepsins in normal aging (Green et al., 1981), compared to early AD

2.3. Theories of Alzheimer's disease

There are two major and widely accepted types of AD; familial (inherited) and sporadic. The two types are discussed in detail in this section. However, much emphasis is placed on the sporadic which is the most common. Under this theory the neurotransmitter systems involved are discussed.

2.3.1 Neurotransmission hypotheses.

The leading hypothesis of AD is that it results from a progressive synaptic failure prior to dramatic cellular changes such as cell death. Hence, the neurotransmitter and the receptors involved in the synaptic communication are perhaps the best candidates for this theory and will be the major focus in this Chapter.

a) Glutamatergic System

Glutamate is the most abundant neurotransmitter in the human brain and the major mediator of excitatory neurotransmission in the central nervous system (CNS). Glutamate receptors can be classified as metabotropic or ionotropic depending on the effects of their activation; G-coupled second messengers or ion gating, respectively. In particular the ionotropic glutamate receptors have been shown to mediate excitatory synaptic transmission in the vertebrate central nervous system (Cotman and Monaghan 1987; Monaghan *et al.*, 1989; Boulter *et al.*, 1990). Moreover, the glutamatergic transmission has been implicated in the

long lasting plasticity changes that enhance the communication between neurons (Nakanishi, 1992; Hollmann and Heinemann, 1994; Conti and Weinberg 1999). Such communication plays a significant role in long term potentiation (LTP), a form of synaptic plasticity thought to underlie memory and learning (Bliss and Collingridge 1993). Traditionally, the ionotropic glutamate receptors that transmit this communication have been classified into three subtypes according to their preferred pharmacological agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate. The expression of long-lasting plasticity is mediated through AMPA receptors (Muller and Lynch 1988; Isaac et al 1995; Durand *et al*., 1996; Ben-Ari *et al*., 1997) resulting in synaptic activity that transiently activates NMDA receptors (Bliss and Collingridge 1993). Of particular interest to researchers is the pathophysiological relationship between ionotropic glutamate receptors and the neurodegeneration observed in several disorders of learning and memory including Alzheimer's disease.

Studies show that working memory performance is diminished by reducing glutamate release or a decrease in its postsynaptic action on AMPA receptors at least in prefrontal cortex neurons of rodents (Romanides et al., 1999). In addition, NMDA receptor agonists and antagonists were shown to either enhance or reduce the working memory respectively in monkeys (Dudkin et al., 1997). Suprisingly, exposure to apoptotic insults of amyloid β-peptide in cultured hippocampal neurons results in proteolytic degradation of AMPA receptor

subunits (GluR1, GluR2/3, and GluR4) in neurons (Chan et al., 1999). The degradation of the AMPA receptor subunits is prevented by caspase inhibitors indicating that caspases mediate the proteolysis of the receptor subunits. Although caspases can be activated in response to physiological stimulation of glutamate receptors (Mattson et al., 1998a) it is still not clear whether caspase-mediated cleavage of AMPA receptor subnits occurs during or is involved in synaptic plasticity. However, actin and spectrin are known to be substrates of caspases (Martin et al., 1995; Kayalar et al., 1996; Wang et al., 1998) and these cytoskeletal proteins may modulate synaptic plasticity (Furukawa et al., 1997). Interestingly, changes in actin polymerization can affect NMDA induced currents, voltage-dependent calcium currents, and calcium responses to glutamate in cultured hippocampal neurons (Furukawa et al., 1995, 1997).

The complexity of glutamate receptors including multiple subtype configurations offers a challenge as to which of these elements is the likely cause of cognitive decline in AD. Several drugs in trial now target both AMPA and NMDA receptors. Pioneer studies focusing on glutamatergic enhancement were performed utilizing pyrrolidinones (e.g., aniracetam, piracetam) and benzothiadiazines (e.g., cyclothiazide). These studies show that these chemicals can positively modulate AMPA receptor-mediated currents by suppressing the desensitization process (Ito et al., 1990; Copani et al., 1992; Yamada and Tang, 1993). Although the mechanism of desensitization is still not clear, experimental observations suggest that aniracetam and cyclothizide suppress the AMPA

desensitization through different mechanisms. While cyclothiazide may potentiate AMPA receptors by directly slowing the onset rate of desensitization and by increasing agonist affinity, (Yamada and Tang, 1993; Partin et al., 1994, 1996), aniracetam may potentiate AMPA receptors by directly slowing the rate of deactivation which indirectly slows the onset of desensitization, without a change in agonist affinity (Partin et al., 1996).

A new class of AMPA modulator, CX516, has been used in clinical studies to enhance AMPA receptor function. Indeed, ampakine has been shown to increase memory at doses that do not produce adverse effects (Lynch, 2002). The functional consequence of AMPA modulators is to augment glutamatergic excitatory postsynaptic potentials (EPSPs) by increasing ion flux through AMPA receptors (Ito et al., 1990; Yamada and Tang, 1993). Although direct modulation of NMDA receptors by members of the ampakine family has not been demonstrated, positive AMPA receptor modulators can facilitate NMDA receptor-dependent LTP in the CA1 region of the hippocampus (Staubli et al., 1994a). These findings may contradict the use of AMPA modulators and NMDA antagonist in the treatment of the cognitive impaired patient. However, the enhancement of LTP in the CA1 region is perhaps via mechanisms that involve an increase in the expression of neurotrophins such as brain-derived neurotrophic factor (BDNF), which may influence synaptic plasticity (Lauterborn et al., 2000). Indeed, the induction of LTP in CA1 neurons has been shown to depend on elevation of intracellular BDNF (Patterson et al., 1996). Hence, the use

of AMPA modulators and NMDA antagonist may still be a valuable therapeutic intervention in the treatment of cognitive impairment. However, fairly recent studies by Baumbarger et al., 2001 indicate that one of the AMPA modulators LY404187 augmented the NMDA receptor component of the EPSP in conjunction with potentiation of the AMPA receptor, and that a secondary consequence of positive AMPA modulation is the recruitment of voltage-dependent NMDA receptor activity, which may concomitatly increase synaptic strength.

The idea of targeting AMPA receptors in the treatment of AD arise from previous studies in animal models as well as in human patients. A reduction in AMPA binding was reported in the hippocampus of AD brain (Dewar et al., 1991; Geddes et al., 1992). Experiments also reported a decrease [3H]-AMPA binding in the subiculum and CA1 which correlated with neuronal degeneration and cell loss (Dewar et al., 1991; Geddes et al., 1992). Further studies indicate that a strong interaction exists between aggregated Aβ1–42 peptide and Calcium-permeant AMPA/NMDA ionotropic receptors (Blanchard et al., 1997, 2000). Data suggest that NMDA, AMPA and Aβ25–35 rapidly inhibit fast axonal transport (Hiroma et al., 2003). These studies further suggested that NMDA and AMPA receptors inhibit axonal transport via calcium ion influx, whereas the effect of Aβ25–35 occurs via polymerization and aggregation of intracellular actin. An age related decline in NMDA has been reported in monkeys (Wenk, et al., 1989; 1991) and in rodents (Cohen and Muller, 1992; Kito et al., 1990). In addition a significant age-related decrease in NMDA receptor subunits NR1 and

NR2B, but not NR2A, has been observed (Magnusson, 2000). The observed modification of subunit expression may alter the binding and physiological properties of these receptors (Priestley et al., 1995; Gallagher et al., 1996). Experimental observations suggest that AMPA receptors may be more resistant to age-related changes than NMDA receptors at least in certain brain regions such as cerebral cortex (Tamaru, et al., 1991).

A possible question is whether the decrease in AMPA and NMDA receptors in both aged and AD brain are fully responsible for cognitive decline. This is not necessarily so given that other parameters come into play when it comes to excitatory synaptic function. First, the amount of glutamate released from the post-synaptic site may be increased to counteract the loss of receptors (Kitamura et al., 1992). Second, the receptors may undergo a conformational change to attain a higher affinity status (Cohen and Muller, 1992; Peterson and Cotman 1989). Finally, the activity of the receptors may be enhanced to compensate for the lost receptors. However, it is not known whether these compensatory mechanisms are enough to maintain a normal excitatory synaptic function. Although it is difficult to determine the AMPA and NMDA receptors function in AD patients, animal and slice models of neurodegeneration offer a good tool to study these events. Unfortunately, this area has not been fully explored.

Like many other proteins, AMPA and NMDA receptors undergo several post-translational modifications before they become fully functional receptors. Of

these changes, phosphorylation is perhaps the most common form of post-translational modification for both AMPA and NMDA. Experiments have established that phosphorylation can modulate not only the intracellular receptor trafficing but also receptor functional characteristics such as open probability and mean open time (see review by Carvalho et al. 2000; Barry and Ziff 2002; Malinow and Malenka 2002; Song and Huganir 2002; Gomes et al. 2003). Indeed, phosphorylation of AMPA receptors by protein kinase A (PKA) increases the amplitude, frequency, mean open time and decay time of spontaneous excitatory post-synaptic currents (sEPSC) in cultured hippocampal pyramidal neurons (Greengard et al. 1991). Furthermore, recent findings indicate that phosphorylation of GluR1 is necessary for plasticity, learning and memory (Lee et al. 2003). Interestingly, there seems to be an imbalance between phosphorylation and dephosphorylation processes mediated by kinases and phosphatases, respectively, in AD brain. Whether AMPA and NMDA phosphorylation mechanisms are affected by this imbalance is still not clear.

Since glutamatergic pathways play a significant role in synaptic changes that enhance learning and memory they might be a candidate system for cognitive decline in several neurodegenerative diseases (Choi, 1988, 1992; Pellegrini-Giampietro et al., 1997). Specifically, the NMDA receptors may play a dual role in normal neuroplasticity as well as neurodegeneration (Rogawski et al., 2003; Obrenovitch, et al 1997; Riedel et al., 2003). NMDA receptors, which are permeable to calcium and also sodium ions, may be overactivated to cause

excitotoxicity of neurons (Albin and Greenamyre 1992). Indeed, calcium influx has been linked with neuronal excitotoxicity. In addition, although the majority of AMPA receptors in adult brain are calcium impermeable, a subpopulation of calcium permeable receptors may be responsible for the excitotoxicity (Palmer and Gershon, 1990). For such calcium permeable AMPA receptors the expression of the GluR2 subunit with an edited Q/R site confers Ca^{2+} impermeability. Immunohistochemical studies have shown changes in staining intensity and distribution of the AMPA receptor subunits (GluR1 and GluR2/3) in the AD hippocampus (Armstrong and Ikonomovic, 1996; Armstrong et al., 1994; Ikonomovic et al., 1995a &b; Wakabayashi et al., 1999; Yasuda et al., 1995).

b) Cholinergic System

In the last two decades there has been an increased interest in the functions of the central cholinergic systems. This interest is driven by the neuropathological demonstrations that cholinergic markers in the cerebral cortex are reduced in postmortem AD brain and this correlates with the degree of cognitive impairment (Bowen et al 1976, Perry et al 1978). Indeed, dramatic reduction of cholinergic transmission was the first observation to be well defined and to be implicated, among all neurotransmitter systems, in the neuropathological condition of AD. Studies show that the cholinergic system is critically involved in the control of cognition (Everitt et al., 1997). A transient loss of short term memory was observed following the blockade of the muscarinic acetylcholine

receptors (Sutherland et al., 1982; Roldan et al., 1997; Coyle et al., 1983). As a result of these and other similar findings, first generation drugs that target the acetylcholine neurotransmitter system were developed. However, the minimal effectiveness of this first class of drugs that includes the acetylcholine esterase inhibitors suggest that this system is not the only cause of cognitive decline observed in AD. Despite the minimal effectiveness of drugs that target the acetylcholine neurotransmitter, they still enjoy popularity in treating AD pathology.

One of the most fundamental and consistent features of AD is believed to be the severe degeneration of cholinergic neurons projecting from basal forebrain to cortical and hippocampal areas (Whitehouse et al., 1982; Coyle et al., 1983). Indeed, the basal forebrain cholinergic system has been shown to play a significant role in attention and cognition (Everitt & Robbins, 1997; Sarter & Bruno, 1997; Sarter et al., 2001; McGaughy et al., 2002). As will be discussed under animal models of AD, lesioning of basal forebrain cholinergic neurons in rats with toxins results in impairment of spatial memory tests and attentional functions (Waite et al., 1995; Baxter et al., 1995; Mcgaughyet al., 2002). Studies show evidence of significant loss of nicotinic ACh receptors and certain types of muscarinic ACh receptors in the cortical and hippocampal regions which correspond with neurodegeneration in AD brains (Giacobini, 1990; Greenamyre, et al., 1993; Perry, et al., 1995). Amyloid beta peptides have been suggested to be responsible for suppression of acetylcholine synthesis at least in some

experimental models (Auld, et al., 1998; Hoshi, et al., 1997; Pedersen et al., 1996). Suprisingly, stimulation of nAChRs was shown to inhibit amyloid beta and glutamate-induced cytotoxicity (Kihara et al., 1997; 1998; 2001; Shimohama et al., 1996). These findings are intriguing and it is not clear whether nAChR downregulation precedes neurodegeneration.

Compared to glutamate, the acetylecholine neurotransmitter system is even more complex with more than eight receptor subtypes characterized to date. The complexity is made even more intricate by the fact that acetylcholine neurons make contact with and activate other neurons in different region of the brain. Cholinergic inputs into the hippocampus can activate glutamate receptors in this region. Interestingly, in addition to activating excitatory glutamate receptor rich neurons, acetylcholine may also activate inhibitory neurons such as GABA (Ma et al., 2003). Such inhibition is via inhibition of PKC which in turn blocks the mAChR enhancement of spontaneous IPSC amplitudes (Zhong et al., 2003).

c) GABAergic System

GABA receptors can be subdivided into the GABAA and GABAB subtypes. Just as glutamate is the major excitatory neurotransmitter, GABA is the major inhibitory neurotransmitter in the cerebral cortex (Krnjevic et al., 1997). In fact glutamate and GABA transmitters predominate in the cortical neurons. Furthermore, the GABAergic neurons interaction with cholinergic neurons (Ma

et al., 2003) provides insight into why these receptors may be implicated in AD. The activation of mAChRs has been shown to significantly increase the amplitude of GABAergic spontaneous inhibitory postsynaptic current (sIPSC) in prefrontal cortex pyramidal neurons from wild-type animals but not in APP transgenic mice (Zhong et al., 2003). In essence, the interactions between cortical cholinergic and GABAergic inputs, and their interrelated effects on cortical neuronal excitability, are immensely complex and remain poorly understood. As such there has been an increasd interest in elucidating the role of the GABAergic system in neurodegeneration of AD.

Previous studies on the role of the GABAergic neurotransmitter system in learning and memory provide the basis for GABA-mediated effects on cognitive decline. For example, GABA antagonists are known to enhance learning and memory (Nyitrai et al., 1999; Olpe et al., 1993, Mondadori et al., 1993, 1996; Carletti et al., 1993; Nakagawa et al., 1997; Froestl et al., 2004). Other studies provide evidence of age related alterations in GABAA receptor subunits mRNA levels in rats (Mhatre et al., 1992; Rissman et al., 2000). Surprisingly, the hippocampal GABAergic system has been found to be relatively resistant to insult in AD (Rossor et al., 1982; Mountjoy et al., 1984). However, recent detailed biochemical analysis of individual GABA subunits indicates that the α5subunit of the GABAA receptor subunits are reduced in the AD brain while the α1 remain relatively unchanged (Rissman et al., 2003).

d) Other Neurotransmiter Systems

In addition to the neurotransmitters discussed above, many other neurotransmitters and their receptors apparently become involved at least in the late stages of the disease. Indeed, neurotransmitters such as norepinephrine, dopamine and serotonin may exhibit reductions of up to 50% in the late stages of AD. Serotonin (5-HT) plays an inhibitory role on motivated behaviors and other neurotransmitter systems, and hence loss of inhibiting tone from the 5-HT system may be important in behavioral and psychological symptoms of dementia (Herrmann and Lanctot 1997; Lanctot et al. 2001). Acute depletion of tryptophan, the precursor to serotonin has been reported to cause the impairment in working memory in Alzheimer-type senile dementia patients (Porter, et al., 2003). Selective serotonin reuptake inhibitors (SSRIs), 5-HT1A partial agonists (Bergman et al. 1983; Burke et al. 1994; Pollock et al. 1997) and serotonin norepinephrine reuptake inhibitors have been suggested for trial treatment for BPSD (Lawlor et al. 1994; Sultzer et al. 1997) and in AD patients (Lanctot et al. 2002; Mintzer et al. 1998).

Although the specific functions of norepinephrine neurons in certain brain regions are not well known, there are suggestions that these neurons mediate reward related conditioning which pertains to cortical learning (Gratton & Wise, 1988). Reductions in both serotonergic and/or norepinephrine

neurotransmission have been found to affect learning and memory in humans and rats (Parvizi, et al., 2001; Madhyastha, et al., 2002; Myhrer, 2003; Porter, et al., 2003; Collier, et al., 2004). Indeed, the norepinephrine neurons of the locus coeruleus and the raphe neurons of the dorsal and central raphe nuclei appear to be selectively disrupted by the AD process in a manner corresponding to the Ach system. Other findings indicate that the combined loss of serotonergic and cholinergic neurons leads to a spatial learning deficit in rats (Richter-Levin, 1989; Nilsson, et al., 1990)

Despite implication of other neurotransmitter systems in AD, it is worthwhile to note that the neurotransmission disruption is so selective that a particular neurotransmitter can be affected when it belongs to one neural system, but on the other hand remain intact in a different system or even different cortical projection. In addition the general pattern of disruption of the neurotransmitter systems also provides support for the notion that memory mechanisms are selectively attacked by the AD process.

2.3.2. Genetic Hypotheses.

The familial type of AD is relatively rare, accounting for less than 5% of all AD cases. The familial type is also characterized by early onset in individuals less than 50 years of age. Familial AD has thus been associated with rapid cognitive decline and shorter time to death. On the other hand the sporadic AD usually has a late onset usually starting in the late 60s. According to the genetic theory,

familial AD can be transmitted genetically in an autosomal dominant fashion. Three candidate gene loci have been proposed to contribute to familial AD. These genes include βAPP on chromosome 21, PS1 on chromosome 14, and PS2 on chromosome 1 (Goate et al., 1991; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrigton et al., 1995; Selkoe and Podlisny 2002). The clinical consequence of these mutations is mainly through altering APP processing. Although the mutations in tau gene have been proposed, the clinical significance in AD has not been established.

2.4. The role of lysosomal and ubiquitin systems in Alzheimer's disease

2.4.1 Lysosomal System

The lysosomal pathway consists of a family of organelles that are involved in recycling of cellular ingredients as part of a house keeping role to maintain normal and healthy cells. To perform this role lysosomes are equipped with over 80 hydrolytic enzymes, including the cathepsins family, calpains, caspases and other proteases. Although these enzymes are active within a small range of pH, there are some such as cathepsin D that function over a wide range of pH ranging from physiological to neutral pH. Part of the lysosomal system (early and late endosomes) may carry out limited proteolysis to generate new functionally important proteins while the lysosome carries out most of the autophagy and endocytosis processes. The early endosome is thought to carry out three major sorting functions on the lysosomal pathway. First, the early

endosome receives extracellular materials from the cell surface. Second, it is involved in recycling of some receptors back to the plasma membrane with a major implication in the synaptic region. Third, it directs ligands and some receptors to late endosomes (Clague, 1998). Due to this intergrated function of the lysosomal pathway, a dysfunction of this system may have a great impact on the normal function of the cells. Interestingly, lysosomal function progressively destabilizes in a manner that correspond to the aging process in an animal (Brunk and Brun 1972; Nakamura et al. 1998). The increased lysosomal system instability may contribute to the atrophy and eventual lysis of the neuron. The induced apoptosis or a mixed apoptotic/necrotic pattern in various cell systems is thought to be a result of leakage of cathepsins (Roberg and Ollinger 1998; Hellquist et al., 1997; Fossel et al., 1994; Brunk et al., 1997). Such leakage of the enzyme into the cytosol follows Aβ1-42 accumulation in lysosomes.

Lysosomal dysfunction, as indicated by accelerated hydrolases turnover in the neocortical pyramidal neurons, has been implicated in a variety of pathogenic events that consequently result in age related neurodegeneration. Thus, lysosomal disturbance is perhaps among the earliest markers of metabolic dysfunction in AD (Cataldo et al., 1990. Cataldo et al., 1991; Nixon et al., 1992). Indeed, studies implicate lysosomal disruption in the development and accumulation of beta amyloid fragments (Bahr et al., 1994; Hajimohammadreza et al 1994; Nixon et al., 2000) and tau deposits (Takauchi et al., 1995; Bi et al., 2001; Bendiske et al., 2002). The endosomal-lysosomal pathway may therefore be

a candidate site for the amyloid precursor protein cleavage into smaller β-amyloid containing peptides (Kohnken et al., 1995; Ladror et al., 1994; Haass et al., 1992; Golde et al., 1992). This may cause amyloidogenic events in different ways. First, re-internalized APP into endosomal lysosomal system may be converted into carboxyterminal fragments which are long-lived due to suppressed hydrolase activity during lysosomal perturbation. Second, as a result of lysosomal pathway disturbance, APP trafficking may be altered which promotes availability for processing in the *trans* golgi network.

The implication of the lysosomal system in neurodegeneraion is perhaps mainly due to its role in internalization and initial processing of proteins linked to AD. Of particular interest are the APP and ApE proteins which have been shown to play a major role in AD pathology and whose function depends mainly on their internalization. In addition, early endosomes are also a major site of Aβ production in normal cells and mediate the cellular uptake of Aβ and soluble APP. Following lysosomal dysfunction the accumulation of amyloidogenic fragments and neurofibrillry tangles may lead to early pathological events that target synapses (Bahr et al. 1994, 2002; Bendiske et al. 2002). The emerging synaptopathogenesis causes a decline in synaptic markers such as synaptophysin and GluR1 together with their respective mRNA species. As a result there is a concomitant disruption of synapse structure and functionality (Bahr et al. 1998; Hsia et al. 1999; Mucke et al. 2000; Kim et al. 2001; Walsh et al. 2002). Declines in synaptic markers have been reported as one of the characteristics of AD brains,

thus implicating synaptic loss as a major and early contributor to cognitive impairment (Terry et al. 1991; Mesulam 1999). It is not clear whether lysosomal dysfunction directly impairs memory, but at least the indirect link between lysosomal dysfunction and synaptic pathology has been identified in AD brains (Callahan et al. 1999; Masliah and Licastro 2000).

As mentioned earlier the lysosomal hydrolases such as cathepsin D increase with age. Immunocytochemical studies indicate that lysosomal dysfunction begins early in adult life and occurs in brain regions that are associated with critical pathologies in the aged human brain. Indeed, the cytosolic cathepsin D activity increases significantly from 2 to 6 months of age in rat brain and is twice that found within the lysosomal fraction by 36 months (Nakamura et al., 1989). This corresponds well with studies that show evidence of increased intraneuronal levels of cathepsin D in AD vulnerable regions prior to the onset of overt pathology (Troncoso et al 1998; Cataldo et al., 1991). Such increased levels of cathepsin D protein correlate with depressed levels of the vesicular protein synaptophysin as well as the presence of intraneuronal neurofibrillary tangles (Callahan et al., 1999). Despite the increased levels of cathepsin D with age, other cathepsins such as cathepsin B activity remain at a stable level (Nakamura et al., 1989; Nakanishi et al., 1997). On the other hand, the activity of cathepsin L decreases by 90% from 2 to 28 months in rat brain (Nakanishi et al., 1994). Surprisingly, selective suppression of cathepsins B and L causes rapid increases in cathepsin D in addition to proliferation of lysosomes in the basal pole of

pyramidal cells (Bednarski et al., 1997; Bi et al., 1999). Furthermore, there are recent observations that chloroquine, a compound that elevates intra-lysosomal pH, increases cathepsin D concentrations while blocking cathepsin L, and marginally reducing cathepsin B activity (Bednarski and Lynch 1998)

Since lysosomal dysfunction correlates with brain aging and AD pathology, experimental induction of lysosomal disturbance in brain slices or whole animal may be a powerful tool to study disease progression. Intial experimental studies began with infusions of two functionally distinct, broad spectrum inhibitors; leupeptin or chloroquine (a general lysosomal enzyme inhibitor) into the ventricles of young, adult rats (Ivy et al., 1984). Both drugs caused a rapid and dramatic increase in the number of lysosomes followed shortly by intraneuronal accumulations of hyperphosphorylated tau and distended initial axon segments (Ivy et al., 1989). Recent studies as dicussed later in this project, have utilized hippocampal slice cultures in which lysosomal dysfunction was induced by application of chloroquine (Bahr et al., 1994; Bednarski, et al., 1997; Bi et al., 1999).

2.4.2. Ubiquitin system

While protein degradation in the lysosomal pathway is non-specific, the degradation in the proteosomes is specific requiring tagging by ubiquitin (Ub). Thus, the covalent attachment of ubiquitin is a signal that targets proteins for destruction by proteasome. Ubiquitin is regarded as a stress protein and has been

implicated in the ATP-dependent degradation of short-lived proteins or the removal of abnormal or damaged proteins (Hershko and Ciechanover 1998). The ubiquitin-proteasome system (UPS) was first implicated in AD through the immunohistochemical observation of Ub-conjugated tau protein in neurofibrillary tangles (Mori et al., 1987; Perry et al., 1987) and later confirmed by other investigators (Morishima-Kwawashima et al., 1993; Lowe et al., 1993). In addition, there is a diminished ubiquitin-proteasome system activity in the cortex and hippocampus of AD brain that may further decrease with aging (Keller et al., 2000a, 2000c; Lopez et al., 2000). Recent findings implicate mutations of ubiquitin proteosome system components in neurodegenerative diseases (Ciechanover and Brundin, 2003).

Ubiquitin was first described by Goldstein and collegues (1975) as a highly conserved ubiquitous protein. It is a cytosolic protein of 76 amino acids but can also be found in the nucleus of cells, and is synthesised in all eukaryotic cells. Hence, the UPS controls the levels of most cytosolic and nuclear proteins, while the lysosomal system is responsible for the removal of secretory and internalized proteins. The ubiquitin-mediated regulation of protein structure and function may play a significant role in several processes, including synaptic plasticity. As a result the ubiquitin proteosome system has been implicated in hippocampal long term potentiation in mice (Jiang et al., 1998) and in growth of presynaptic nerve terminals in *Drosophila* (DiAntonio et al., 2001). In addition, long term facilitation of synaptic strength in *Aplisia* is dependent upon UPS degradation of

the regulatory subunit of the cyclic AMP-dependent protein kinase (Hegde et al., 1997; Chain et al., 1999). Perhaps more evidence comes from the findings that ubiquitin-mediated endocytosis of AMPA subtype of glutamate receptors regulates the strength of synaptic transmission (Burbea et al., 2002; Turrigiano, 2002). Indeed, previous studies indicate that regulated AMPA receptor abundance at the synapses by insertion and removal from the postsynaptic membrane is required for the expression of long term potentiation (Lledo et al., 1998) as well as long-term depression (Luscher et al., 1999; Luthi et al., 1999; Man et al., 2000; Wang and Linden, 2000).

Proteins can be either mono-ubiquitinated or poly-ubiquitinated in a process that requires ubiquitin activation by an enzyme (E1). Down the cascade, ubiquitin is then activated by a large group of ubiquitin conjugating enzymes (E2), which either act alone or in concert with ubiquitin–protein ligase (E3) (Hochstrasser, 1996). Although it is not well known whether mammalian AMPA receptors also require mono-or poly-ubiquitination for endocytosis, which might implicate a role in AD, polyubiquitin-mediated degradation of proteins that normally prevent internalization of glutamate receptors may indirectly promote AMPA receptor internalization (Colledge et al., 2003). Interestingly, as is in the case of lysosomes, proteasome expression, activity and response to oxidative stress are impaired during ageing (Bulteau, et al., 2000; Keller et al., 2000a; Merker and Grune, 2000). Notwithstanding, the proteasome activity also appears to be reduced in Alzheimer's disease brains (Keller et al., 2000b). Support for

these findings come from studies that indicate proteasome inhibition alters APP processing, thereby causing an increase in Aβ production (Lopez, et al., 2000; Nunan et al., 2001; Yamazaki et al., 1997; Marambaud et al., 1998; Zhang et al., 1999). Whether UPS works in collaboration with lysosomes to excute the process of cognitive decline in AD is still not clear and remains a subject of further investigation.

2.5. Animal and tissue models for Alzheimer's disease

The fact that AD naturally occurs in humans with no known case in other animals, makes it impossible to examine the progressive changes in neuropathological conditions of the disease in humans. Hence, animal models that mimic the disease conditions have gained popularity in recent years. One major advantage of the use of animal tissue models for AD is the possibility to investigate molecular changes occurring at an early stage prior to the overt signs of the disease. Each animal or tissue model is designed to mimic different aspects of pathological events observed during neurodegeneration. Most of these models have focused on the production of two major hallmarks of AD, hyperphosphorylated tau and beta amyloid protein. However, some investigators have utilized the same hallmark proteins such as amyloid beta, which are introduced into an animal or slice culture. Such studies aim to study the advanced stage of the disease with the cardinal point of studying the effects of these proteins. Two major groups of animal models include the lesioned and

the transgenic animals. As will be discussed here each has its own strength and shortcomings. Transgenic rats carrying Alzheimer's disease-linked mutations in amyloid precursor protein (APP) and presenilin 1 (PS1) have been used to investigate hippocampal proteomic alterations in a pre-plaque stage. Lesioned animals utilize neurotoxins or physical damage of a particular part of the brain usually the ones associated with learning and memory. The slice model is perhaps one of the *in vitro* models that may withstand a lot of manipulation and will also be considered in this section. Long before the onset of plaque formation and/or cognitive impairment, the expression level of many hippocampal proteins that play a role in learning and memory formation may be altered and some of these models may be used to investigate these early changes.

2.5.1. Organotypic slices cultures

The introduction of long term organotypic slice cultures has provided a break through for *in vitro* studies in which slices can withstand external physical and chemical manipulation to produce a desired effect. This advantage coupled with the fact that the cultures can be maintained for many weeks to months make this technique a valuable research tool which has found a wide spectrum of application in physiology, pharmacology, morphology and development of neuronal circuits. Given the basic requirements such as nutrients in the culture media, sufficient oxygen and temperature, organotypic slice cultures can differentiate and develop to a tissue organization that closely resembles that

observed *in situ*. Slice cultures are usually prepared from early postnatal animals (P0 to P7) because at this stage the essentials of the cytoarchitecture are already established in most brain areas, the post-natal brain is larger and easier to dissect, and the early post-natal nerve cells survive explantation more readily. In essence, slice cultures have become an attractive alternative and complement to acute slices, and the possibility of having different culturing methods for different applications has considerably expanded their use.

Since the development of the organotypic slice culturing technique by Gahwiler (1981), the method has undergone rapid modification with an aim of maintaining viable and stable cells over a period of time. Thus, although the initial steps in the preparation of slice cultures from the brain may be similar, several modifications of culturing technique have been invented. These include: the *roller-tube method* (Banker and Goslin, 1988) where the tissue is embedded in either a plasma clot or in a collagen matrix on glass coverslips and then subjected to continuous slow rotation, *culture dishes method* (Bahr, Vanselow and Thanos, 1988) where the tissue is placed either directly on plastic dishes coated with collagen or in Petriperm dishes that contain a gas-permeable membrane and then covered with the medium and lastly the *membrane culture method* (Stoppini, Buchs and Muller, 1991) where the slices are placed on semiporous membranes at the air–medium interface and remain stationary during the entire culturing process. Interestingly, these three major culturing techniques are useful in different

paradigms and are still used currently. However, the method of choice will depend on the intended manipulation and expected results.

Of particular interest to this project is hippocampal slices cultured on millicell inserts which retain the cytoarchitecture of the tissue. In general, the initial steps in the preparation of slice cultures involves the removal of the brain tissue from the skull followed by tissue sectioning into slices of about 400 μm in thickness by means of a tissue chopper or a vibratome. All preparations are done in ice cold buffer of a well balanced salt. The next step involves attaching the slices to a substrate which is a semipermiable membrane (milicell inserts) where the slices remain for a period of time while being fed with nutrients provided in the culture medium. The pyramidal cells in this tissue slice not only display normal synaptic transmission (Debanne, *et al.* 1995), but also exhibit several forms of short- and long-term synaptic plasticity (Bonnhoefer et al., 1989; Debanne, et al., 1994; Muller, *et al.* 1996). Perhaps one of the most important advantages of slice cultures over acute slices is the expression of monosynaptically connected neurons. Indeed 50% of CA3 pyramidal cells and 75% of CA3–CA1 pyramidal cells are monosynaptically connected in slice cultures, at least ten times the percentage that has been reported for acute slices.

2.5.2. Cholinergic animal model

The cholinergic system is one of the most important and complicated modulatory neurotransmitter systems in the brain, forming network connections with the

major regions of the brain. The cholinergic system is therefore distributed in a variety of nuclei. There are however two major groups of cholinergic neurons: Those found in the basal forebrain within the medial septal nucleus, limb nuclei (composed of the vertical diagonal band and horizontal diagonal band) and those found in the nucleus basalis magnocellularis, also called the nucleus basalis of Meynert in human beings, a site associated with profound neurodegeneration in Alzheimer's disease. Of particular interest are the medial septal connections to the hippocampus otherwise termed as septal-hippocampal innervation. The subcortical cholinergic neurons that provide hippocampal innervation have been shown to undergo degeneration as a result of aging in a variety of species and in human patients with AD (Altavista, et al., 1990; Coyle et al., 1983; Fisher, et al., 1991; Fisher, et al., 1989; Geula, et al., 1994).

In order to mimic the events that occur during neurodegenration of AD cholinergic lesioned animal models have been developed using several excitotoxins with varying selectivity. Lesioning of basal forebrain cholinergic neurons in rats with toxins results in impairments of spatial memory tests and attentional functions (Waite et al., 1995; Baxter et al., 1995; Mcgaughyet al., 2002). Although the excitotoxins used previously such as ibotenic acid, AMPA and NMDA agonists, may be nonselective to specific fiber passages which makes their interpretation difficult, they have been undoubtedly an improvement over physical lesioning methods.

Since the cholinergic neurons may comprise only a fraction (20–30%) of the total number of neurons in any particular region (Gritti et al., 1993, 1997), non-specific lesioning agents may lesion other non-cholinergic neurons. Most affected could be the GABAergic neurons that may be twice the density of cholinergic neurons in the basal forebrain nuclei (Gritti et al., 1993, 1997). Due to the non-selectivity of the physical and excitotoxin lesions, there has been an effort to develop a more selective method of lesioning. As a result a more selective and perhaps most effective approach to eliminating basal forebrain cholinergic neurons has been introduced (Wiley et al., 1991) and used in several studies by other workers (Book et al., 1994; Lee et al., 1994; Waite et al., 1995; Leanza et al., 1996; Milner et al., 1997). This approach utilizes an immunolesioning technique, whereby an antibody to the low-affinity p75 neurotrophin receptor 192 IgG, is conjugated to the plant-derived protein saporin. The 192 IgG-saporin complex after being taken into the cell, is thought to be transported retrogradely to the soma where it shuts down the protein synthesis by inactivating the ribosomes leading to neuronal death (Wiley *et al.*, 1991; Wiley and Lappi, 1993; Curtis *et al.*, 1995).

The specificity of immunolesioning with 192 IgG-saporin is due to the fact that p75 receptors are more localized in the cholinergic neurons than in GABAergic at least in the basal forebrain. However, a subpopulation of neurons that express these receptors may exist elsewhere outside the basal forebrain region (Bothwell, 1995; Carter and Lewin, 1997). Nevertheless, with precise

injection of the 192 IgG-saporin, diffusion to other unwanted regions is a rare occurrence. Hence, 192 IgG-saporin is considered to be the most selective and potent lesioning agent and was used in this project to lesion the septal-hippocampal cholinergic pathway to create an AD animal model.

2.5.3. Transgenic animal model

Several transgenic animal models have been engineered to express certain genes that have been implicated in AD. Indeed, animal models that express genes of interest such as APP, presenilins and tau have provided a valuable tool to study the disease progression (Janus and Westaways 2001; Janus et al., 2001; Van Leuven 2000; Duff and Rao, 2001). However, transgenic animals are more often than not incomplete in developing the full pathological features of AD. For example, transgenic mice for APP can only develop neuritic plaques and can exhibit learning and memory deficit but do not show evidence of neurofibrillary tangles. This is also the case for transgenic mice for tau that exhibit tangle formation but not neuritic plaques. These findings are intriguing and further complicate the question whether tau tangle or neuritic plaques are responsible for learning and memory impairment.

Despite these deficiencies transgenic mice give information on the genetic contribution of each gene and help to decipher the risk factors in familial AD. More importantly they can be used to study the progressive stages of the disease. Thus transgenic mice become more useful when *in vivo* studies are required.

Current studies on transgenic animals aim to engineer animals that fully exhibit most if not all the pathological features of AD.

6. Summary

The mechanism of cognitive decline in several neurodegenerative diseases including AD is still not clear. Progress has been made in elucidating the process behind neurodegeneration and the observed cognitive impairment. Among the possibilities are the tau and abeta proteins which accumulate in neurons probably leading to their dysfunction. However, it is not well known how abeta and/or tau result in memory loss in AD. Other possibilities include the presenellin family of proteins that may be important for familial AD. Also, there appears to be some similarity between cognitive diseases and age-related changes in protein metabolism which may be studied to determine various factors that promote neurodegeneration.

Several neurotransmitter systems have been implicated in neurodegenerative diseases and the resulting cognitive impairment. Among them are the cholinergic, glutamate and GABAergic systems. However, the contribution of each to memory and neuronal loss is still under investigation. An important neurotransmitter system to study is the glutamatagic system since it has been directly implicated in learning and memory. Indeed, the observed memory and cognitive decline may be directly related to tau and/or abeta

proteins or indirectly via receptors involved in memory acquisition. The kinetic properties of these receptors are probably altered concomitantly with or prior to receptor downregulation

To better understand the process behind the memory impairment, it is important to unravel the early events of cognitive impairment. There are suggestions that some of the early events include lysosomal dysfunction. Indeed, lysosomal perturbation has been observed in normal brain without signs of memory loss. In this study we induced lysosomal dysfunction in slices to study the early events prior to overt signs of neurodegeneration. As mentioned above, lysosomal dysfunction may be accompanied by a functional impairment of glutamate receptors and thus functional properties of these receptors were investigated under the current study.

Several animal and slice models have been used to study the neuropathology, physiology and pharmacology in neurodegenerative disorders. The model of choice depends on the problem to be addressed since each model has its own advantages and disadvantages. However, combining several models may be a powerful tool whereby the models complement each other. In this project we combine the slice culture model with the cholinergic lesioned animal model to study the glutamatergic function in early events of AD.

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SYNAPTIC GLUTAMATE RECEPTOR DYSFUNCTION IN AN ORGANOTYPIC SLICE CULTURE MODEL OF ALZHEIMER'S DISEASE.

Abstract

The expression and functional properties of ionotropic glutamate receptors play an important role in cellular mechanisms of cognition. The glutamate receptors may therefore be associated with impaired cognition observed in neurodegenerative disorders such as Alzheimer's disease (AD). Such disorders and especially AD have also been associated with accelerated production of lysosomal enzymes implying a dysfunction of these organelles. It is thought that lysosomal dysfunction may be among the earliest markers of cognitive decline that occurs prior to overt signs. Hence, it is important to establish the link between lysosomal dysfunction and glutamate receptor function to elucidate the early events of cognitive decline. Here, we have utilized organotypic hippocampal slice cultures exhibiting a progressive lysosomal dysfunction induced by chloroquine to study the functional properties of synaptic AMPA and NMDA receptors. We performed electrophysiological recordings of AMPA and NMDA mediated sEPSCs and mEPSCs in hippocampal slice cultures treated with chloroquine for 3, 6, and 9 days. Our results show a progressive decline in

AMPA and NMDA receptor mediated sEPSCs and mEPSCs properties corresponding to the degree of lysosomal dysfunction. Studies on single channel properties of AMPA and NMDA receptors in isolated synaptosomes reconstituted into lipid bilayers further supported the progressive decline in receptor function observed in whole cell recordings in slices. The single channel data showed a decrease in the probability of channel opening and dwell open times in slices exhibiting lysosomal dysfunction. Western blot analysis revealed a decrease in postsynaptic marker NR1 but no change in GluR1. Put together our data indicate that lysosomal dysfunction is followed by a decrease in the functional properties of synaptic AMPA and NMDA receptors.

Introduction

The efficiency of excitatory synaptic transmission in the central nervous system depends heavily on uncompromised glutamate receptors mediated communication between neurons (Cotman et al., 1986; Monaghan et al., 1989; Boulter et al., 1990; Song-Hai et al., 1999). Both the regulated release of the glutamate transmitter from the pre-synaptic cleft and the concomitant activation of glutamate receptors in the postsynaptic site are important parameters of synaptic transmission in the glutamatergic system (Nakanishi, 1992; Hollmann and Heinemann, 1994; Conti and Weinberg 1999). The glutamatergic transmission is comprised of two types of receptors; the metabotropic and ionotropic glutamate receptors. Of particular interest to the current study are the

ionotropic receptors which are further subdivided into alpha-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and Kainate depending on their preferred agonist (Muller and Lynch 1988; Isaac et al 1995; Durand et al., 1996; Ben-Ari et al., 1997). AMPA and NMDA receptors have been shown to play a significant role in the induction and expression of long term potentiation (LTP), respectively. LTP is a phenomenon thought to underlie some forms of learning and memory (Bliss and Collingridge 1993), and is impaired in many cognitive disorders. The functional diversity of both NMDA and AMPA receptors arising from altered channel properties is thought to be responsible for memory impairment in many cognitive disorders. Indeed, a wealth of studies implicate altered AMPA and NMDA receptor function in the neurodegeneration observed in Alzheimer's disease (AD) and Parkinson disease (Carlson et al., 1993; Weihmuller et al., 1992; Dewar et al., 1991)

A well established characteristic of AD is the accumulation of amyloid beta protein and tau isoforms both of which form the major hallmarks of this disorder. The role played by amyloid beta in promoting cytotoxicity in AD is found to be partly via NMDA receptors (Cowburn et al., 1994, 1997; Schulz et al., 1996). In addition, the down regulation of the AMPA receptor population in an AD vulnerable region has been suggested (Dewar et al., 1991, Yasuda et al., 1995; Ikonomovic et al., 1997). Although much is known about the later stages of this cognitive disorder, the events that take place prior to overt signs are still not clear. In addition, it is not well know what causes the accumulation of aberrant

proteins such as amyloid beta and tau proteins. An important observation which may provide an insight to the cause of aberrant protein formation is lysosomal perturbation in disorders of cognition (Adamec et al., 2000; Cataldo et al., 1995, 1996; Li et al., 1999). Indeed, several studies link lysosomal dysfunction with both amyloid beta and tau formation and to some extent synaptic dysfunction (Bahr et al., 1995; Cole et al., 1989; Nixon et al., 2001 Takauchi et al., 1995; Bi et al., 2001; Bendiske et al., 2002). Furthermore, lysosomal dysfunction has been suggested as an early stage of neurodegeneration (Cataldo et al., 1990a; 1990b; 1996a; 1996b).

Lysosomal dysfunction may not only be responsible for the accumulation of amyloid beta proteins and tau proteins but may also play a significant role in altering synaptic glutamate receptor function. Determining whether lysosomal dysfunction promotes the alteration of AMPA and NMDA receptor kinetics requires an animal or slice model that mimics the events that take place during neurodegeneration. Previous studies have utilized a hippocampal slice model exhibiting lysosomal dysfunction induced by a lysosomotropic agent such as chloroquine to study various parameters of neurodegeneration (Bahr et al., 1994, 1995). The current study therefore utilized the established hippocampal slice model to study whether AMPA and NMDA receptor channel properties are altered during the early stages of neurodegeneration. Hippocampal slice cultures treated with chloroquine were utilized to study the properties of these two receptors in both whole cell recordings in slices and single channel recording in isolated synaptosomes. The use of these two techniques is a valuable tool that

fully characterizes receptor activity at the cellular and molecular level. The current study also utilized an immunological technique to analyze the level of lysosomal dysfunction and how it correlates with electrophysiological recording of cultured hippocampal slices.

Materials and Methods

Animals and Chemicals

Sprague-Dawley rats (mother with pups) were obtained from Charles River Breeding Laboratories. All salts and reagents were purchased from Sigma Chemical Co unless specified. The following drugs and chemicals reagents were purchased from Gibco Co.: Horse serum, Hanks balance salts, Earl's balance salt, MEM, penicillin/streptomycin, fungizone and glutamine. Culture plates, membrane inserts, Micro-filters and sterile pipettes were purchased from Fisher Scientific.

Antibodies

Monoclonal antibodies against synaptophysin and beta amyloid were obtained from Sigma Chemical Company (St. Louis, MO) while those of cathepsin D, GluR1 and NR1 were obtained from Upstate USA Inc (Charlottesville, Virginia). Monoclonal antibodies against GluR2 and Tau-1 were obtained from Chemicon (Temecula, CA). The secondary antibodies were obtained from Amershem Bioscience (Piscataway, NJ).

Organotypic Slice Cultures

This technique relies on the principal that hippocampal slices prepared from young rodents can be maintained in culture for many weeks. Due to their high neuronal connectivity, slice cultures provide a very useful tool for studying the properties of synaptic transmission between monosynaptically coupled cell pairs. Organotypic hippocampal slice cultures were prepared according to the procedure of Stoppini with slight modifications (Stoppini et al 1991). Briefly, the whole brain was isolated from 5-7 days postnatal Sprague Dawley rats and placed in ice cold Hank's balanced salt solution. The brain tissue was then submerged in the buffer and sliced into sections of 400 μm in thickness by means of a vibrotome. Individual hippocampal slices were separated from the cortex and midbrain region for culturing. Hippocampal slices were placed at the air–medium interface of humidified semi-porous membrane inserts that rested on a 6 well culture plate containing 1 ml culture media in each well. The culture media was composed of 50% Earle's balanced salt, 50% MEM and 25% heat-inactivated horse serum. The media was supplemented with 1 mM glutamine and 36 mM glucose. The antibiotics penicilline/streptomycin and fungizone were also added in a proportion of 1:100 of the media (1%). The culture media was finally sterile filtered with 0.22 μm pore filters before use. Culturing was done in an incubator at 36 ^{o} C, 100% humidity and 5% carbon dioxide. Under these conditions, nerve cells continue to differentiate and to develop a tissue

organization that closely resembles that observed in situ. The media was changed every other day. Treatment with chloroquine (60 μM) began after allowing the slices to recover for 10 days in culture. Chloroquine treatment corresponded to 3, 6 and 9 days.

Synaptosomes Preparation

The slices were harvested after 3, 6 and 9 days of chloroquine treatment. Tissues were isolated from the insert membranes by scraping with a spatula and then homogenizing in a modified Krebs-Henseleit buffer (mKRBS). Slices from the same treatment groups were pooled together and homogenized in 100 μl of ice cold mKRBS buffer in an Eppendorf tube. The mKrebs buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO4, 2.5 mM CaCl2, 1.18 mM KH2PO4, 24.9 mM NaHCO3, 10 mM dextrose, 10 mg/ml adenosine deaminase and pH was adjusted to 7.4 by bubbling with 95:5 O_2:CO_2. To minimize proteolysis the following were included in the buffer: 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM benzamide. After homogenizing with five turns of a Teflon hand-held pestle the homogenate was diluted with 350 μl of additional ice-cold mKRBS buffer. The mixture was then filtered through a 13 mm diameter Millipore syringe filter holder using a 1 cc Tuberculin syringe. The diluted filtrate was then forced over three layers of nylon (Tetko, 100 μm pore size) pre-wetted with 150 μl of mKRBS, and collected in a 1.5 ml Eppendorf tube. The pre-filtered mixture was loaded into another 1 cc tuberculin

syringe and forced through a pre-wetted 5 mm Millipore nitrocellulose filter. The filtrate was then spun at 1000 x *g* for 15 min in a microfuge at 4°C. The supernatant was removed, and the pellet (synaptosomes) was resuspended in 20 µl of mKRBS buffer for electrophysiology and Western blot analysis.

Single Channel Recordings in Synaptosomes.

Isolated synaptosomes were reconstituted into a lipid bilayer for electrophysiological recording. Briefly, the patch pipette pulled from borosilicate glass capillaries was filled with intracellular solution containing: 110 mM KCl, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 2 mM MOPS. To determine the pipette resistance the glass electrode was dipped into a micro beaker containing 300 µl of extracellular solution which was composed of: 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4 and 5 mM Tris HCl. 10 µl of phosphatidylcholine (PC) dissolved in hexane was added to the extracellular solution in the beaker. Phospholipid bilayer membrane was formed by tip dip method to separate the two aqueous solutions; the internal solution inside the borosilicate glass electrode and the external solution contained in a micro-beaker. The resistance of the membrane was monitored using Axopatch 200B (Axon Instruments, Union City, CA) and pclamp 6 software. Once a stable membrane was formed 10 µl of synaptosomes was introduced. The small micro-beaker allowed rapid introduction of the proteins into the immediate proximity of the membrane in a volume of only 300 µl.

AMPA currents were activated by addition of 290 nM AMPA (Tocris, Ellisville, MO) to the *cis* side of the bilayer in the presence of 10 μM APV. Using axopatch 200 B amplifier recordings were made with a varying holding potential at different voltages which were applied through an Ag-AgCl reference electrode placed directly into the extracellular solution. Membrane capacitance and resistance were monitored continuously to ensure the formation and stability of reproducible membranes. To verify that the recordings were from AMPA channels the events were blocked with 1 μM CNQX. Single channel events were recorded in a video tape for further computer analysis using pclamp 9 software. Recorded signals were filtered at 5 kHz and digitized between 5–25 kHz.

To isolate NMDA currents, recordings were performed in the presence of 3 μM NMDA, 1 μM CNQX (AMPA antagonist), 1 μM SYM2081 (Kainate antagonist), 100 μM picrotoxin (GABA antagonist), 2 μM TEA (potassium antagonist), and 1 μM TTX (sodium antagonist). NMDA currents were confirmed by the addition of 10μM of APV at the end of each experiment.

Immunobloting

Hippocampal culture slices from controls and each treatment group were collected and homogenized in 50 mM Tris, pH 7.5; 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 60 mM octyl-glucoside, and protease inhibitors (Roche). Protein content was determined using the *DC* Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of each homogenate (30 μg) were diluted

with equal amounts of sample buffer containing 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue. Samples were boiled for 5 min, and then subjected to SDS-PAGE according to the method of Laemmli (Laemmli, 1970) using 4-12% polyacrylamide gradient gels (Bio-Rad). Proteins were electrophoretically transferred onto nitrocellulose membranes as described by Towbin et al. (Towbin et al. 1979). After transfer, nitrocellulose membranes were blocked in 5% non-fat dry milk (NFDM) in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween-20 for 1 hour at room temperature. Primary antibody incubations were carried out in 1% NFDM in TBS plus 0.1% Tween-20 overnight at 4° C. The antibodies used were GluR1 (1:1000), capthesin D (1:200), tau-1 (1:500), and actin (1:5000). After overnight incubation, membranes were washed with 1% NFDM in TBS plus 0.1% Tween-20. Membranes were then incubated with either anti-mouse or anti-rabbit IgG (1:2000 – 1:10,000) for 1 hour at room temperature. After washing 3X in 1% NFDM in TBS plus 0.1% Tween 20 (10 min. each), the blots were developed via enhanced chemiluminescene using ECL Plus (Amersham Biosciences).

Whole Cell Electrophysiological Recordings in Slices

Slice cultures were harvested for electrophysiological recordings after 3, 6, and 9 days of chloroquine treatment. The hippocampal slices were isolated from the insert by cutting the membrane around the tissue. Slices were then transferred to a submerged-type recording chamber and held between two nylon nets.

Throughout the recording the slices were perfused with oxygenated (95 % O_2 / 5% CO_2) artificial cerebral spinal fluid containing NaCl, 130 mM; KCl, 3 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 2.5 mM; $NaHCO_3$, 26 mM; KH_2PO_4, 1.25 mM; glucose, 10 mM (pH 7.4. and osmolarity of 310 mOsmol). Cells were identified from the slices using an Olympus BX-51WI upright microscope equipped with an infrared camera. Whole-cell recordings were made from hippocampal CA1 neurons using patch electrodes (tip resistance 5-10 Mega ohms) pulled from thick-walled borosilicate glass. The patch electrodes were filled with intracellular solution containing 122.5 mM Cs-Gluconate 10 mM HEPES, 2 mM $MgCl_2$, 20 mM KCl, 1 mM EGTA, 2 mM Na_2-ATP, 2 mM QX-314, 0.25 mM Na_3-GTPx$3H_2O$ and pH 7.4. The AMPA mEPSC were recorded in continuous voltage-clamp at a holding potential of -65 mV using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA), in the presence of GABA receptor antagonist picrotoxin 50 µM, TTX 1 µM and of an NMDA antagonist 2-amino-5-phosphonovalerate (APV) 50 µM in order to isolate pure AMPA responses. AMPA sEPSC were recorded using the same conditions but in the absence of TTX. Access resistance and cell input resistance were monitored throughout the experiment; no series compensation was used in order to maximize the signal-to-noise ratio. Access resistance below 15 MOhms was accepted for analysis, otherwise the experiments were rejected. Offline analysis of mEPSCs and sEPSCs were detected and analyzed using a Windows-based analysis program (Mini Analysis, Synaptosoft, Inc, Decatur, GA).

The recordings of NMDA mEPSC and sEPSC were similar to those of AMPA except that APV was replaced with 4µM CNQX to block AMPA currents. The extracellular solution for these recordings contained (in mM) 160 NaCl, 2.5 KCl, 0.2 $CaCl_2$, 10 HEPES, 10 Glucose and 0.2 EDTA.

Results

Reduction in pre- and post-synaptic markers

In order to verify that lysosomal dysfunction occurred in hippocampal slices used for electrophysiology, Western blot studies were performed on treated and control synaptosomes isolated from the slices. The study was designed to measure the lysosomal enzyme cathepsin D that usually appears to be upregulated following lysosomal perturbation. Our experimental data show that cathepsin D was increased in a manner in the third day of chloroquine treatment but decreased in the 6th and 9th day of chloroquine treatment (fig. 3.1B). The Western Blot experiments were also performed to determine the level of synaptic proteins synaptophysin, NR1 and GluR1. The data indicate a quantitative reduction in the level of NR1 subunit but no change in the level of GluR1 subunits in chloroquine treated slices.

Effect of Chloroquine on AMPA-mediated Miniature EPSCs and Spontaneous EPSC

To determine the effects of chloroquine on the AMPA component of miniature EPSC and spontaneous EPSC, electrophysiological recordings in hippocampal slices were performed on 3, 6 and 9 days chloroquine treated slices. We first recorded the spontaneous currents in the absence of TTX. The unitary properties of excitatory synaptic transmission mEPSCs were recorded in the presence of 4 μM TTX to block action potential-mediated synaptic activity and 100 μM picrotoxin to block inhibitory GABA currents. After each recording the traces were blocked with 4 μM CNQX. Representatives of gap free recordings of membrane ionic currents at holding potential of -65 mV are illustrated in Figure 3.2A & B. Chloroquine treatment resulted in a reduction in both miniature and spontaneous current frequency in a time-response manner corresponding to the days of treatment. The mEPSC in slices after 3 days treatment resulted in only a slight but insignificant decrease in frequency when compared with control (3.5 ± 0.5 Hz treated vs. 4.4 ± 0.8 Hz control). The 6 and 9 days treated slices recorded a significant change in frequency ranging from values of 0.4 Hz to 0.8 Hz for 6 days and 0.13 Hz to 0.33 Hz for the 9 days. Additionally, as indicated in Figure 3.2Db the AMPA mEPSC from 6 and 9 days exhibited prolonged inter-event intervals compared to control. In addition to reduced AMPA mEPSC frequency in 3, 6 and 9 days chloroquine treated slices, the average mEPSC amplitude was also significantly reduced ($p < 0.05$, n=10) with a greater change observed in 6

and 9 days treated culture slices. The average mEPSC waveforms are shown in figure 3.2C. The average amplitude for the respective days of treatment of 3, 6 and 9 days was 19.6 ± 1.5, 12.4 ± 2.3 and 8.2 ± 1.4, respectively. The comparative analysis of cumulative fractions of mEPSC amplitude and inter-event for each number of days of chloroquine treatment are illustrated in figure 3.2D. There was no significant difference in either amplitude or frequencies between the controls (Fig 3.2E & F)

Similar to the alterations seen with AMPA miniature currents after chloroquine exposure, the spontaneous currents were also significantly altered in a time-response manner corresponding to the days of chloroquine treatment. The average amplitude of the sEPSC in control, 3, 6 and 9 days chloroquine treated slices was 47.2 ± 6.5 pA, 35.3 ± 4.3 pA, 21.1 ± 2.7 pA and 14.4 ± 3.1 pA, respectively. On the other hand the average sEPSC frequency (table 3.1) for control, 3, 6 and 9 days treated slices were 7.4 ± 1.2 Hz, 5.2 ± 0.7 Hz, 0.8 ± 0.3 Hz and 0.4 ± 0.2 Hz, respectively.

We investigated the consequence of chloroquine treatment on the decay and the rise times of AMPA mEPSCs. Our results indicate that none of the treatments groups showed an appreciable difference in decay kinetics. Furthermore, there was no significant difference in rise time for the three treatments. In addition, neither the slight changes in decay nor the insignificant changes in rise time correlated with the progress of chloroquine treatment.

Alteration of Single Channel Properties of AMPA Receptors in Synaptosomes

To determine whether the observed changes in amplitude observed in AMPA mEPSC were due to changes in AMPA receptor properties we investigated the single channel kinetics in chloroquine treated slices. The synaptosomes isolated from chloroquine treated and untreated slices were incorporated into lipid bilayer and single channel recordings were performed using the axopatch 200B amplifier. Reconstituted synaptosomes in a lipid bilayer expressed single channel current fluctuations upon application of 290 nM AMPA (Fig. 3.3). This activity was ultimately blocked by addition of an antagonist for the AMPA glutamate receptors, CNQX (1 μM). Since synaptosomes are composed of other synaptic receptor channels, isolation of AMPA channels were performed by introducing 10 μM APV (and other blockers as explained in the methods) into the extracellular solution to block NMDA channels. Closed and open levels were analyzed and amplitude histograms were constructed by Gaussian fitting. The resulting data, analyzed with pclamp 9, indicate a reduction in the probability of channel opening (Po) for the treated slices (Fig. 3.3). In addition, the amplitude histograms show that the number of events for open state in chloroquine treated samples decreased dramatically compared to controls. There was a remarkable and progressive reduction in the probability of channel opening corresponding to the days of treatment 3, 6 and 9 days (Fig. 3.3). Although there was no significant reduction in the probability of channel opening between synaptosomes from the control and three days treated slices the later did not

exhibit the occasional bursting activity occasionally observed in control (not shown). The data also indicate that there was no change in the conductance of AMPA channels for the three treated groups compared with the control.

Single channel currents were determined from the experimental open time distributions of all the digitized points in current traces at 96 mV. The number of observations of AMPA currents was plotted as a function of open dwell times at 96 mV. Inspection of exponential fittings (2/3 exponential fittings) of open and closed dwell levels constructed from each treatment group suggests that chloroquine and consequently lysosomal dysfunction results in reduction of open time and a concomitant increase in closed time for AMPA channels. The unitary AMPA currents from control synaptosomes remained in the open state longer than the treated groups with transient or brief closures. However, in chloroquine treated slices, the channels opened for shorter periods and were characterized by prolonged closures. Collectively, these observations suggest that lysosomal dysfunction decreases the AMPA channel activity by decreasing the mean open time.

Effect of Chloroquine on NMDA-mediated mEPSCs and sEPSCs

We conducted electrophysiological recordings in chloroquine treated and untreated slices to determine the NMDA component of mEPSC and sEPSC. To isolate the NMDA component of GluR- mediated mEPSC, 10 μM of CNQX was added to the extracellular solution to block the AMPA mEPSCs while the sEPSC

were performed in the absence of TTX. The representative of sEPSC and mEPSC traces of each treatment group are shown in figure 3.4 A and B, respectively. Our data indicate that like AMPA, the NMDA component was significantly altered in a trend corresponding to the time period of chloroquine treatment. However, in comparison to the AMPA changes, the range difference for the NMDA component between each treatment group amplitude and frequency was smaller. In addition, there was no statistical significant difference between the average amplitudes of the control and those of 3 days chloroquine treated slices ($P<0.05$, $n=10$). The mEPSCs in control slices recorded an average amplitude of 14.9 ± 2 pA while the 3 days treated slices had average amplitude of 13.6 ± 1pA. Similarly, the 6 and 9 days treated slices recorded amplitudes of 7.7 ± 2 pA and 4.8 ± 1 pA respectively. The range between the control and nine days was approximately 10 pA. These data therefore suggest that although the average amplitude of NMDA in control slices is approximately half of those of AMPA, NMDA receptors are slightly resistant to chloroquine insults and the accompanying lysosomal dysfunction. The average traces of the amplitudes are shown in figure 3.3C while their cumulative amplitudes are shown in figure 3.3F. A summary of the NMDA mEPSC and sEPSC data is shown in table 3.2.

The significant attenuation in the frequency of the NMDA component of both mEPSC and sEPSC in chloroquine treated slices suggests a pre-synaptic alteration in either the release probability or the quantal release of the pre-synaptic glutamate vesicles. On the other hand, the alteration in the amplitude

suggests that the post-synaptic impairment may be attributed to either a change in the number of the receptors or their kinetics. Such kinetics can be resolved by conducting single channel experiments which are discussed below.

Further investigation into the rise and decay kinetics of the NMDA component did not show any significant changes ($P < 0.05$, n=10) and the slight changes that were seen did not correlate with the days of chloroquine treatment. The mean decay times and rise time for both sEPSCs and mEPSCS are shown in table 3.2.

Alteration of Single Channel Properties of NMDA Receptors in Synaptosomes

Reconstitution experiments were conducted in lipid bilayer to determine whether the observed changes in amplitude was in part due to the alteration in the single channel kinetics of NMDA receptors. Synaptosomes isolated from all the three treatment groups (3, 6 and 9 days) and the control groups were incorporated into a pseudo-lipid bilayer and recordings were performed using axon instruments. NMDA receptors currents were isolated by blocking AMPA currents with 4 μM CNQX and application of NMDA agonist in the extracellular solution. A preparation of 10 μM APV was applied to the extracellular solution at the end of each experiment to verify that the recordings were from NMDA and indeed in most cases the NMDA currents were blocked. Results indicate that NMDA currents were remarkably altered in chloroquine treated samples (Fig.

3.5). Single channel analysis indicates that the probability of channel opening was reduced from 3 to 9 days of chloroquine treated synaptosomes compared with controls. Thus the open probabilities for the control, 3, 6 and 9 days treated slices were 0.26, 0.18, 0.05 and 0.03, respectively. The single channel data also indicates that the mean open time decreased with days of chloroquine treatment whereas the mean closed time was increased.

Discussion

The current study utilized a combination of electrophysiological analysis of single channel recordings of isolated synaptosomes and measurement of synaptic currents in hippocampal slices to investigate the effects of chloroquine and the resulting lysosomal dysfunction on glutamatergic synaptic transmission. The channel properties of glutamate receptor subtypes AMPA and NMDA were investigated in control and 3, 6 and 9 days chloroquine treated slices. In addition, quantification of the pre- and post-synaptic proteins was also investigated by western blot analysis. Previous studies indicate that such synaptic markers are modified following lysosomal dysfunction in hippocampal slices (Bahr et al 1994; 1995). In the current study these synaptic proteins levels were determined to validate lysosomal perturbation and to confirm whether the slices used for electrophysiology mimic the major hallmarks observed in AD. Thus, our approach utilized organotypic slice cultures exhibiting lysosomal dysfunction as an AD slice model to investigate the early events prior to overt signs of

neurodegeneration. Indeed, previous studies implicate lysosomal disruption in the development and accumulation of beta amyloid fragments (Bahr et al., 1994; Hajimohammadreza et al 1994; Nixon et al., 2001) and tau deposits (Takauchi et al., 1995; Bi et al., 2001; Bendiske et al., 2002) within neurons. The endosomal-lysosomal pathway has therefore been proposed as a potential candidate site for the amyloid precursor protein cleavage into smaller β-amyloid containing peptides (Kohnken et al., 1995; Ladror et al., 1994; Haass et al., 1992; Golde et al., 1992). To study the progressive events of the early stages of AD, we treated cultured hippocampal slices with chloroquine for 3, 6 and 9 days. After each treatment, slices were harvested and either used for whole cell recordings or processed for synaptosomal preparations for bilayer electrophysiological recordings and western blot analysis.

Our results confirm earlier reports that chloroquine induces lysosomal dysfunction in hippocampal culture slices and that the dysfunction is followed by modifications in synaptic proteins (Bahr et al. 2002; Bendiske et al. 2002). Induction of lysosomal dysfunction with the lysosomotropic agent chloroquine resulted in an accumulation of cathepsin D for day 3 and a decrease of the same for 6 and 9 days. Tau protein was also increased in chloroquine treated slices but no change in actin and post-synaptic markers GluR1. Actin protein is a good marker for intact synapse and used to determine the integrity of the cytoskeleton. Conversely the post-synaptic markers GluR1 receptor subunits are good indicators of intact glutamatergic transmission. Immunoblot findings from our

experiments suggest that the overall synaptic currents mediated through AMPA and NMDA receptors may be unchanged owing to the conserved number of receptors. However, this data is inadequate to make such conclusions since the quantal release of glutamate or even single channel conductance may be altered. Thus, investigation into both the whole cell and single channel recordings is therefore paramount in drawing such conclusions.

Both AMPA- and N-methyl-D-aspartate (NMDA)-subtypes of glutamate receptors mediate the excitatory synaptic transmission in the vertebrate central nervous system. The expression of long-lasting plasticity is mediated through AMPA receptors (Muller and Lynch 1988; Isaac et al 1995; Durand *et al*., 1996; Ben-Ari *et al*., 1997) resulting in synaptic activity that transiently activates NMDA receptors (Bliss and Collingridge 1993). The molecular basis for the induced changes in AMPA and NMDA receptor function during early stages of AD is not known but may include changes in channel conductance and frequency of channel opening among other factors. Our results indicate that induction of lysosomal dysfunction in organotypic hippocampal slice culture exhibits a remarkable attenuation of channel properties of both AMPA and NMDA receptors. Experiments conducted in both whole cell recordings in slices and single channel recordings in isolated synaptosomes provide evidence of alteration in channel properties that correlate with the time period of chloroquine treatment. The data from the mEPSC and single channel recordings of the NMDA component is intriguing since the activity of NMDA receptors, which are

believed to play a major role in excitotoxicity that leads to neuronal death, was significantly reduced. The fact that NMDA receptor activity in chloroquine treated slices was decreased in the current study may be due to two reasons; 1) at the early stages the low amount of amyloid beta present does not potentiate NMDA channels and does not cause excitotoxicity. Support of this notion comes from binding studies indicating a selective and direct interaction of amyloid beta with the glutamate recognition sites of the NMDA receptor (Cowburn et al., 1994, 1997). Indeed, amyloid beta has been suggested to enhance the NMDA currents (Schulz et al., 1996) which perhaps at elevated levels may cause excitotoxicity. 2) Our model of lysosomal dysfunction may alter other cellular functions that are required for receptors activity, most importantly the cytoskeletal molecules that may modulate and enhance receptors functions. However western blot analysis of actin, a cytoskeleton marker, did not show any significant change over the time period of chloroquine treatment. This observation excludes other cytoskeletal proteins that may modulate glutamate receptors.

The alterations in AMPA receptor mediated synaptic transmission in CA1 pyramidal neurons correlate with earlier findings that synaptic protein levels may be compromised in AD (Reddy et al., 2005). The intense depolarization of the dendritic spines require the activation of the AMPA receptors to induce long term potentiation, a phenomenon believed to underlie memory encoding. Thus, decreasing the currents through AMPA receptors would be expected to

compromise cognitive function. Although our focus was on the CA1 region of the hippocampus which is more vulnerable to degeneration in AD, a comparison of the miniature currents and spontaneous recordings indicate that the connection between CA1 and the less vulnerable CA3 region was impaired. The infrequent openings of single AMPA and NMDA receptors currents observed in chloroquine treated samples supports data from whole cell recording in slices that chloroquine treatment results in a reduction in amplitude of mEPSC. Data from the present study indicate that a synergestic impairment of both AMPA and NMDA activity may occur and result in drastic reductions in synaptic currents that may alter synaptic communication between neurons.

The sequence of events that leads to neurodegeneration in a cognitive disorder such as AD has been a focus of many studies but are still not well understood. First, it is not well known whether amyloid is the promoter of neurodegeneration and the observed cognitive decline. Second, it is not clear whether amyloid or the tau proteins are upregulated as a compensatory mechanism in response to other cellular insults. Previous studies indicate that formation of amyloidogenic fragments leads to alteration in synaptic structure and function (Bahr et al. 1998; Mucke et al. 2000; Kim et al. 2001, Walsh et al. 2002). The data from this study uncovered sequential early events of AD which includes disruption of lysosomal function followed by glutamatergic synaptic dysfunction. This information can be useful in development and selection of

drugs that selectively target specific glutamate receptor function and thereby help delay the neurodegenerative process.

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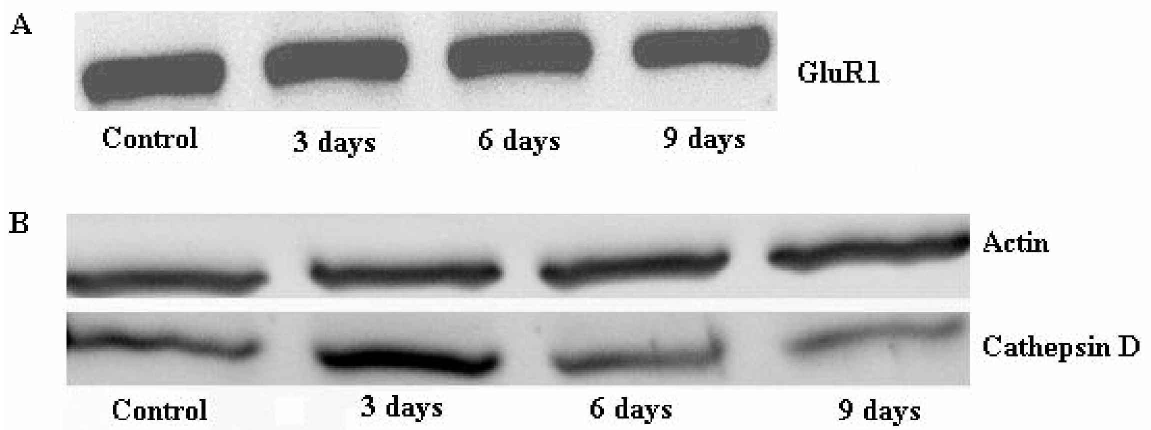
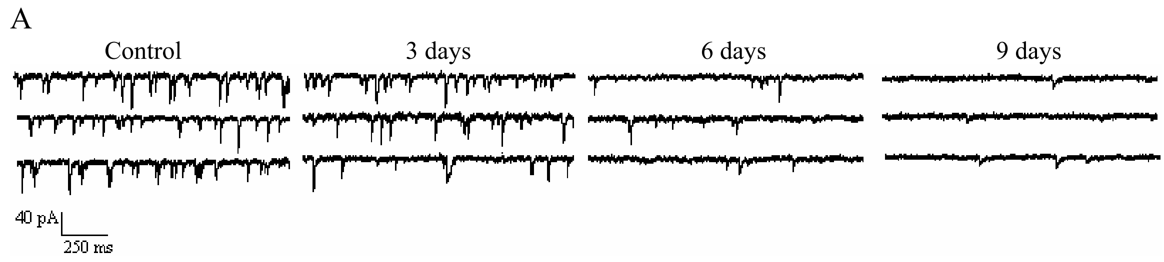
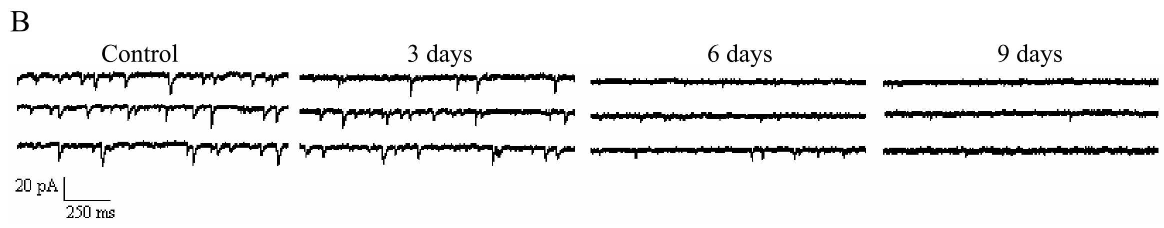


Fig.3.1. Effect of chloroquine on synaptic proteins and lysosomal enzyme cathepsin D. A. Chloroquine treatment did not result in any change in GluR1 subunits. B Chloroquine treatment did not result in any significant change in actin levels. Cathepsin D on the other hand increased in the third day and decreased after 3, 6 and 9 days of chloroquine treatment.


A
Control
3 days
6 days
9 days
40 pA
250 ms


B
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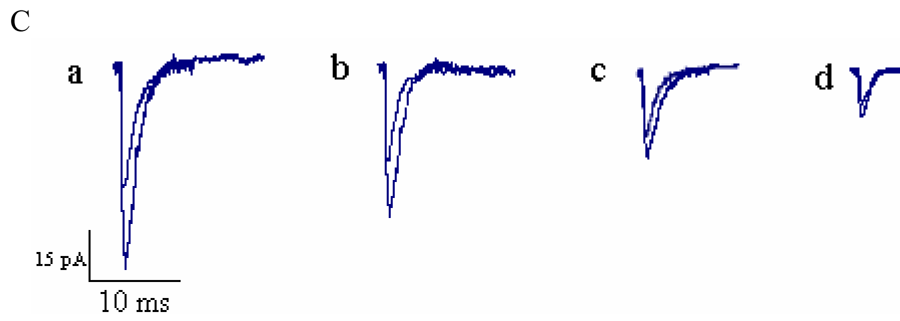
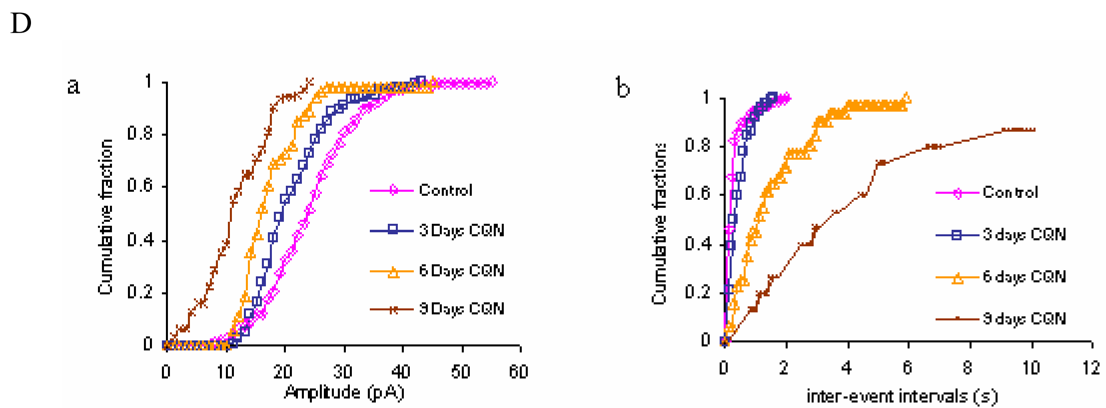
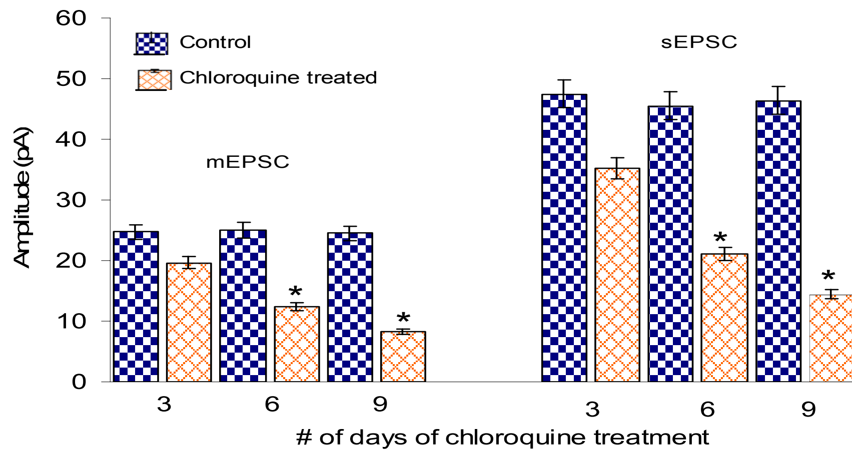
250 ms


C
a
b
c
d
15 pA
10 ms


D
a
Cumulative fraction
Amplitude (pA)
Control
3 Days CQN
6 Days CQN
9 Days CQN
b
inter-event intervals (s)
Control
3 days CQN
6 days CQN
9 days CQN

E



F

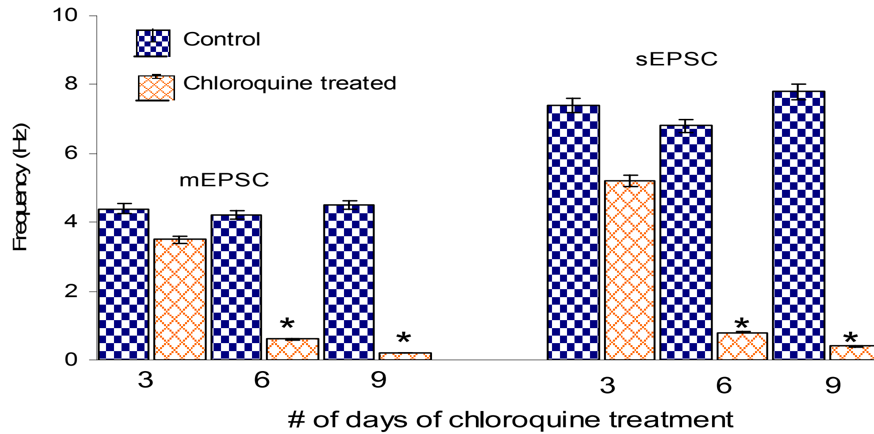


Fig.3.2. Effects of Lysosomal dysfunction on AMPA mediated sEPSC and mEPSCs on hippocampal slice cultures. Lysosomal dysfunction depresses the AMPA component of GluR-mediated miniature and spontaneous EPSCs recordings in hippocampal cultured slices in control, 3 days, 6 days and 9 days of chloroquine treatment. A. Representative recordings of AMPA spontaneous EPSCs in hippocampal slice cultures in the absence of TTX and in the presence of NMDA blockers. Figure B shows representative recordings of AMPA receptor-mediated mEPSCs in slices before chloroquine (CQN) treatment and after 3, 6 and 9 days of CQN treatment. C. chloroquine treatment on hippocampal slices resulted in reduction in peak amplitude. The figure Ca, Cb, Cc and Cd represents average traces of 50 seconds from culture slices treated with chloroquine for 0, 3, 6 and 9 days, respectively. “0”days treatment represents control. The recordings were performed in the presence of TTX, APV and picrotoxin at membrane potential of -65 mV. Cumulative fraction of amplitude (Da) and inter-events intervals (Db) of AMPA mediated mEPSCs in control, 3, 6 and 9 days chloroquine treated hippocampal slice cultures. Chloroquine treatment resulted in a shift of the curve to the right representing a decrease in the amplitude corresponding to the number of days of chloroquine treatment. Graph E and F represent the amplitudes and frequencies respectively of AMPA sEPSC and mEPSC in control and in 3, 6 and 9 dayS chloroquine treated slices

Table 3.1. Effects of lysosomal dysfunction on AMPA mediated mEPSC and sEPSC.

	Frequency (/sec)		Amplitude		Decay τ1, ms		Decay τ2, ms		Rise time, ms	
	mEPSC	sEPSC	mEPSC	sEPSC	mEPSC	sEPSC	mEPSC	sEPSC	mEPSC	sEPSC
Control	4.4± 0.8	7.4±1.2	24.7± 3.2	47.2±6.5	2.5±0.2	2.9±0.4	12.0±1.2	11.8±1.4	1.6±0.3	1.5±0.2
3 days	3.5± 0.5	5.2±0.7	19.6± 1.5	35.3±4.3	2.3±0.1	3.0±0.2	11.8±1.3	12.1±0.9	1.4±0.6	1.4±0.7
6 days	0.6± 0.2*	0.8±0.3*	12.4± 2.3*	21.1±2.7*	2.6±0.1	2.8±0.3	12.3±1.5	13.2±1.1	1.7±0.4	1.7±0.2
9 days	0.2± 0.1*	0.4±0.2*	8.2± 1.4*	14.4±3.1*	2.4±0.2	2.5±0.2	12.1±1.4	11.5±0.6	1.5±0.3	1.6±0.2

A summary of AMPA kinetics observed in the mEPSC and sEPSC in chloroquine treated slices at different time periods (3, 6 and 9 days). There was no significant change in the decay and rise time kinetics between control and treated groups.
* Significant level at $P < 0.05$. All values are expressed as mean ± SE.

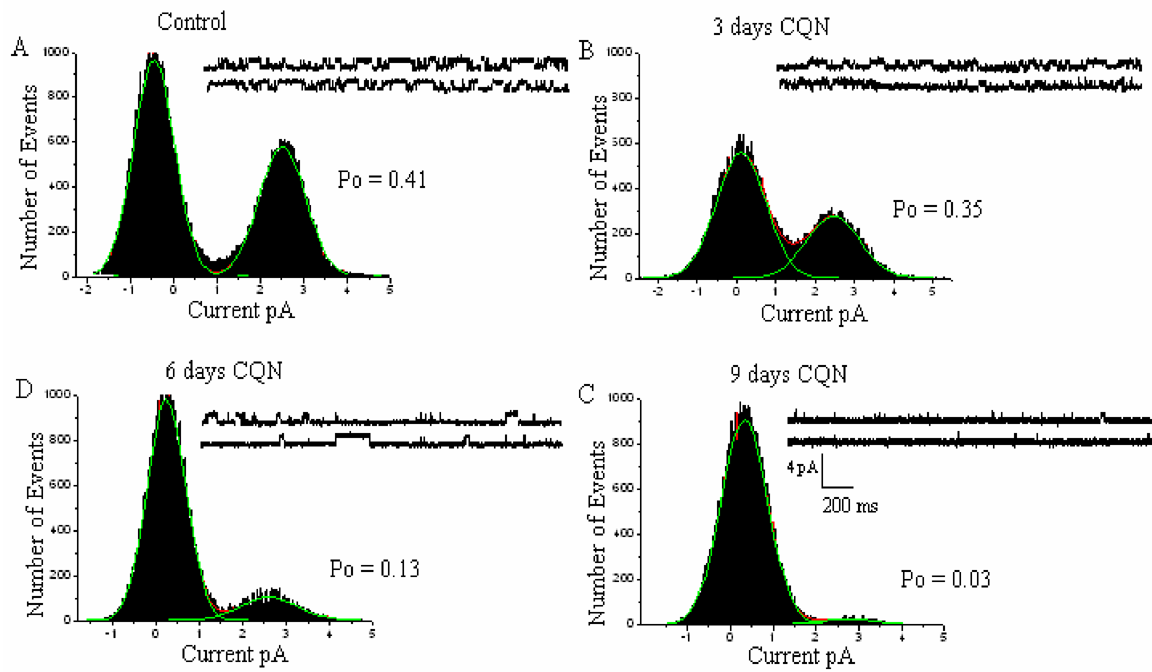
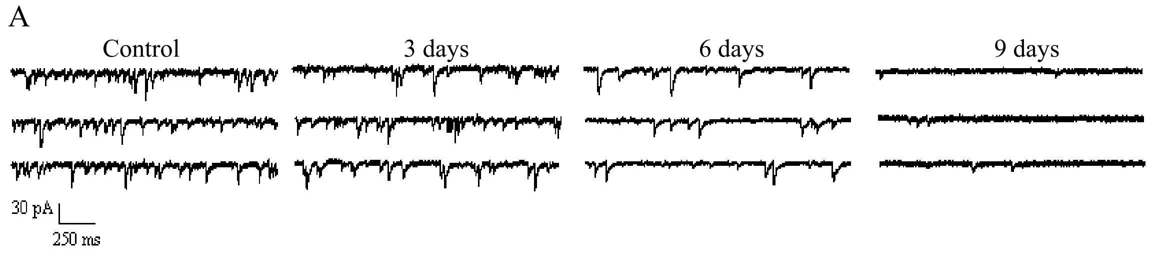


Fig.3.3. Lysosomal dysfunction alters the single channel properties of AMPA receptors. Isolated synaptosomes from control slices and those of slices treated with chloroquine for 3, 6 and 9 days were incorporated into lipid bilayer. The amplitude histogram shows a reduction in the open probabilities (Po) of AMPA receptors in control, 3, 6 and 9 days chloroquine treated hippocampal slices. The traces represent 2 second long recordings of AMPA elicited currents at a holding potential of 96 mV in the presence of 290 nM AMPA . Blockers such as APV and picrotoxin were also included

A



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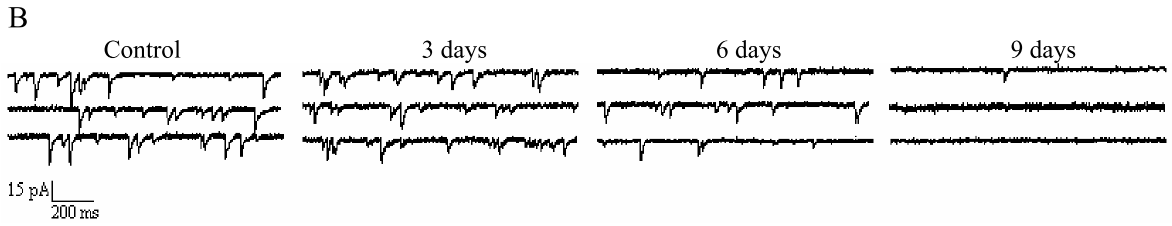
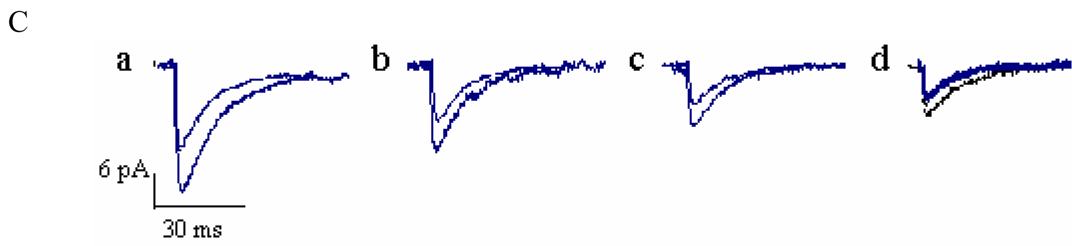
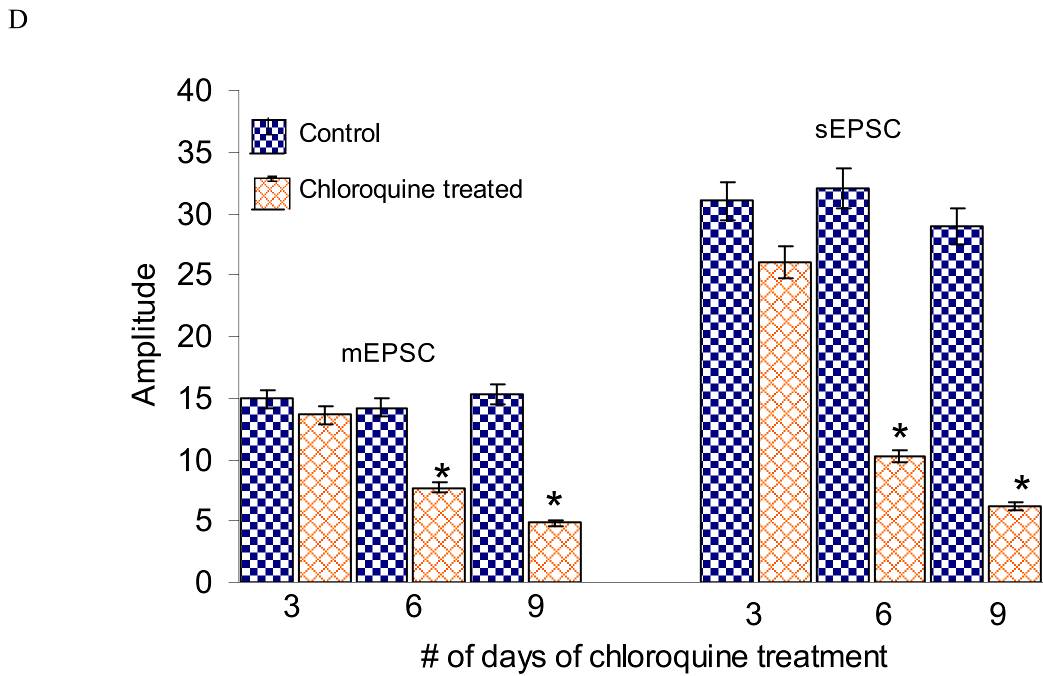


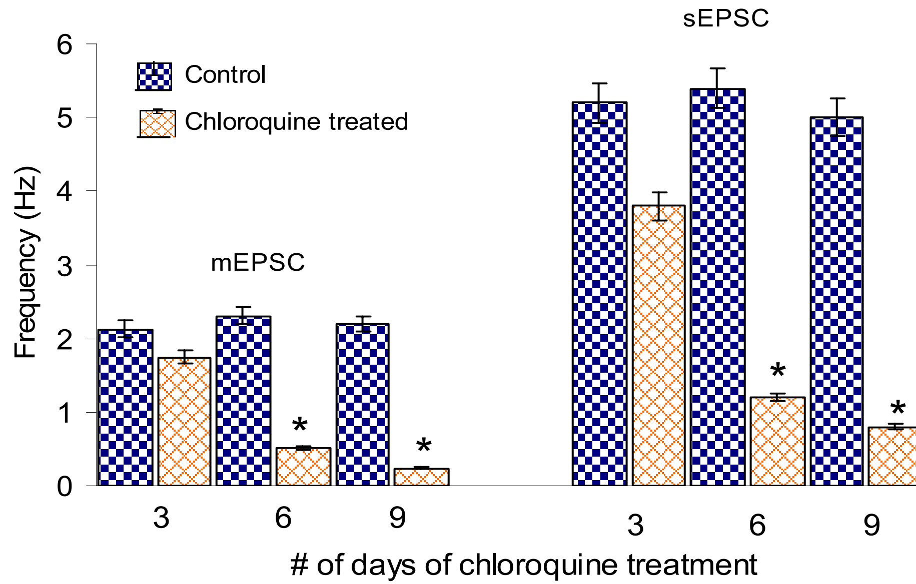
C



D



E



F

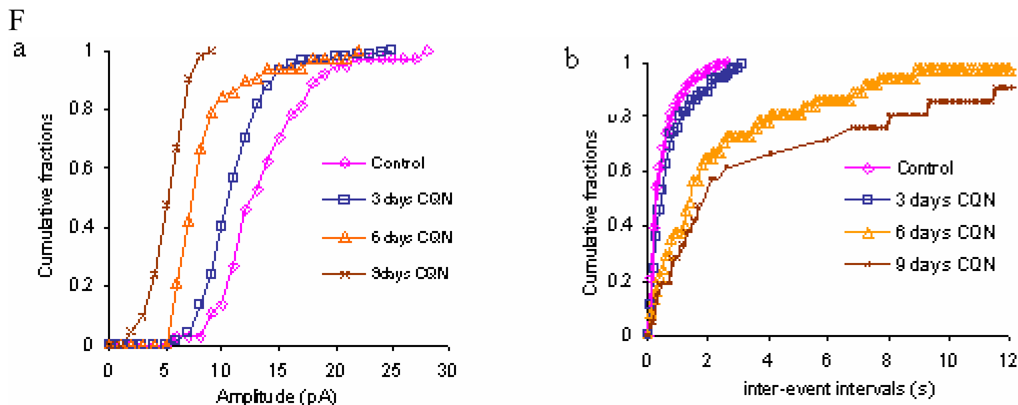


Fig. 3.4 Effect of lysosomal dysfunction on NMDA receptor-mediated currents. Chloroquine treatment on hippocampal slices results in a decrease in the NMDA component of sEPSCs (A) and mEPSC (B) recordings. Figure A and B represent 3 second long traces of NMDA recordings in slice cultures treated with chloroquine for 3, 6 and 9 days and in untreated slices (control). The representative traces in both sEPSC and mEPSC indicate a change in the frequency and the amplitude of NMDA mediated currents. Figure C (a,b,c and d) shows superimposed average traces (from 1 min long trace) of amplitude of NMDA mediated sEPSC and mEPSC in control, 3, 6 and 9 days chloroquine treated hippocampal slices, respectively. All recordings were performed at a membrane potential of – 40mV in the presence (mEPSC) and absence (sEPSC) of TTX. CNQX and picrotoxin were also added to block AMPA and GABA mediated currents. Cumulative fraction of amplitudes (Fa) and of inter-event intervals (Fb) in control, 3, 6 and 9 days chloroquine treated hippocampal culture slices. A shift to the left in (Fa), represents a decrease in the NMDA mEPSC amplitude corresponding to the number of days of chloroquine treatment. Likewise a shift to the right in the inter-events interval graph (Fb) depicts a decrease in the frequency of NMDA mediated mEPSC with the increase in the number of days of chloroquine treatment. Graph D and E represent the amplitudes and frequencies, respectively, of AMPA sEPSC and mEPSC in control and in 3, 6 and 9 dayd chloroquine treated slices

Table 3.2. Effect of lysosomal dysfunction on NMDA mediated mEPSC and sEPSC.

	Frequency (/sec)		Amplitude		Decay τ1 (ms)		Decay τ2 (ms		Rise time (ms)	
	mEPSC	sEPSC	mEPSC	sEPSC	mEPSC	sEPSC	mEPSC	sEPSC	mEPSC	sEPSC
Control	2.13± 0.7	5.2±0.9	14.9± 2.6	30±5.2	28.4±3.8	30.3±2.1	142.2±18.3	140.1±25.2	4.2±0.4	3.9±0.5
3 days	1.74± 0.3	3.8±1.1	13.6± 1.8	25±3.9	30.2±2.4	29.4±1.9	150.1± 13.1	160.3±12.8	3.8±0.2	3.8±0.8
6 days	0.52± 0.1*	1.2±0.3*	7.7± 2.1*	10±2.4*	26.9±3.2	28.0±2.2	139.7±20.2	150.2±15.1	5.4±0.6	5.7±0.4
9 days	0.24± 0.1*	0.8±0.2*	4.8± 1.3*	6.2±1.7*	31.1±2.9	29.6±2.3	148.6±14.5	141.3±21.6	4.7±0.1	5.1±0.2

A summary of the effect of chloroquine treatment on the kinetics of NMDA mEPSC and sEPSC. The table shows a decrease in both the frequency and amplitude of NMDA mEPSC and sEPSC but not the decay and rise time kinetics in the treated groups.
* significant level of $P < 0.05$, n= 12, The values are expressed as mean ± SE.

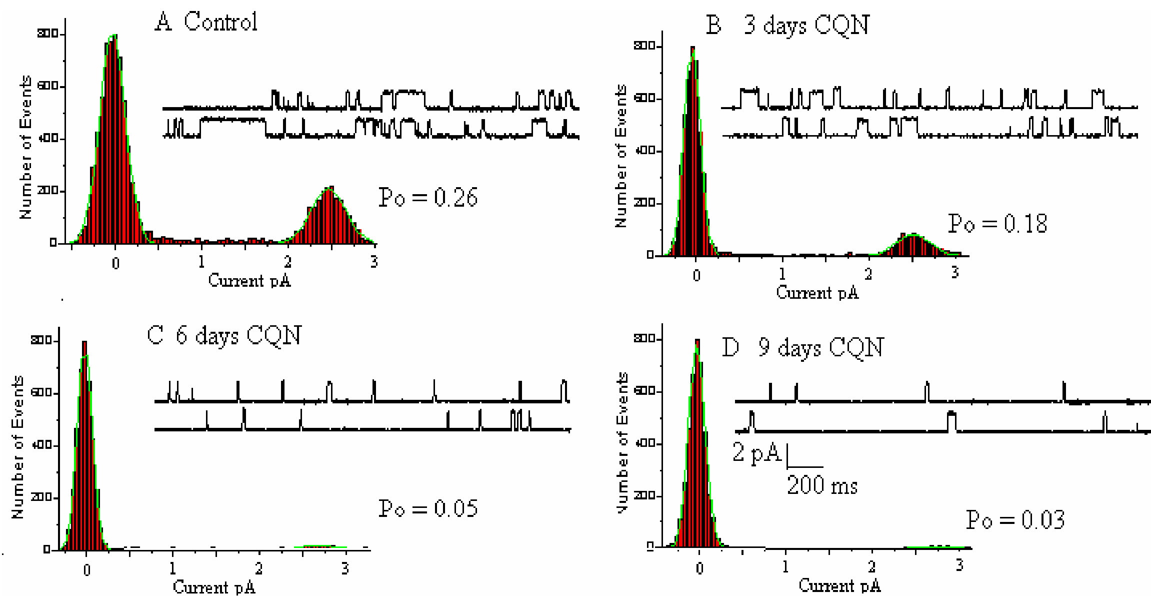


Fig. 3.5. Lysosomal dysfunction results in a decrease in open probability (Po) of NMDA single channel in isolated synaptosomes. The decrease in Po correlates with the time period of chloroquine treatment. Representatives of amplitude histograms A, B, C and D showing the Po of NMDA currents in control, 3, 6, and 9 days of chloroquine. The insets are representatives of 2 second long traces recorded at a holding potential of 54 mV in the presence of AMPA and kainate blockers.

THE EFFECT OF AMAPAKINE CX516 ON SYNAPTIC AMPA RECEPTOR PROPERTIES IN ORGANOTYPIC SLICE MODEL OF ALZHEIMER'S DISEASE.

Abstract

The role played by the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors in the expression of long term potentiation (LTP), a proposed mechanism of learning and memory, makes it a good candidate for therapeutic intervention of cognitive disorders. Indeed the ampakines family of drugs offers a promising solution if not cure of certain cognitive disorders. Members of the ampakines family of drugs are known to positively modulate the AMPA subtype of glutamate receptors. Such modulation is important in re-enhancing the lost neuronal communication between neurons and consequently restoring the cognitive function. We have previously shown that hippocampal slice cultures exhibiting lysosomal dysfunction are characterized by a decrease in AMPA receptor function. The current study therefore utilized this slice model to investigate the compensatory mechanisms of ampakine CX516 on the altered AMPA channel function. We performed whole cell recordings of AMPA mediated mEPSC in hippocampal slice cultures that had been treated with chloroquine for 9 days to induce lysosomal dysfunction. The 9 days chloroquine

treated cultured hippocampal slices exhibited a decrease in the frequency and amplitude of the AMPA mediated mEPSCs and these conditions were reversed by application of 30 μM of CX516. In addition, the single channel recordings of isolated synaptosomes that were incorporated into lipid bilayer showed a significant recovery of the single channel open probability and mean open times of AMPA receptors that had been reduced following the experimentally induced lysosomal dysfunction. Our data therefore not only support earlier findings on the effect of CX516 on synaptic AMPA receptor channels, but also indicate that the drug can compensate for the altered AMPA receptor properties following lysosomal dysfunction.

Introduction

Members of the ampakine family of drugs enhance glutamatergic transmission in the central nervous system of the mammalian brain. Specifically, the alpha-amino-3-hydroxy-5-methyl-4-isooxazolepropionic acid (AMPA) subtype of glutamate receptors, which mediate fast excitatory synaptic transmission in the central nervous system, appear to be the primary target for modulation. Reports indicate that positive modulation of AMPA receptors by the ampakines can improve channel function by increasing receptor responsiveness to endogenous ligand and specific agonists (Tang et al., 1991; Arai et al., 1996; Suppiramaniam et al., 2001). Indeed, the initial class of allosteric modulators

including pyrrolidinone analogues aniracetam and piracetam and benzothiadiazines such as cyclothiazide were shown to positively modulate AMPA receptor-mediated currents via a mechanism that at least partially involves suppressing the desensitization of AMPA receptors (Ito et al., 1990; Copani et al., 1992; Yamada and Tang, 1993). However, the mechanisms involved in desensitization suppression may vary from one modulator to another. For example, cyclothiazide potentiates AMPA receptors by directly slowing the onset rate of desensitization and by increasing agonist affinity, (Yamada and Tang, 1993; Partin et al., 1994, 1996), but aniracetam potentiates AMPA receptors by directly slowing the rate of deactivation which indirectly slows the onset of desensitization, without a change in agonist affinity (Partin et al., 1996).

Fairly recent derivatives of the benzoylpiperidine compounds such as 1-(quinoxalin-6-ylcarbonyl)-piperidine (CX516), which have the ability to cross the blood brain barrier (Staubli et al., 1994a, b; Arai and Lynch, 1998a,b), have been developed. Studies show that these ampakines, like their predecessors, positively modulate AMPA receptors resulting in memory enhancement in both rodents and humans (Granger et al., 1996; Ingvar et al., 1997; Larson et al., 1995). However, these ampakines appear to be generally more effective than cyclothiazide in prolonging response decay upon glutamate removal thus slowing the rate of deactivation, but are less effective in blocking receptor desensitization. Recent findings now indicate that through allosteric modulation

by ampakines, AMPA receptor responses can be enhanced in such a way as to strengthen survival signaling and a concomitant reduction in hippocampal damage (Munirathinam et al., 2002). These findings confirm earlier reports indicating that low-level stimulation of AMPA receptors by endogenous glutamate enhances neuronal survival and promotes synaptic maintenance (Bambrick et al., 1995; McKinney et al., 1999). Indeed, AMPA receptors have been shown to be linked to the neuroprotective mitogen-activated protein kinase (MAPK) pathway which can be positively modulated (Bahr et al., 2002).

The existing literature therefore implicates ampakines in modulating AMPA receptor function with a potential application in therapeutic intervention of cognitive disorders. Thus, understanding the mechanistic basis of AMPA modulators in channel gating properties during synaptic transmission and the concomitant effect on cognition is of particular interest. By utilizing various animal and slice models, the effect of AMPA modulators on the kinetic properties of AMPA receptors that may be altered in a disease state can be studied. In this study we have utilized a unique slice model for Alzheimer's disease (AD) to study the effects of CX516 on AMPA receptors channel properties following lysosomal dysfunction. Experimental induction of lysosomal dysfunction in hippocampal slice cultures has been used by other investigators (Bahr et al., 1994; Bendiske et al., 2002). Furthermore, the slice model was used in these studies because of its features characteristic of the adult

brain, including the circuitry, integrity, and organization of neuronal subfields (Bahr et al., 1995). We performed whole cell recordings on hippocampal slices to study the compensatory effect of CX516 on altered AMPA mediated synaptic transmission in AD model slices. To investigate how the changes in single channel properties of synaptic AMPA receptors would contribute to changes in synaptic transmission, we utilized isolated hippocampal synaptosomes incorporated into lipid bilayer for single channel recordings of AMPA receptors as described elsewhere (Vaithianathan et al., 2004).

Materials and Methods

Salts and reagents

Unless specified, all salts and reagents were purchased from Sigma Chemical Co. Horse serum, Hanks balance salts, Earl's balance salt, MEM, penicillin/streptomycin, fungizone, glutamine, culture plates and membrane inserts were purchased from Gibco.

Organotypic slice cultures

Hippocampal slices were prepared using conventional methods (Stoppini et al., 1991). Briefly 5-7 days postnatal Sprague-Dawley rat pups (Charles River Breeding Laboratories, Wilmington MA) were sacrificed and whole brain was isolated from the skull and submerged in ice cold Hanks balance salt containing:

137.9mM NaCl, 5.33 mM KCl, 0.41mM $MgSO_4$, 0.49 mM $MgCl_2$, 1.26 mM $CaCl_2$, 0.44 mM KH_2PO_4, 4.17 mM NaHCO3, 0.34 mM Na_2HPO_4 and 10 mM glucose. Transverse brain slices (400 μM) were sectioned using a vibrotome and the hippocampal sections were isolated from the rest of the brain. Then 4 to 6 hippocampal slices were placed on insert membranes that rested on a 6 well culture plate containing the culture media. The culture media contained 50% Earle's balanced slat, 50% MEM and 25% heat-inactivated horse serum. The culture media was also supplemented with 1 mM glutamine and 36 mM glucose which was sterile filtered before adding to the media. Antibiotics such as penicillin/streptomycin and fungizone were added in case of any infection. Slices were incubated at 37° C, 100% humidity and 5% carbon dioxide to maintain the pH. The media was changed every other day.

Chloroquine treatment started after the slices were maintained in the cultures for 2 weeks. This time was selected since the slices have recovered and are in a healthy condition. A 50 μM solution of chloroquine was used to induce lysosomal dysfunction in slices for 9 days. This treatment did not affect protein glycosylation, protein synthesis and secretion of various proteins including amyloid precursor protein (Caparaso et al., 1992). Chloroquine being a weak base disturbs the proton gradient in lysosomes (Poole et al., 1981) thereby disrupting protein degradation (Wibo and Poole 1974).

Synaptosomes preparation

Slices harvested after 9 days of chloroquine treatment were isolated from the insert membranes by scraping with a spatula. Pools of slices from the same treatment group were then homogenized in 100µl of homogenizing buffer (mKRBS) using a Potter homogenizer with 10 strokes. The mKrebs buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose and 10 mg/ml adenosine deaminase. The pH was adjusted to 7.4 by bubbling with 95:5 O_2:CO_2. The buffer was also supplemented with 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM Benzamide to minimize proteolysis. The homogenate was filtered through a 13 mm diameter Millipore syringe filter holder which was attached to a 1 cc Tuberculin syringe followed by filtration through three layers of nylon filters (Tetko, 100 µm pore size) and finally collected in a 1.5 ml Eppendorf tube. The filtrate was then loaded into another 1 cc tuberculin syringe and forced through a pre-wetted 5 µm Millipore nitrocellulose filter followed by spinning at 1000 x g for 15 min in a microfuge at 4°C. The supernatant was removed, and the pellet which contained synaptosomes was resuspended in 20 µl of mKRBS buffer for electrophysiology and Western blot analysis.

Electrophysiological Recordings in Slices

After 9 days of chloroquine treatment slices were subjected to whole cell electrophysiological recordings. The slices were first isolated from the inserts by cutting the insert membrane around the slice and then transferred into a perfusion charmber containing extracellular solution (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1mM NaH_2PO_4, 26 mM $NaHCO_3$ and 11 mM dextrose). Individual pyramidal neurons from CA1 region were visually selected using Nomarski differential interference contrast Olympus microscope (BX51Wi). Patch pipettes pulled from borosilicate capillaries (9-12 MΩ) were filled with pseudo-intracellular solution containing:100 mM K-gluconate, 0.6 mM EGTA, 5 mM $MgCl_2$, 2mM ATP x Na x $3H_2O$, 0.3 mM GTP-Na and 40mM HEPES. Using the patch pipetter, cells were patched by first forming a giga ohm seal achieved by applying a slight negative pressure while the electrode was attached to the membrane. Intracellular recordings of miniature currents (mEPSCs) were performed with an axopatch 200 B instrument.

The AMPA miniature currents were recorded in the presence of 4 μM TTX. To isolate AMPA currents, recordings were performed at -65 mV holding potential in the presence of 4 μM TTX, 50 μM APV and 100 μM picrotoxin. AMPA mediate mEPSCs were verified by applying 40 μM CNQX to the extracellular solution. Recordings were accepted if there was a complete block of the responses.

Single channel recording of AMPA

Synaptosomes were incorporated into an artificial phospholipid bilayer for electrophysiological recordings. Patch pipettes, with a resistance of 100MΩ, were pulled using borosilicate glass capillaries and filled with intracellular solution containing: 110mM KCl, 4mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS) at a pH of 7.4. The extracellular solution consisted of: 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris HCl. The bilayer was formed using 1,2-diphytanoyl-sn-glycero-3-phosphocholine (PC), (Avanti Polar-Lipids Inc., Alabaster, AL), dissolved in anhydrous hexane (Aldrich Chemical Co., Milwaukee, WI). Approximately 5-7 μL of PC was added to a micro-beaker containing 300 μL of extracellular solution. After tip dipping the pipette into the extracellular solution containing the PC a few times, a bilayer will be formed at the tip of the pipette. Successful formation of the bilayer was confirmed by measuring the resistance using the axopatch 200B (Axon Instruments, Union City, CA) and pclamp 9 software. About 2 μL of synaptosomes were added to the extracellular fluid with continuous stirring to enhance the synaptosomal fragments to fuse with the bilayer. AMPA was then added and channel recordings were made at different voltages. Single channel events were recorded on a video tape for off-line analysis with pclamp 9 software. Recorded signals were filtered at 5 kHz and digitized between 5-25 kHz. AMPA currents were activated by 300nM of AMPA

(Tocris, Ellisville, MO) to the cis side of the bilayer in the presence of 50μM APV (NMDA antagonist), 1μM SYM2081 (Kainate antagonist), 100μM picrotoxin (GABA antagonist), 2μM TEA (potassium antagonist), and 1μM TTX (sodium antagonist). At the end of the experiments the AMPA elicited currents were blocked by the addition of 1μM CNQX.

Data analysis

The data recorded in pclamp 9 software (Axon instruments) was subject to kinetic analysis utilizing clampfit 9 (axon instruments) for single channel analysis and mini analysis program (Synaptosoft, Decatur, GA) for mEPSC recordings. The detection threshold mEPSC was set at ≥ 2.5 to detect the smallest AMPA mEPSCs. All measurements were expressed as mean ± SEM. Paired t-test was used for single comparison and a P value less than 0.05 was considered significant. For the purpose of presentation Orign 6.0 software was used.

Results

Effect of CX516 on AMPA mediated mEPSC in organotypic slice cultures.

Organotypic slice cultures were allowed to recover for 2 weeks after surgery which is the time they exhibit adult characteristics (Stoppini et al., 1991; Bahr et al., 1994, 1995). Slices were then treated with chloroquine for 9 days to induce lysosomal dysfunction. Previous reports indicate that chloroquine

treatment induces feature characteristics including accumulation of aberrant proteins similar to those observed in Alzheimer's disease (Bahr et al., 1994; 1995). The nine days time point was selected as the optimum effect of chloroquine induced lysosomal dysfunction resulting in modified AMPA receptor function. We have previously observed (Kanju et al., 2002) an alteration in AMPA receptor channel properties after chloroquine treatment of hippocampal slice culture in slices and isolated synaptosomes. In the current study we report that the chloroquine induced impairment of AMPA channel kinetics observed previously can be reversed by treatment with CX516.

Hippocampal slices treated with chloroquine for nine days exhibited a decrease in AMPA receptor activity. Specifically, both the amplitude and the frequency (fig. 4.1) were significantly (t-test, $P < 0.05$, $n = 10$) reduced in 9 day treated slices compared to control. The amplitude of AMPA mediated mEPSC was reduced from 24.2 ± 2.3 pA to 6.3 ± 0.8 pA resulting in a shift to the left observed in the cumulative fraction graphs (Fig. 4.1A). The frequency of the AMPA miniature currents (mEPSC) were decreased from 3.8 ± 0.3 Hz in control to 0.3 ± 0.1 Hz in 9 day treated slices. This is represented by a shift of the inter-events intervals to the right in the cumulative probability graphs (4.1B).

Addition of 30μM CX516 resulted in a compensatory increase in the amplitude of the AMPA mediated mEPSCs in 9 day chloroquine treated slices. The amplitude was increased from 3.8 ±0.3 pA to 21 ±1.6 pA. This shows a

significant ($P < 0.05$, n=11) reversible effect of CX516 on AMPA mediated currents. Unexpectedly, application of CX516 in the extracellular solution resulted in a significant increase ($P < 0.05$, n=10) in the frequency of AMPA mediated mEPSC. Previous studies indicate that CX516 directly modulates AMPA receptor function (Arai et al., 2002). Conventionally an increase in frequency implies a change in pre-synaptic release of glutamate neurotransmitter. However, this may not reflect the already established mechanism of action of CX516 on AMPA modulation. It is possible that CX516 may play an indirect role to enhance the release probability. Our results indicate that the frequency of AMPA mediated mEPSC was increased from 0.3 ± 0.1 Hz to 3.1 ± 0.5 (Fig. 4.1)

Effect of CX516 on single channel currents of AMPA receptors in isolated synaptosomes

Whole cell recordings of AMPA-mEPSCs provide generalized information of effects of CX516 on postsynaptic AMPA receptors. However, detailed alterations in the kinetic properties of single synaptic AMPA receptors that are responsible for the changes in amplitude and time course is essential to elucidate the mechanism of action of CX516. We therefore advanced our search into the measurement of single channel properties of synaptic AMPA receptors utilizing isolated synaptosomes. Single channel activity of AMPA receptors were elicited

by application of 290nm of AMPA followed by 3 μM CX 516 to the external solution in the presence of sodium, potassium, NMDA, kainite and GABA channel blockers. The channel activity was blocked by application of CNQX (1 μM) to the external solution at the end of each experiment. Our results indicate that the single channel properties of synaptic AMPA receptors in 9 day treated slices were significantly altered. Specifically, the probability of channel opening (Po) was reduced from 49% to 8% (Figure 4.2). In addition the dwell distributions of closed and open levels show a significant reduction in 9 day chloroquine treated preparations compared to control ($P<0.05$, n=12). The data show that the mean open times of tau 1 and tau 2 were significantly reduced from 0.34 ± 0.05 ms and 3.11 ± 0.07 ms in controls to 0.15 ± 0.02 ms and 2.30 ± 0.06 ms for synaptosomes from 9 day treated slices, respectively. We did not observe a significant change in the conductance of AMPA receptors ($P< 0.05$, n= 12).

Addition of CX516 in synaptosomes isolated from 9 day treated slices resulted in a complete reversal of the altered AMPA currents. Specifically, the probability of channel opening was increased from 8% to 45%. In addition, the mean open times (tau) fitted with two exponential decay fittings were significantly increased. The values for tau 1 and tau 2 were increased from 0.15 ± 0.02 ms and 1.41 ± 0.04 ms in the absence of CX516 to 0.32 ± 0.03 ms and 2.30 ± 0.06 ms in the presence of CX516. Concomitantly, the mean closed times were

significantly reduced ($P<0.05$, n=12) by the addition of CX516. A summary of both the mean open times and closed times are illustrated in table 4.1.

To fully utilize the data from the single channel and that of whole cell recordings in slices we compared the percentage change in the expected amplitude (Conductance X Probability of channel opening (Po) with the percentage change in the mEPSC currents in the absence and in the presence of CX516. Our data indicate that the percentage change in both cases was 84 ± 2%. Thus the change in single channel currents of AMPA receptors in isolated synaptosomes correlate with the mEPSC recordings of hippocampal slices.

Discussion

The carefully controlled AMPA receptor function is required for proper excitatory neuronal transmission in the mammalian brain. Therefore, dysfunction of these receptors may compromise the synaptic plasticity and memory acquisition. Indeed, the channel properties of AMPA receptors may be altered in cognitive disorders such as AD. Therefore, positive modulation of AMPA receptors may act to improve the channel function and may have an important role in maintaining normal synaptic mechanisms required for learning and memory. The ampakine class of compounds has been shown to significantly enhance the AMPA receptors responsiveness to endogenous ligand and specific agonists (Tang et al., 1991; Arai et al., 1996; Suppiramaniam et al., 2001; Arai et

al., 2002). Consistent with these findings are behavioral studies that indicate CX516 and other AMPA modulators improved performance in a variety of memory tasks in rodents. (Pontecorvo and Evans, 1985; Staubli et al., 1994b; Hampson et al., 1998).

Findings from the current study demonstrate that the channel properties of synaptic AMPA receptors are significantly altered in organotypic slice cultures exhibiting lysosomal dysfunction. The current study also suggests that the altered synaptic AMPA channel properties are compensated in the presence of 30 μM CX516. Our results are in agreement with a previous study that ampakines such as CX516 modulate the activity of synaptic AMPA receptors (Lynch et al., 1996). We combined electrophysiological recordings of isolated synaptosomes that were incorporated into lipid bilayer and those of whole cell recordings in cultured slices to elucidate the modulation of AMPA receptors by CX516. Although whole cell recordings of mEPSC in slices provide valuable information for the neuronal connectivity and communication, such information is limited in providing kinetic details of single synaptic receptors.

Due to the complexity of neuronal functions the relative impact of ampakines such as CX516 on large-scale neuronal networks and on various forms of synaptic plasticity including long-term potentiation (LTP) is still not clear. However, although ampakines directly modulate AMPA receptors, the repetitive activation of AMPA receptors may transiently activate NMDA

receptors which in turn triggers long-lasting plasticity. Indeed, CX516 has been shown to facilitate the induction of NMDA-dependent LTP in the hippocampus in vivo (Staubli et al., 1994a). The observed facilitation may be a result of the consequential activation of NMDA receptors arising from depolarization produced by the AMPA receptors potentiated by the ampakine (Staubli et al., 1994a, b). However, our data did not substantially prove that modulation of AMPA receptors have an effect on NMDA mediated miniature synaptic currents. The change in AMPA miniature currents may not be large enough to induce a significant change in NMDA mEPSC. However, large currents evoked from the pre-synaptic site do produce a significant increase in the NMDA currents at the postsynaptic site.

Our results indicate that there were significant changes in the decay kinetics, but not rise time, of AMPA mEPSC after addition of CX516. The decay time represents the desensitization and deactivation of receptors. Previous reports indicate that desensitization does not play a major role in shaping AMPA receptor-mediated synaptic currents (Arai and Lynch 1998a;b). These studies showed that addition of cyclothiazide which caused a decrease desensitization of AMPA receptors and thus an increase in decay time did not result in a significant increase in extracellularly recorded excitatory postsynaptic potentials. Although changes in desensitization kinetics may not have contributed to the AMPA

synaptic currents, the deactivation of the AMPA receptors may account for the increased decay time we observed in the current study.

Surprisingly, our results indicate that CX516 increased the frequency of AMPA mediated mEPSC in chloroquine treated hippocampal slices for nine days. These findings appear to be controversial since CX516 acts directly on AMPA receptors which imply that the most probable response would be an increase in amplitude and not frequency. Traditionally, an increase in the frequency is thought to represent a pre-synaptic effect while a change in amplitude represents a post-synaptic effect. However, reports indicate that this is not always the case (Choi et al., 2003; Malinow and Malenka, 2002). Some reports provide evidence of pre-synaptic AMPA receptors that may regulate glutamate release (Patel et al., 1997). Indeed, application of AMPA increased glutamate release from the rat neostriatum in a dose-dependent manner, and the increase was blocked by competitive AMPA antagonists (Patel et al., 2001). In addition, AMPA-evoked glutamate release from striatal glutamatergic terminals has been shown to be potentiated by β-adrenergic receptor-mediated cAMP accumulation (Dohovics et al. 2003). Therefore, the observed increase in frequency of AMPA mediated miniature currents was probably the result of pre-synaptic potentiation of AMPA receptors by CX516. However, our data cannot prove that the observed increase in mEPSC frequency was a result of pre-synaptic AMPA receptors modulation by the ampakines. Furthermore, it is not well known whether pre-

synaptic AMPA receptors exist in the CA 1 region of hippocampal which was our region of interest. In addition, it is still not clear whether the modulation mechanism of CX516 is the same for both pre- and postsynaptic AMPA receptors. Our data can only suggest that such modulations are possible.

A previous study utilizing hippocampal slice cultures indicated that CX516 failed to offer neuroprotection induced by lysosomal dysfunction (Bahr et al., 2002). On the other hand, the same study demonstrated that decreased neuronal markers induced by excitotoxicity can be attenuated by application of CX516. The AMPA receptors are also linked to the neuroprotective mitogen-activated protein kinase (MAPK) pathway (Wang and Durkin, 1995; Hayashi *et al.*, 1999; Bahr *et al.*, 2002; Limatola *et al.*, 2002). Hence, the neuroprotection offered by CX516 may be via glutamate-mediated survival response against neuropathogenesis. More specifically, these studies may imply a direct role of CX516 in neuroprotection via modulating AMPA receptors function. In addition, the fact that Bahr et al (2002) did not observe a CX516 induced neuroprotective effect against nonexcitotoxic pathology support our findings that AMPA receptors activity is significantly impaired following lysosomal dysfunction. However, lysosomal induced neuronal injury may be due to a host of impaired parameters that require more than CX516 modulation of AMPA receptors to reverse the pathology. Our electrophysiological data indicate that although CX516 is incapable of reversing the lysosomal mediated insults as observed by

Bahr et al (2002) its role in reversing the altered AMPA channel kinetics may compensate for the lost synaptic communication.

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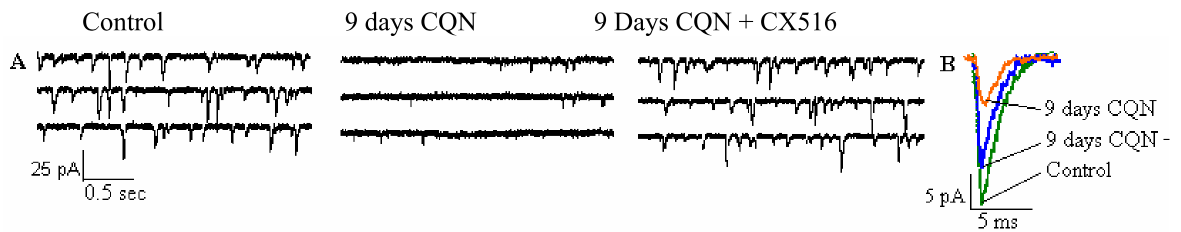
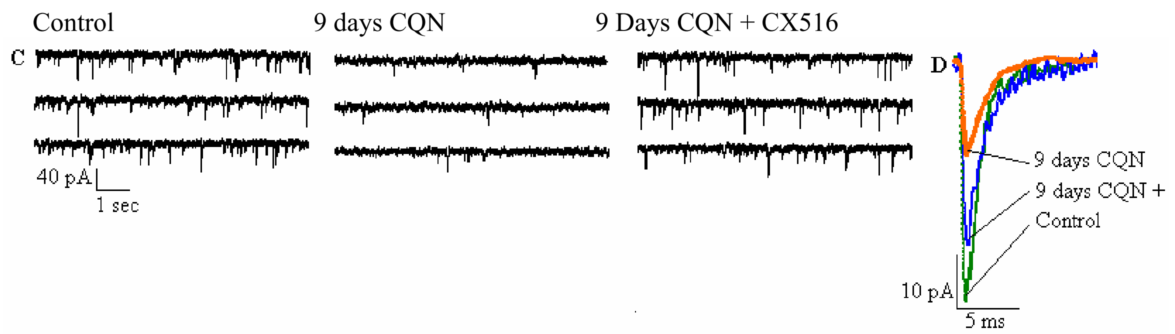

Control
9 days CQN
9 Days CQN + CX516
A
25 pA
0.5 sec
B
9 days CQN
9 days CQN -
Control
5 pA
5 ms


Control
9 days CQN
9 Days CQN + CX516
C
40 pA
1 sec
D
9 days CQN
9 days CQN +
Control
10 pA
5 ms

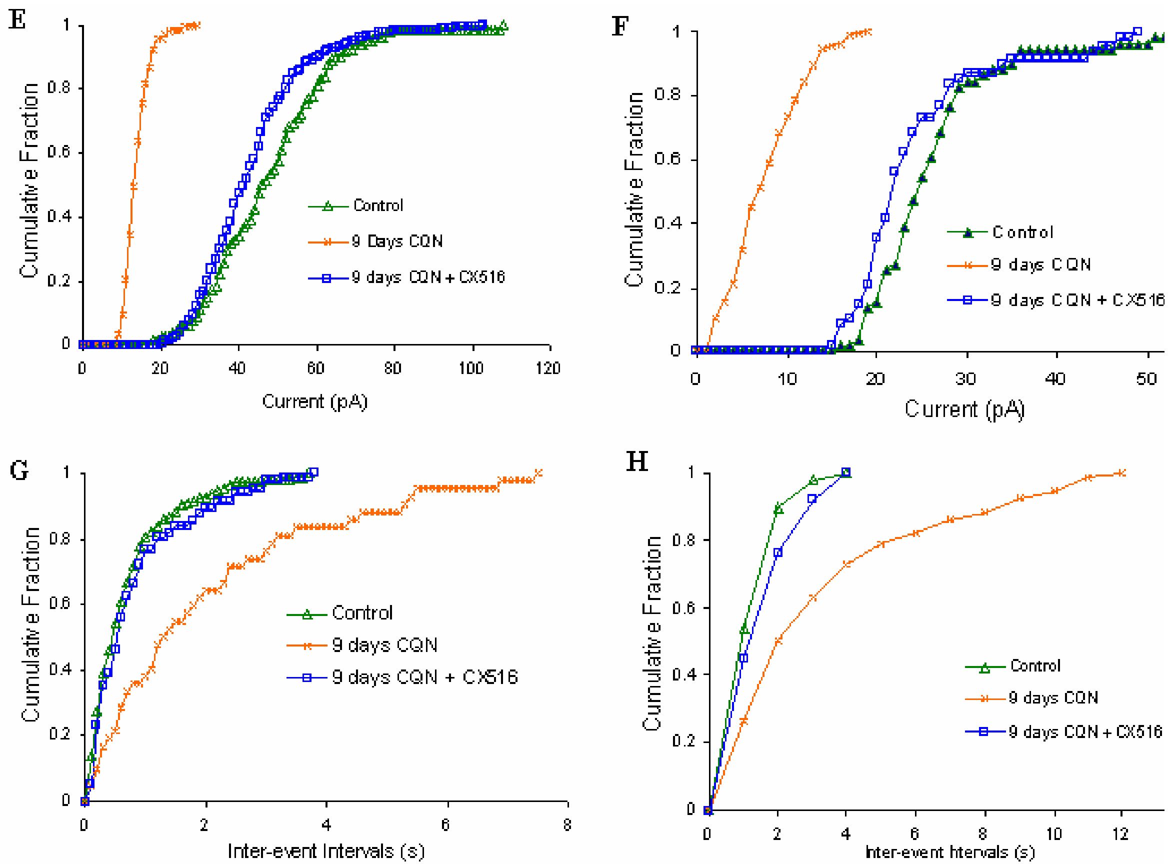


Figure 4.1 Alteration of AMPA mediated mEPSC and sEPSC in hippocampal cultured slices after lysosomal dysfunction. Representative traces of mEPSC (Panel A) for control and after 9 days of chloroquine treatment indicate a reduction in event frequencies in 9 day CQN treated slices. The ability of 30µM CX516 to reverse the chloroquine effect is also shown in Panel A. The superimpose average amplitudes for control, 9 days chloroquine treated and 9 days chloroquine plus CX516 are in panel B. AMPA meadiated mEPSC recordings were performed at a membrane potential on – 65 mV in the presence of TTX, APV and picrotoxin. The AMPA mediated sEPSC are shown in panel C while their superimpose average amplitudes for control and in 9 days chloroquine with or without addition of CX516 are shown in D. The cumulative fractions of the amplitude for the AMPA mediate sEPSC and mEPSC in controls and 9 days chloroquine slices in the presence and absence of CX516 are shown in E and F respectively. The cumulative fraction of both mEPSC and sEPSC recordings indicate a significant (t-test, $P<0.05$, $n=12$) shift in the amplitude to the left after chloroquine treatment and a compensated shift to the right after addition of 30µM CX516. The inter-events intervals for the sEPSC and mEPSC are shown in G and H respectively where increase in the inter-events intervals (decreased frequency) in the 9 days treated slices was compensated by addition of 30µM CX516.

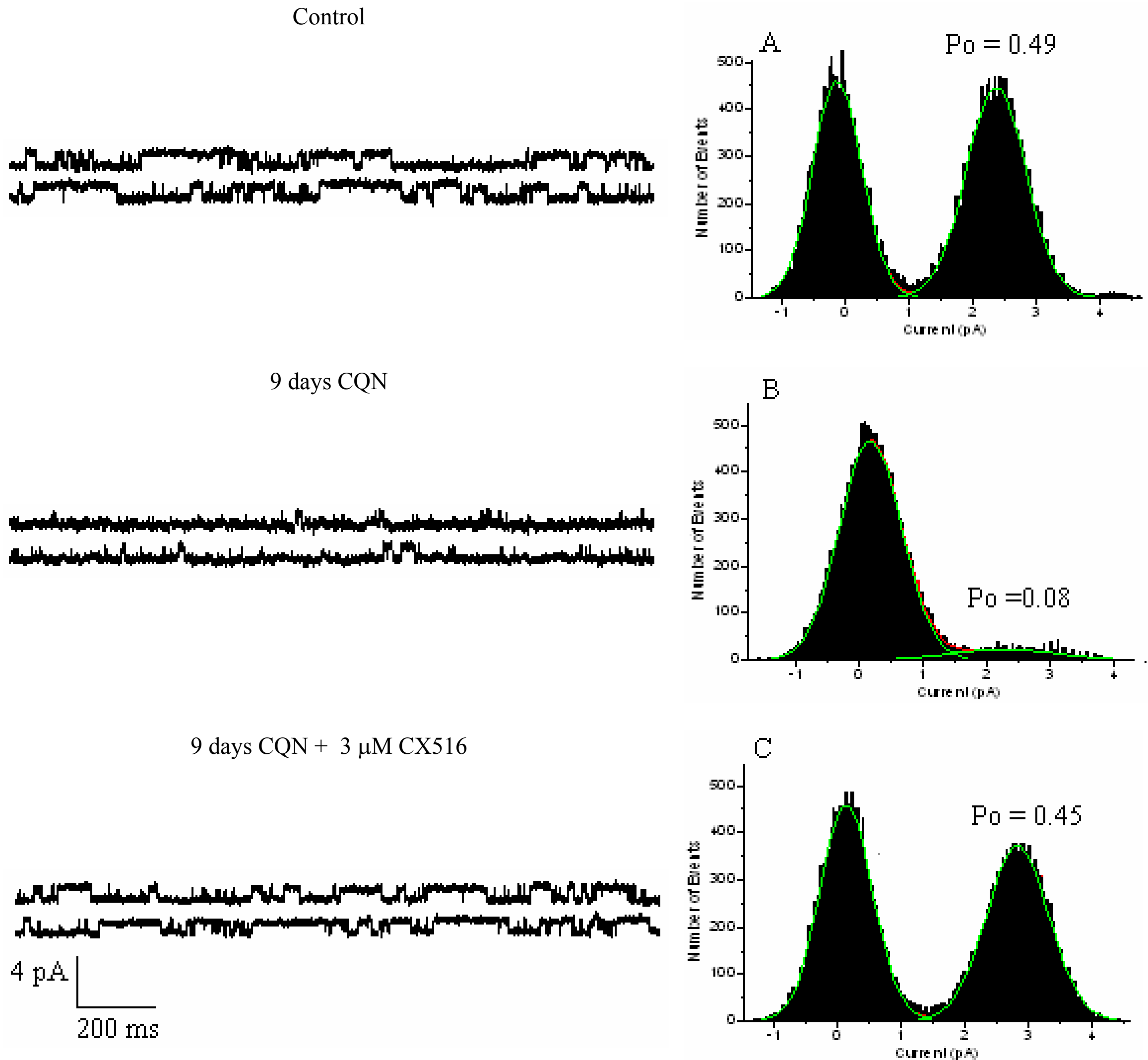


Fig.4.2: Effects of CX516 on AMPA elicited currents in isolated synatpsomes from hippocampal cultured slices after lysosomal dysfunction. AMPA mediated single channel recordings in isolated synaptosomes in control, 9 days chloroquine treated slices and in 9 days of chloroquine treated slices after addition of 3 µM CX516. The corresponding amplitude histograms, A, B and C indicate the open probability in control , 9 days chloroquine treated slices and in 9 days of chloroquine treated slices after addition of CX516 respectively. Application of CX516 to the extracellular solution resulted in an increase in probability of channel opening (Po) of 0.45 representing over 85% restoration. Addition of the same concentration of CX516 that restored the Po did not have any significant change in the AMPA channel conductance (t-test, $P< 0.05$). The recordings were performed in lipid bilayers at holding potential of +68 mV.

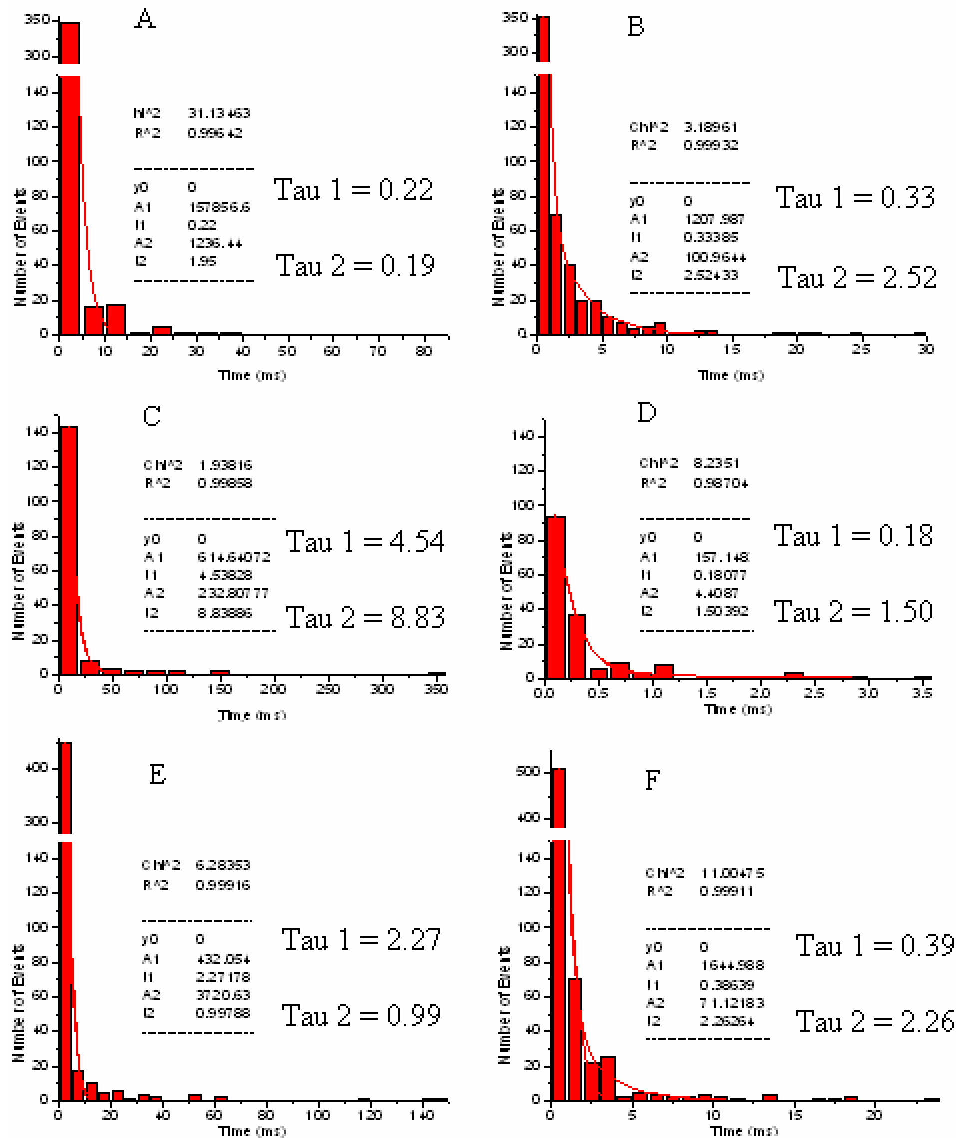


Fig.4.3**:** Effects of CX516 on dwell times of single channel of AMPA receptors. Exponential decay fittings of dwell level 0 (A, C and E) and dwell level 1 (B, D and F) for the control, 9 days chloroquine treated slices in the absence and in the presence of 30 μM CX516, respectively. Application of CX516 resulted in an increase in mean open time of AMPA currents that had been altered in 9 days chloroquine treated slices. Concomitantly, CX516 decreased the mean closed time of AMPA elicited responses in synaptosomes. Notice the different x-axis scale. The insets show the statistics of the second order exponential decay fittings. The tau values (mean open time in ms) are also shown in each inset.

Table4.1: The kinetics of mean open, closed times and open probabilities of AMPA channels

	Po	Dwell 0 (ms)		Dwell 1 (ms)	
		Tau 1	Tau 2	Tau 1	Tau 2
Control	0.47 ±0.02	0.20 ±0.03	0.15 ±0.06	0.34 ±0.05	3.11 ±0.07
9days CQN	0.06 ±0.03	4.45 ±0.12	8.63 ±0.21	0.15 ±0.02	1.41 ±0.04
9days CQN + CX516	0.43 ±0.02	1.21 ±0.09	0.89 ±0.11	0.32 ±0.03	2.30 ±0.06

The table illustrates the open (Dwell1) and closed (dwell 0) times in ms observed in control and in synaptosomes from 9 days treated slices in the presence or absence of CX516. The open probabilities (Po) are also shown for the three groups. All values are expressed as mean ± SD of 12 experiments.

SYNAPTIC GLUTAMATE RECEPTOR DYSFUNCTION IN CHOLINERGIC LESIONED ANIMALS.

Abstract

Accumulating evidence supports the role of septohippocampal cholinergic projections in learning and memory mechanisms. Hence, a complete and selective destruction of the septal cholinergic neurons projecting to the hippocampus by immunotoxin 192 IgG-saporin may result in memory impairment. Alterations in NMDA and AMPA receptor binding properties have been previously reported following septohippocampal cholinergic denervation. A decrease in NMDA binding and an increase or no change in AMPA binding was observed seven days after lesioning. Therefore, it is important to study the effects of cholinergic lesioning on functional properties of synaptic glutamate receptors. This study investigated the electrophysiological properties of synaptic AMPA and NMDA receptors 4 to 6 days after medial septal lesioning. Selective medial-septal lesioning was performed in rats with the immunotoxin 192 IgG-saporin. Whole cell recording of mEPSC and sEPSC were performed in CA1 hippocampal region in slices from lesioned and sham lesioned animals. The single channel recordings of synaptosomes isolated from hippocampi of these

animal groups incorporated into lipid bilayer were also performed. Our results indicate a reduction in the frequency and amplitude of AMPA and NMDA mediated mEPSCs and sEPSCs of animals lesioned with 192 IgG-saporin. Furthermore, single channel recording of isolated synaptosomes demonstrate a reduction in channel open probability and conductance. Collectively, our results indicate that synaptic AMPA and NMDA receptor functions are altered 4-6 days following medial septal lesioning.

Introduction.

Selective lesioning of the cholinergic basal forebrain by immunotoxin 192 IgG-saporin provides a valuable model to study the neurophysiological paradigms of learning and memory. The rat basal forebrain comprises important cholinergic neuronal network such as the nucleus basalis of Meynert and the septal nucleus of the diagonal band of Broca (Fibiger 1982; Sofroniew et al. 1982) which projects to the entire cortex (Wenk et al., 1980). Of particular interest are the septohippocampal acetylcholinergic projections that may play a significant role in learning and memory processes. Indeed, hippocampal-dependent learning and memory has been associated with an increase in hippocampal extracellular acetycholine levels (Fadda et al. 1996, 2000; Orsetti et al. 1996; Ragozzino et al., 1998; Nail-Boucherie et al. 2000; McIntyre et al. 2002; Chang and Gold 2003). Other studies demonstrate that basal forebrain lesions induce deficits in memory,

and that septal infusion of drugs such as pregnenolone sulfate (Darnaudery et al. 2002), and glucose (Ragozzino et al., 1998) enhance memory by increasing the level of extracellular hippocampal acetylcholine. Although the septal infusion of cholinergic agonists may enhance memory (Givens and Olton 1995; Pang and Nocera 1999), a septal infusion of cholinergic agonists was shown to decrease hippocampal extracellular acetylcholine levels (Gorman et al. 1994). This inconsistency indicates that there may be another neurotransmitter that is enhanced by cholinergic agonists which causes the observed cognitive enhancement.

The glutamatergic system mediates the majority of the excitatory neurotransmission in the central nervous system and has been widely known to play a major role in learning and memory (Francis et al., 1993, Lynch, 1998; Myhrer, 2000; Riedel and Micheau, 2001). Furthermore, glutamatergic neurotransmission has been implicated in mechanisms of synaptic plasticity such as long term pontentiation (LTP), which is thought to underlie learning and memory (Baudry and Lynch, 2001; Scannevin and Huganir, 2000). Of particular interest is the alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors which dominate the hippocampal region of the brain. Glutamate receptor function in the hippocampus may be altered following cholinergic denervation induced by immunotoxins such as 192 IgG-saporin. Indeed, neurotoxic lesioning of the

medial septum results in profound impairment of learning and memory tasks that are also impaired by hippocampal damage (Decker, et al., 1992; Janis etal., 1998; Nilsson et al., 1992; Walsh et al., 1996). The link between glutamate receptors and acetylcholine is strengthened by the finding that acetylcholine enhances NMDA-dependent LTP (Segal and Auerbach, 1997; Aramakis et al., 1997). However, other reports indicate that, while immunolesioning resulted in a decrease in NMDA binding, it caused an increase in AMPA and Kainate binding seven days after inmmunolesioning (Rossner et al., 1995). In addition, Nicolle et al found a decrease in NMDA binding and no significant change in AMPA or Kainate binding 30 days after immunolesioning (Nicolle et al., 1997). Another study by Jouvenceau et al., (1997) reported that both NMDA and Non-NMDA mediated responses were potentiated 4 weeks after selective lesioning with 192 IgG-saporin.

Previous findings therefore suggest that the effects of selective lesioning with 192 IgG-saporin are not only dependent on the magnitude of cholinergic denervation but are also time dependent. Effects of cholinergic denervation on NMDA receptors appear to be transient and diminish with time while the AMPA receptor binding may increase as a compensatory mechanism due to the lost NMDA receptor function. Whether the function of AMPA or NMDA receptors is altered at the early stage of immunolesioning is still not clear and is the subject of this study. Previous studies indicate that lysosomal disturbance may be one of

the early markers of neurodegeneration (Bi et al., 1999). The current study therefore investigated whether cholinergic denervation is accompanied by lysosomal dysfunction. Hence, we combine the electrophysiological recordings for AMPA and NMDA receptors and immunological studies for synaptic markers and lysosomal disturbance to elucidate the early events that take place following medial septum lesioning. Specifically we performed electrophysiological recordings of miniature and spontaneous excitatory postsynaptic currents (mEPSCs and sEPSCs) in slices obtained from lesioned and unlesioned rats. To test whether the changes in synaptic currents are due to altered functional properties of individual synaptic receptors, we isolated synaptosomes and incorporated them into lipid bilayers and measured the single channel properties.

Materials and Methods

Surgical Procedure

Female rats weighing 230–300 g were used in this study. The animals were individually caged and kept in a 12-h light and dark cycle. Three independent groups were used in the current study: Two control groups were utilized, one control group was noninjected rats and the other control group received the vehicle. The treated groups received the 192 IgG-saporin. The injections were performed on the medial septum region of the brain after the animals were

anaesthetized with Ketamine 87 mg/kg and Xylazine 13mg/Kg intraperitoneal. Lesioning was performed on a stereotaxis frame which held the head rigidly in place. After dorsal midline skin incision was made over the skull, a small burr hole was drilled and medial septal lesions were performed by stereotaxic infusion of the toxin or vehicle (0.4 μl/min for 5 min) at the following stereotaxic co-ordinates (Bragma coordinates from Paxinos and Watson atlas (Paxinos and Watson 1998): AP 0.2 mm; DV 6.0 mm ML 0 mm). The syringe needle was left in place for 5 min to allow proper diffusion. The lesioned animals were monitored and allowed to recover. Four to six days after surgery the rats were sacrificed and the hippocampus was quickly removed and placed in ice cold cutting solution. Slices 400 μm thick were cut with a vibrotome and incubated for at least 30 min in phosphate buffer. Slices were either subjected to electrophysiology or preserved for synaptosomal preparation, western blot and histochemistry.

Histology

Hippocampal slices were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. The slices were cryoprotected at 4°C in 30% buffered sucrose and coronal sections of 40 μm thick were cut on a freezing microtome, collected in 0.1 M phosphate buffer. The slices were then processed immediately for histochemistry. Destruction of cholinergic terminals in the hippocampus were determined by choline acetyltransferase (ChAT)

immunostaining with mouse polyclonal antibody (Chemicon International, Temecula, CA, USA)

Electrophysiological recordings in slices

A single slice was transferred to the recording chamber and was held between two nylon net, which was submerged beneath and continuously superfused with oxygenated extracellular solution (95% O_2 and 5% CO_2). The composition of extracellular solution was 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1mM NaH_2PO_4, 26 mM $NaHCO_3$ and 11 mM Dextrose. Individual CA1 pyramidal neurons were visually selected utilizing a Nomarski differential interference contrast microscope (Olympus BX51Wi). Conventional intracellular recordings from the selected neurons were obtained using glass micropipettes (9-12 MΩ) that were filled with a pseudo-intracellular solution containing:100 mM K-gluconate, 0.6 mM EGTA, 5 mM $MgCl_2$, 2mM ATP x Na x $3H_2O$, 0.3 mM GTP-Na and 40mM HEPES. Intracellular recordings of miniature excitatory postsynaptic currents (mEPSCs) and spontaneous excitatory postsynaptic currents (sEPSC) were performed with axopatch 200 B instruments.

To isolate AMPA mEPSC, recordings were performed in the presence of the GABA receptor antagonist picrotoxin 50 μM, TTX 1 μM and an NMDA antagonist 2-amino-5-phosphonovalerate (APV) 40 μM . The recording of NMDA mEPSC were similar to those of AMPA except that APV was replaced with 4μM

6-cyano-7-nitroquinoxaline-2, 3-dione CNQX to block AMPA currents. In addition, the extracellular solution for NMDA recordings contained (in mM) 160 NaCl, 2.5 KCl, 0.2 $CaCl_2$, 10 HEPES, 10 Glucose and 0.2 EDTA.

To record sEPSC of both the NMDA and AMPA components the conditions mentioned above were maintained. However, the extracellular calcium magnesium ratio was altered to 4:1 to exploit the release probability and to rule out quantal variability of pre-synaptic glutamate.

Synaptosomal Preparation.

Slices that had been stored at -80° C were thawed for synaptosomal preparation. Synaptosomes were prepared following the methods of Johnson et al 1997. Slices were homogenized in an Eppendorf tube with modified Kreb-Henseleit (mKRBS) buffer containing 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO4, 2.5 mM CaCl2, 1.18 mM KH2PO4, 24.9 mM NaHCO3, 10 mM dextrose, 10 mg/ml adenosine deaminase and pH was adjusted to 7.4 by bubbling with 95:5 O_2:CO_2. The solution also contained 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM Benzamide. The homogenate was diluted in 400 μl of mKRBS buffer before being filtered through a 13 mm diameter millipore syringe filter holder. The filtrate was forced through a nylon filter (100 μm pore size). The pre-filtered mixture was loaded into another 1 ml tuberculin syringe and forced through a 5 μm Millipore syringe filter (Millex SV) pre-wetted with

mKRBS buffer. The filtrate was then spun at 1000 x g for 15 min in a microfuge at 4° C. The supernatant was discarded and pellets (synaptosomes) were resuspended in mKREBS buffer for electrophysiology.

Single channel recordings in synaptosomes

Isolated synaptosomes from lesioned and unlesioned animals were incorporated into an artificial phospholipids bilayer for electrophysiological recordings. Patch pipettes (resistance of 100MΩ) were pulled using borosilicate glass capillaries and filled with intracellular solution containing: 110mM KCl, 4mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS). The extracellular solution consisted of: 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris HCl. The successful bilayer formation was performed by tip-dip method using 1,2-diphytanoyl-sn-glycero-3-phosphocholine (PC), (Avanti Polar-Lipids Inc., Alabaster, AL) which was dissolved in anhydrous hexane (Aldrich Chemical Co., Milwaukee, WI). Approximately 5-7 µL of PC was added to a microbeaker containing 300 µL of extracellular solution. Successful formation of the bilayer was confirmed by measuring the resistance using axopatch 200B (Axon Instruments, Union City, CA) and pclamp 9 software. Following the addition of 5 µL of synaptosomes, protein-lipid interaction allowed synaptosomal fragments to fuse with the bilayer. Recordings were made using axopatch 200B amplifier at different

voltages applied through an Ag-AgCl reference electrode, which was placed directly into the extracellular solution. Single channel events are recorded on a video tape for further computer analysis using pclamp 9 software. Recorded signals were filtered at 5 kHz and digitized between 5-25 kHz. AMPA currents were activated by 300nM of AMPA chemical (Tocris, Ellisville, MO) to the *cis* side of the bilayer in the presence of 50μM APV (NMDA antagonist), 1μM SYM2081 (Kainate antagonist), 100μM Picrotoxin (GABA antagonist), 2μM TEA (potassium antagonist), and 1μM TTX (sodium antagonist). At the end of each recordings, AMPA currents were confirmed by the addition of 1μM CNQX. NMDA currents were activated by 3μM of NMDA chemical in the presence of 1μM SYM2206 (AMPA antagonist), 1μM SYM2081 (Kainate antagonist), 100μM Picrotoxin (GABA antagonist), 2μM TEA (potassium antagonist), and 1μM TTX (sodium antagonist). NMDA currents were confirmed by the addition of μM of APV.

Immunobloting.

Hippocampal culture slices from controls and each treatment group were collected and homogenized in 50 mM Tris, pH 7.5; 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 60 mM octyl-glucoside, and protease inhibitors (Roche;). Protein content was determined using the *DC* Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of each homogenate (30 μg) were diluted

with equal amounts of sample buffer containing 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue. Samples were boiled for 5 min, and then subjected to SDS-PAGE according to the method of Laemmli (Laemmli, 1970) using 4-12% polyacrylamide gradient gels (Bio-Rad). Proteins were electrophoretically transferred onto nitrocellulose membranes as described by Towbin et al. (Towbin et al. 1979). After transfer, nitrocellulose membranes were blocked in 5% non-fat dry milk (NFDM) in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween-20 for 1 hour at room temperature. Primary antibody incubations were carried out in 1% NFDM in TBS plus 0.1% Tween-20 overnight at 4° C. The antibodies used were GluR1 (1:1000), capthesin D (1:200), tau-1 (1:500), and actin (1:5000). After overnight incubation, membranes were washed with 1% NFDM in TBS plus 0.1% Tween-20. Membranes were then incubated with either anti-mouse or anti-rabbit IgG (1:2000 – 1:10,000) for 1 hour at room temperature. After washing 3X in 1% NFDM in TBS plus 0.1% Tween 20 (10 min. each), the blots were developed via enhanced chemiluminescene using ECL Plus (Amersham Biosciences).

Results

Immunohistochemistry

To determine the efficacy of the lesions, immunohistochemical experiments were performed in slices from positive and negative controls and in 192 IgG-saporin–

treated rats. Loss of ChaT-positive fibers was observed in medial septum region of 192 IgG-saporin–treated rats.

Western Blot.

Investigation into the synaptic protein immunoreactivity indicates that the level of GluR1 remain unchanged in the lesioned animals compared to control. The levels of lysosomal enzyme cathepsin D also remain unchanged (Fig. 5.1).

Effects of cholinergic denervation on AMPA mediated sEPSC and mEPSC

AMPA mediated sEPSCs were recorded in the presence of APV (40μM) and in the absence of TTX to allow the action potential-driven presynaptic stimulation. The ratio of calcium to magnesium was increased to ensure maximum release of glutamate from the vesicles. For the positive and negative controls used in these experiments, the data indicate that there was no statistically significant difference in the amplitude and the frequency of the AMPA sEPSCs ($P>0.05$, $n= 6$). Results indicate that the amplitude of the AMPA mediated spontaneous currents in hippocampal slices from the injected and non-injected control animals was 45.6 ± 4.2 pA and 44.1 ± 5.7 pA, respectively, whereas their frequencies was 8.2 ± 2.5 Hz and 7.8 ± 2.9 Hz respectively (Fig. 5.2). On the other hand in the 192 IgG-saporin treated animals we observed a significant reduction in the amplitude and frequency of AMPA mediated spontaneous currents. The average amplitudes

and frequencies of AMPA mediated sEPSC in hippocampal slices of 192 IgG-saporin treated animals was 24.6 ± 3.7 pA and 2.3 ± 0.8 Hz respectively.

Although sEPSC recordings provide a more physiological nature of synaptic transmission, combining this technique with the mEPSC recordings provide a valuable tool to study synaptic communication. The mEPSC recordings enable visualization of synaptic current events in the absence of presynaptic stimulation that enhances the vesicular release. We therefore studied the AMPA mediated mEPSC in CA1 of the hippocampus region in controls and cholinergic lesioned animals. Results indicate that both the frequency and amplitude of AMPA mediated mEPSC are significantly reduced in slices from animals subjected to cholinergic denervation (Fig. 5.2). The mean amplitude of the AMPA mediated mEPSC for the control injected and control non-injected was 18.7 ± 3.2 pA and 18.4 ± 3.6 pA, respectively, whereas the frequency was 3.6 ± 1.2 Hz and 3.2±1.4Hz, respectively. Thus there was no significant difference between the positive and the negative controls ($P > 0.05$, $n = 6$). In 192 IgG-saporin treated animals, the amplitude and the frequency of the AMPA mediated mEPSC were significantly altered (8.5 ± 2.6pA and0.6 ± 0.3 Hz respectively). The complete block of these mEPSCs by CNQX application (10 μM) indicated that only the AMPA receptors were recorded under previous condition ($n = 5$, not illustrated)

Effects of cholinergic denervation on NMDA mediated sEPSC and mEPSC

We investigated the effect of 192 IgG-saporin induced cholinergic denervation on the functional properties of NMDA receptors. The recordings were performed in the presence of AMPA and Kainate antagonist and in the absence of TTX. The extracellular solution contained a low concentration of magnesium ions. Our results indicate that the NMDA component of sEPSC was significantly reduced (Fig. 5.4). The average amplitudes in the positive and the negative control were 22.3 ± 2.6 pA and 21.5 ± 1.9 pA respectively. The frequency of the NMDA mediated spontaneous events was 7.3 ± 2.7 Hz and 7.1 ± 2.4 Hz in hippocampal slices of injected and non-injected animals, respectively. Hence, there was no significant difference between the positive and the negative controls. In hippocampal slices from 192 IgG-saporin treated animals the data show a statistically significant decrease in both the frequency and the amplitude of NMDA mediated sEPSCs. The average amplitude and frequencies in this case was 11.3 ± 2.6 pA and 2.8 ± 0.9 Hz, respectively.

Investigation into the NMDA mediated mEPSCs performed in the presence of TTX indicates a reduction in the average amplitude and frequency (fig. 5.4B). There was no significant difference in the amplitude and frequency of NMDA mediated miniature currents in hippocampal slices from injected and non-injected animals. The average mEPSC amplitude in hippocampal slices of the injected and non-injected animals was 12.4 ± 1.7 pA and 11.8 ± 1.3 pA,

whereas the frequencies were 2.3 ± 1.1 Hz and 2.1 ± 1.3 Hz. The mEPSCs of the NMDA component recorded in hippocampal slices from 192 IgG-saporin treated animals showed statistically significant changes in the amplitude and frequency. The average current amplitude and the frequency of the mEPSC synaptic events were 5.8±1.6pA and 0.19±0.2Hz respectively.

Effects of Cholinergic Denervation on Single Channel Properties of AMPA and NMDA Receptors in Isolated Synaptosomes.

These experiments were designed to determine whether the single channel properties of synaptic AMPA and NMDA receptors were altered following the cholinergic denervation. Synaptosomes were isolated from the hippocampus region of the control and lesioned animals. Recordings from reconstituted synaptosomes in lipid bilayer reveal an alteration in the single channel properties of AMPA and NMDA receptors. For AMPA elicited currents, recordings done in the presence of sodium, NMDA, GABA and potassium blocker, the dwell time and the probability of channel opening (Po) show a significant reduction. Thus, for the negative and the positive control, which showed no significant difference, the AMPA dwell times for Tau 1 and Tau 2 were 2.425 ± 0.206 ms, 6.567 ± 0.177 ms and 1.693 ± 0.213 ms, 6.687± 0.684 ms respectively (Fig. 5.3). The Po values for the AMPA elicited currents in the positive and negative controls were 0.22 ± 0.02 and 0.20±0.03 respectively. On the other hand AMPA single channel recordings

from synaptosomes isolated from 192 IgG-saporin lesioned animals recorded a decreased dwell time of the open level as well as the Po. The values corresponding to these changes were 0.06±0.03 and 0.257±0.411 ms, 2.434±0.865 ms for Po and dwell time (tau 1 & 2), respectively. Another remarkable difference between lesioned and nonlesioned animals was alteration in the bursting activity of AMPA elicited currents (Table 5.1). The number of bursts was decreased from 84 ± 9 in control to 6 ± 2 in lesioned animals whereas the interburst duration increased from 0.373 ± 0.226 ms in controls to 19.796 ± 3.544 ms in lesioned animals. For all AMPA elicited currents, the responses were completely blocked by addition of 1 μM CNQX.

To record and isolate NMDA currents in synaptosomes incorporated into lipid bilayer, recordings were performed in the presence of glycine, NMDA agonist and blockers for AMPA, Kainate, sodium, potassium and GABA. The NMDA elicited currents show significant changes in the dwell time (fitted with two exponential fittings) and Po values (fitted using Gaussian function). Thus, for the positive and negative controls the reported dwell times for tau 1 and tau 2 of the open level were 2.7526 ± 0.3079 ms, 8.7120 ± 2.0642 ms and 2.5815 ± 0.7291 ms, 8.1608 ± 2.4631 ms, respectively, whereas their Po values were 0.23 ± 0.03 and 0.22 ± 0.02, respectively (Fig. 5.5). Hence, these results clearly demonstrate that there was no significant difference in either the dwell times or the Po values between the negative and the positive controls. On the other hand, the dwell

times and Po values for the NMDA elicited current in synaptosomes from 192 IgG-treated animals were significantly reduced ($P<0.05$). The average dwell times (tau 1, 2) and the Po values reported in this group were 0.7152 ± 0.1821 ms, 3.8207 ± 1.0854 ms and 0.09 ± 0.02, respectively. The bursting activity of NMDA elicited current was also significantly reduced. The number of bursts were drastically reduced from 123 ± 11 in control to 12 ± 6 in lesioned animals while the interburst duration was prolonged from 0.297 ± 0.311 ms in control to 14.872 ± 3.276 ms in treated animals (Table 5.1). The observed NMDA elicited currents were completely blocked by addition of APV (data not shown).

Discussion.

The current study investigated the functional properties of glutamate receptors following immunolesioning of the septohippocampal cholinergic neurons. Specifically, the electrophysiological properties of AMPA and NMDA subtype of glutamate receptors were studied in both whole cell recordings in slices and single channel recordings from isolated synaptosomes. Recordings were performed in hippocampal CA1 region 4 to 6 days after medial septal lesioning with 192 IgG-saporin. This time point was selected since at this stage the cholinergic neurons show significant neurodegeneration and it is a time point prior to the onset of compensatory mechanisms of degeneration. This study was also designed to elucidate whether medial septal cholinergic denervation is

accompanied by lysosomal dysfunction. We have previously shown that lysosomal dysfunction leads to alteration in glutamate receptor function (Kanju et al., 2002). Hence, the current study investigated the link between cholinergic denervation, lysosomal dysfunction and glutamate receptor function.

We report here that 192 IgG-saporin injections into the medial septum resulted in alteration of AMPA and NMDA receptor function in the hippocampal region. We observed a reduction in both the amplitude and frequency of AMPA and NMDA receptor mediated mEPSCs and sEPSCs. These recordings which were performed 4-6 days after surgery indicate that both the amplitudes and frequencies of AMPA and NMDA receptor currents were drastically reduced after medial septal lesioning. The reduction of sEPSC and mEPSC frequency likely arise from a reduced number of functional synapses or a decrease in the release probability from presynaptic glutamate vesicles. However, the analysis of the amplitude ratio of sEPSC and mEPSC, which reflects changes in number of functional synapses (Hsia et al., 1998), indicate that this ratio was decreased in immunolesioned animals implying a reduction in the number of functional synapses. Although we observed a decrease in the functional synapses, the contribution of this decrease and that of release probability in decreasing the frequency of sEPSC and mEPSC of the AMPA and NMDA components could not be established. Nevertheless, our findings suggest that the septohippocampal cholinergic pathway plays an important role in maintaining the functional

properties of AMPA and NMDA receptors. Our results are supported by earlier findings of activity dependent delivery of GluR1 into the synapse (Ehrlich and Malinow, 2004). This implies that in the absence of cholinergic inputs into the hippocampus the neuronal activity in this region is compromised leading to less delivery of GluR1 into the synapse. The impact of such a deficit will be in part indicated by the reduction in the current amplitudes of the sEPSCs and mEPSCs.

Although extra-synaptic glutamate receptors play an important role in glutamatergic transmission, the majority of synaptic communications depend heavily on synaptic glutamate receptors. Our whole cell recordings in hippocampal slices could not determine whether the observed reduction in AMPA and NMDA receptor function was due to changes in the extra-synaptic or the synaptic receptors. Therefore, to determine the magnitude of reduction of synaptic AMPA and NMDA activity following the ablation of cholinergic septohippocampal projections, we isolated synaptosomes that were incorporated into lipid bilayer for single channel recordings. For the AMPA receptors, the data indicate both the mean current amplitude and the probability of channel opening were drastically reduced. In addition, the dwell time of the open state was decreased significantly in synaptosomes isolated from lesioned animals. The results also indicate that the bursting activity of the AMPA receptor was reduced in 192-IgG-saporin treated animals compared to that observed in controls. For the NMDA receptors, both the amplitude and the probability of channel opening

were significantly reduced. Similarly, the dwell time for open state was reduced in synaptosomes from lesioned animals as compared to control. The data from single channel and whole cell recording were utilized to draw certain conclusions. First, alteration in single channel properties may be responsible for the observed changes in current amplitude observed in slice recordings. This is because the percentile reduction of the expected amplitude calculated from the product of the current amplitude and Po in single channel recordings corresponds to the amplitude in whole cell recordings. Second, since synaptosomes are predominantly composed of synaptic receptors, the reduction in single channel properties of AMPA and NMDA receptors imply that the alteration in the amplitude of sEPSC and mEPSC is mainly due to changes in the synaptic receptors. In general, the single channel recordings of AMPA and NMDA in synaptosomes complement the data from the whole cell recordings in slices.

The alteration of synaptic currents may be due to changes in the expression of certain synaptic proteins. The receptor population in question may be decreased resulting in a decrease of sEPSC and mEPSC. Our data did not show any significant changes in immunoreactivity levels of GluR1 subunits between controls and lesioned animals. These observations suggest that changes in receptor population could not have accounted for the decrease in sEPSCs and mEPSCs. Thus, it is possible that the changes in sEPSC and mEPSC were a result

of single channel properties as shown by single channel recordings in synaptosomes. Western blot studies on cathepsin D levels as a marker for lysosomal dysfunction did not show any significant change between the controls and the 192 IgG-saporin treated animals. Although lysosomal dysfunction may vary in culture slices compared to *in vivo*, hence accounting for the inconsistency, the current study suggests that there may not have been a direct effect on glutamate receptors. However, lysosomal dysfunction may be directly or indirectly responsible for the altered glutamate receptor function observed previously. Our current findings as well as previous studies on slice cultures suggest that the role of lysosomal dysfunction in cognitive decline observed in AD patients is rather complicated and may involve an indirect mechanism of glutamate receptor dysfunction. Indeed, the indirect role of lysosomal dysfunction may be via synaptic proteins that regulate the functional properties of synaptic glutamate receptors.

The decrease in the AMPA and NMDA receptor function may be due to changes in inhibitory currents of GABAergic neurons. However, addition of GABA blockers to the extracellular solution in both control and treated animals eliminated this possibility. Besides the septohippocampal cholinergic innervations are known to control GABAergic interneurons of the CA1 field (Pitler and Alger, 1992). Studies show that the cholinergic control of the GABAergic interneurons disappears in 192 IgG-saporin–treated rats (Jouvenceau

et al., 1994). However, further studies show that the GABAergic inhibitory synaptic events are unchanged in 192 IgG-saporin treated animals compared with controls (Jouvenceau et al., 1997). Hence, the inhibitory currents from GABAergic neurons can not be held responsible for changes in AMPA and NMDA receptor function. Another possible reason for the decrease in glutamate receptor function was changes in receptor responsiveness to glutamate. However, addition of low and high concentrations of AMPA or NMDA chemicals in bilayer reconstituted synaptosomes did not result in any statistically significant change.

Put together, these findings are consistent with previous studies indicating that septohippocampal cholinergic projections play a significant role in hippocampal functions including learning and memory (Stewart and Fox, 1990; Givens and Olton, 1995; Markowska et al., 1995). However, our results are not in agreement with other investigators who showed that selective septohippocampal lesioning fails to cause impairment in a number of behavioral tasks (Baxter et al. 1995, 1996; Baxter and Gallagher 1996, McMahan et al. 1997; Chappell et al. 1998; Kirby and Rawlins 2003; Frick et al. 2004). This poses the question as to whether septohippocampal acetylcholine is necessary for or involved in learning and memory (see review by Parent and Baxter 2004). Although it cannot be proven from the current data that the septohippocampal cholinergic pathway may play a significant role in learning and memory, our

data demonstrate that the septohippocampal cholinergic pathway does play a role in maintaining glutamate receptor function, and thus we propose that the septohippocampal cholinergic pathway may play a significant role in learning and memory. However, this does not disapprove earlier findings that selective ablation of cholinergic septohippocampal projections is largely without effect on hippocampal-dependent learning and memory on certain behavioral tasks. Rather we suggest that in the absence of other compensatory mechanisms learning and memory of such tasks may be impaired.

There are two possible reasons why our results did not agree with those of other investigators. First, the time points selected in the current study were chosen to eliminate the possibility of development of compensatory mechanisms. Such compensatory mechanisms may explain why there was no change in the behavioral paradigm as observed by others. Indeed, this is in agreement with the findings that showed no significant change in NMDA binding seven days after medial septal immunolesioning (Rossner et al 1995). Second, the degree of lesioning may vary depending on the dosage given, and the dose administered may not have produced a sufficient lesion to produce a behavioral effect. Also, the septohippocampal cholinergic afferents may be important in a subset of cognitive processes but have a limited role in influencing other behavioral tasks. Data from the current study indicate that at the molecular level the activity of glutamate receptors, which is required for learning and memory, is

compromised following septohippocampal cholinergic lesioning. Whether this change in glutamate function causes a change in cognitive function cannot be determined from our current study.

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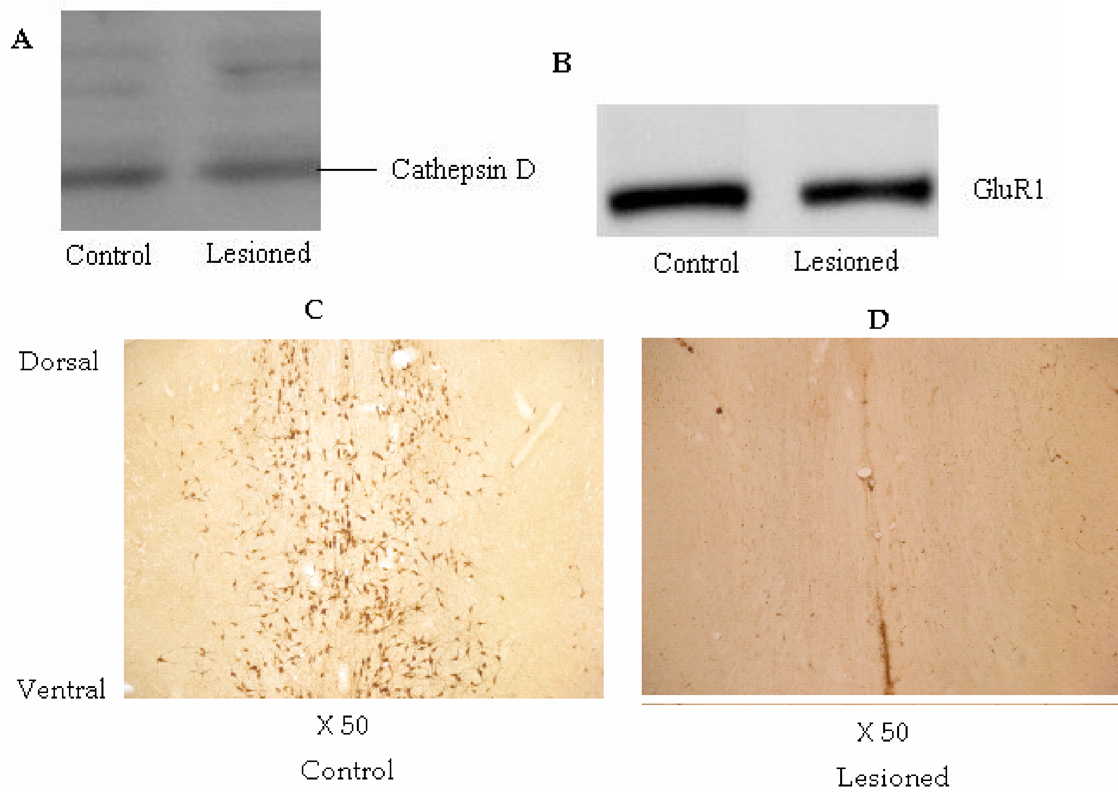

5.1. Synaptic proteins and lysosomal enzymes are unchanged by cholinergic lesioning. A, the levels of cathepsin D remained stable in lesioned animals compared to control. B, GluR1 subunits were conserved in both control and lesioned animals. D show loss of Choline acetyl transeferase positive fibres compared to control D

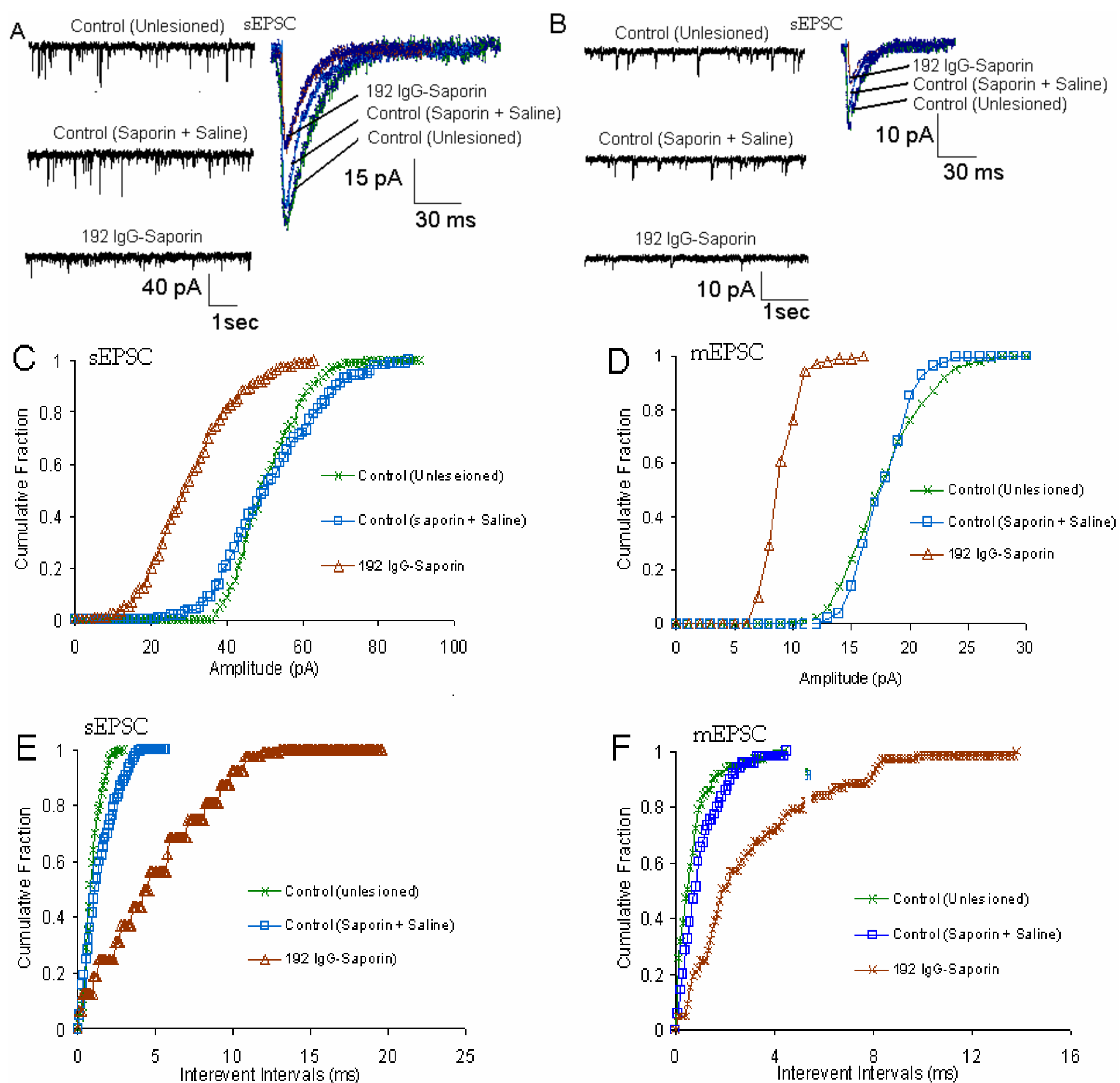

Fig. 5.2. Inhibition of septohippocampal cholinergic pathway decreases the AMPA receptor activity in the hippocampal slices. A, representatives of AMPA sEPSC traces (10 sec long) in controls and 192 IgG-saporin recorded at -65mV membrane potential, in the presence of APV but in the absence of TTX. The adjacent average traces depict the changes in the amplitude in medial septal lesioned animals compared to controls. Notice the insignificant difference between the uninjected and injected controls B, representative traces of AMPA mediated mEPSC in control and 192 IgG-saporin lesioned animals recorded at -65mV membrane potential, in the presence of APV, picrotoxin and TTX. The adjacent average traces show the changes in AMPA mEPSCs amplitude in control and lesioned animals. The cumulative fractions of AMPA sEPSC and mEPSC are shown in C and D respectively. E and F show the cumulative fraction of inter-event intervals of AMPA mediated sEPSCs and mEPSCs repectively.

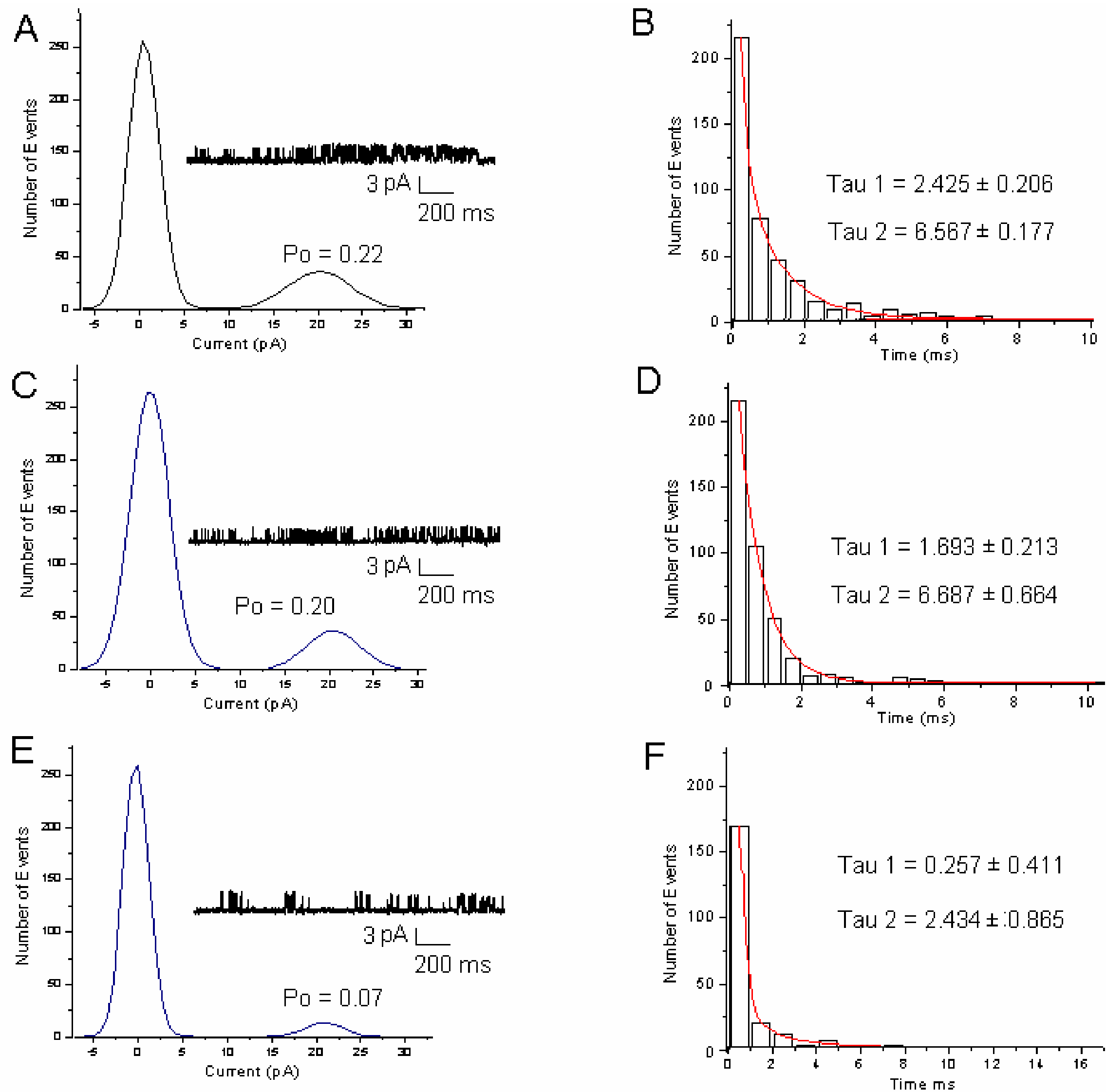

Fig.5.3. Ablation of septohippocampal cholinergic pathway alters the single channel properties of AMPA receptor in hippocampal isolated synaptosomes. The open probability of AMPA single channels in synaptosomes that were incorporated into lipid bilayer are shown in A, C and E for the control (unlesioned), control (saporin + saline) and 192 IgG-saporin treated animals, respectively. The insets show representative current traces of AMPA single channels. The adjoining histograms B, D and F depict the dwell times of the open levels fitted by two exponential fittings for the control (unlesioned), control (saporin + saline) and 192 IgG-saporin treated animals, respectively. All recordings were elicited by 300 nMAMPA chemical and maintained at a holding potential of 100 mV in the presence of GABA, sodium, potassium, and NMDA blockers. The AMPA elicited currents were completely blocked by the addition CNQX (data not shown)

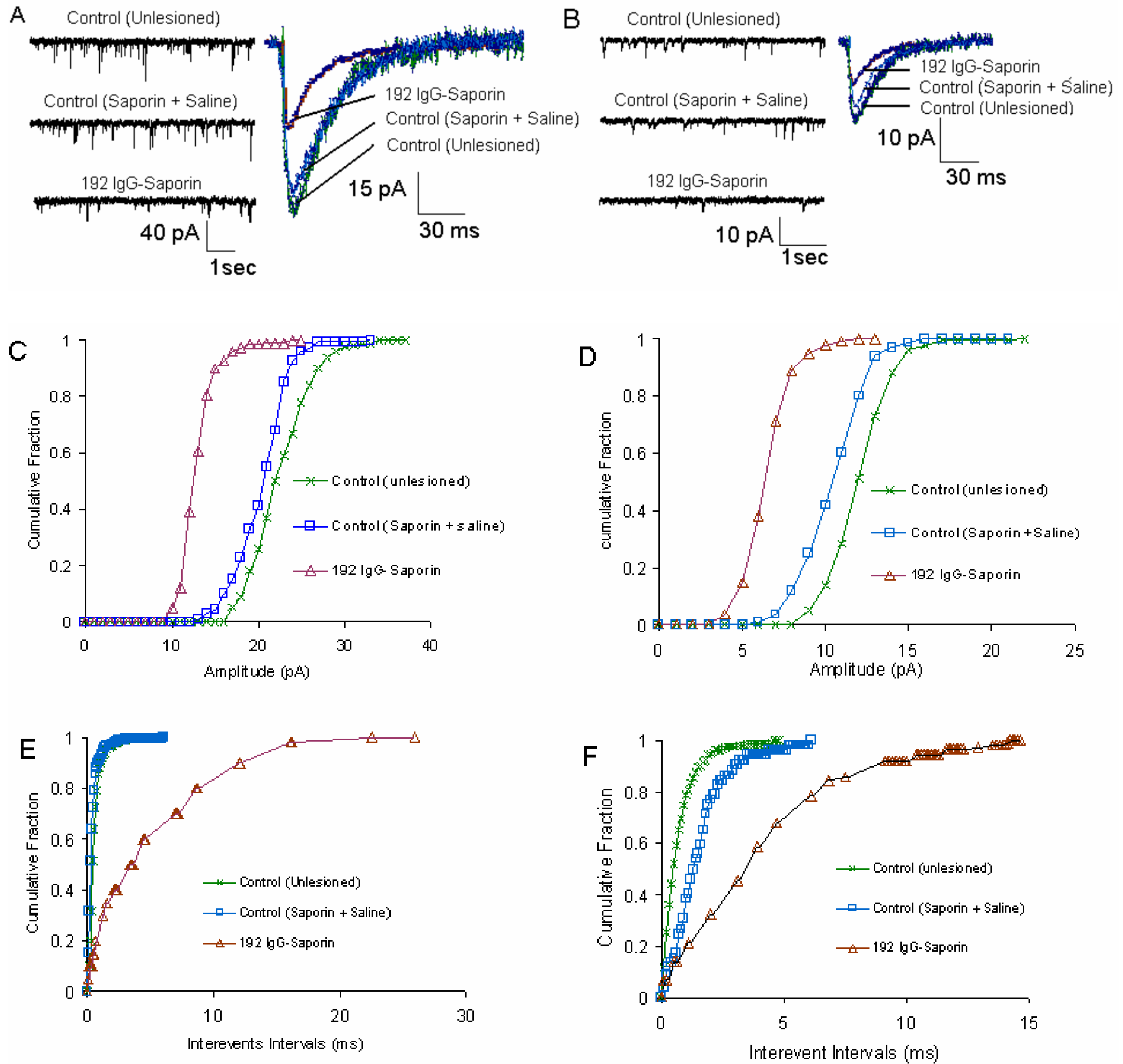

Fig. 5.4. Inhibition of septohippocampal cholinergic pathway decreases the NMDA receptor mediated responses in the hippocampal slices. A, representatives of NMDA mediated sEPSC traces (10 sec long) in positive and negative controls and 192 IgG-saporin recorded at -40mV membrane potential, in the presence of CNQX, but in the absence of TTX. The adjoining average traces shows the average changes in the amplitude in medial septal lesioned animals compared to controls. There was no significant difference between the non-injected and injected controls (P<0.005, n = 12). B, representative traces of NMDA mediated mEPSCs in hippocampal slices of control and lesioned animals. The recordings were performed in the same condition as in sEPSC recording but in the presence of TTX to exclude action potential responses. The adjacent traces represent the average amplitude of NMDA mediated mESPC in control and lesioned animals. Notice the insignificant differences between the positive and negative controls. The cumulative fractions of amplitude for the NMDA mediated sEPSCs and mEPSCs are shown in C and D, respectively. E and F indicate the cumulative fractions of the interevents intervals of NMDA mediated sEPSC and mEPSC, respectively.

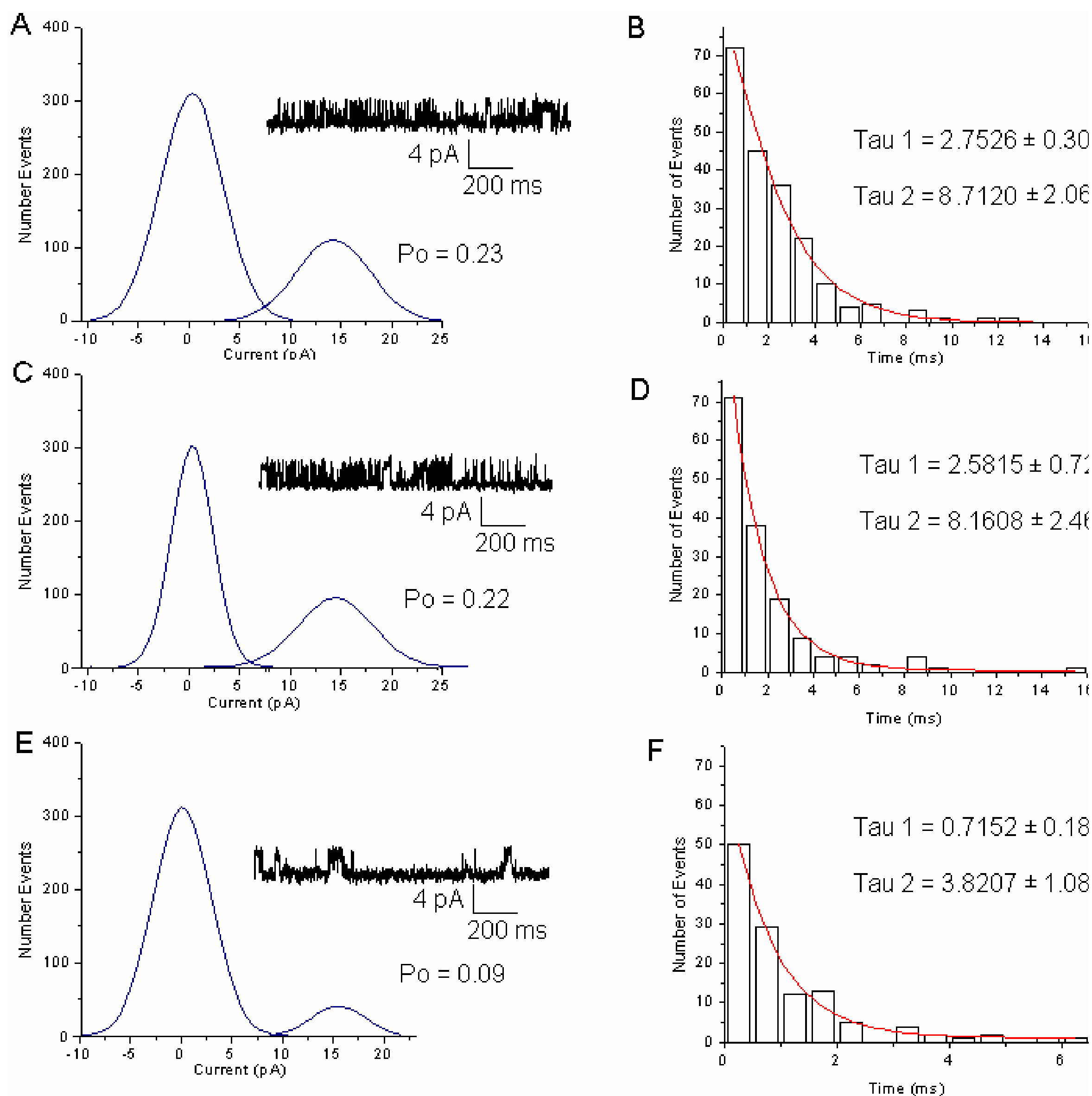

Fig. 5.5. Cholinergic denervation leads to alteration in NMDA elicited currents in isolated synaptosomes. Synaptosomes were isolated from the hippocampal region of the lesioned and controls and then reconstituted into lipid bilayer for single channel recordings of NMDA elicited currents. The open probability of NMDA elicited currents in synaptosomes are shown in the amplitude histograms A, C and E for Control unlesioned, control injected (Saporin + Saline) and 192 IgG-saporin treated animals. The respective insets show representative traces of NMDA single channels in synaptosomes isolated from lesioned and unlesioned animals. The adjoining histograms B, D and F represent the dwell time of the open level fitted by two exponential fittings for the NMDA currents in synaptosomes from unlesioned controls, injected controls and 192 IgG-saporin treated animals. All recordings were performed at a holding potential of 100 mV in the presence of AMPA, Kainate, GABA, Sodium and potassium blockers. The NMDA elicited currents were confirmed by addition of APV to the extracellular solution to completely block these currents (Data not shown)

Table 1. Burst Analysis of AMPA and NMDA Single Channels in synaptosomes

	AMPA			NMDA		
	Injected Control	Non-Injected control	192 IgG-Saporin	Injected Control	Non-Injected control	192 IgG-Saporin
Number of Burst	84 ± 9	91 ± 5	6 ± 2	123 ± 11	132 ± 15	12 ± 6
Mean Interburst duration ms	0.373 ± 0.226	0.413 ± 0.241	19.796 ± 3.544	0.297± 0.216	0.247± 0.127	14.872± 3.276

SUMMARY AND CONCLUSION

The current study utilized a slice and an animal model of Alzheimer's disease to investigate whether alterations in glutamate receptor function, namely AMPA and NMDA, occur at an early stage prior to overt signs of neurodegeneration. Previous studies have shown that neurodegenerative disorders including Alzheimer's disease exhibit lysosomal dysfunction and this might be an early event that occurs well before the symptoms of cognitive decline (Cataldo et al., 1990a; 1990b; 1996a; 1996b). This study was also designed to establish the link between lysosomal dysfunction and the modulation of functional properties of glutamate receptors in neurodegenerative conditions.

To study the progressive changes occurring prior to overt signs of neurodegeneration requires an animal model that not only mimics the disease state in question, but also one that addresses the problem at hand. Previous studies have utilized hippocampal slice cultures exhibiting experimentally induced lysosomal dysfunction as a model for AD (Bahr et al., 1994, 1995). These organotypic slice cultures differentiate and grow connections that mimic those of adult brain. In addition, following induction of lysosomal dysfunction these slice cultures develop feature characteristics of the AD brain with regards to accumulation of amyloid beta and hyperphosphorylation of tau proteins (Bahr et

al., 1994, 1995). Indeed, the accumulation of amyloid beta and tau proteins are the major hallmarks of AD. Therefore, the present study utilized the slice model to study the progressive modifications in glutamatergic function in AD.

Hippocampal slice cultures treated with the lysosomotropic agent chloroquine for 3 days exhibited an accelerated increase in cathepsin D enzyme, an indicator of lysosomal disruption. Electrophysiological recordings of AMPA and NMDA mediated currents in hippocampal slices show a reduction in the receptor activity following lysosomal dysfunction. The reduction of single channel activity in isolated synaptosomes complemented the whole cell data obtained from slice experiments. The current study also investigated whether the observed reduction in the AMPA receptor function can be reversed by the addition of drugs that positively modulate AMPA receptors. CX516 which is one of the AMPA modulators, also known as ampakines, was used in this study. The results indicate that CX516 was capable of compensating for the lost AMPA receptor activity following lysosomal dysfunction on slices treated with chloroquine for up to nine days.

In addition to the *in vitro* model in slice cultures, the current study also utilized an animal model to characterize glutamate receptor function in. cholinergic denervated rats, a known animal model of AD. Previous studies have either shown an increase or no change in glutamate receptor binding following selective septohippocampal cholinergic lesioning. These studies suggested a

possible compensatory mechanism that may result in an increase in glutamate receptor binding. The in vivo study was therefore designed to elucidate the AMPA and NMDA receptor function prior to any compensatory mechanisms. Hence, the aim of this study was to investigate the functional properties of AMPA and NMDA receptors 4 to 6 days after selective lesioning of the medial septum. In addition, this study investigated whether cholinergic denervation is also accompanied by lysosomal dysfunction. Our data indicated altered AMPA and NMDA function four days after cholinergic denervation and it was not accompanied by lysosomal dysfunction. This data suggested that although lysosomal dysfunction may occur in an early neurodegenerative process, it may not be the only mechanism responsible for the modified glutamate receptor function.

Both the *in vivo* and *in vitro* models of AD used in the current study indicated that glutamate receptor function is significantly altered at an early stage well before overt signs of neurodegeneration. In addition, our findings indicate that this reduction may either be accompanied by lysosomal dysfunction as observed in slice culture studies or may be completely independent as noted in animal studies. The findings from the hippocampal slice cultures indicate that the accelerated accumulation of cathepsin D in the third day was sufficient to cause a dysfunction of glutamate receptors. Such dysfunction was not significant on the third day but became more pronounced after six days of chloroquine

treatment. These findings suggest that lysosomal dysfunction is one of the steps in a cascade of events that leads to glutamate receptor dysfunction. Such a cascade may include but is not limited to phosphorylation events of receptors which are important for normal receptor function. Another possibility of this cascade is the recycling events of glutamate receptor in the synaptic sites. The detailed mechanism(s) of glutamate receptor dysfunction following lysosomal disturbances is still not clear and may be a subject of further studies. Although our findings did not establish the mechanism of glutamate receptor dysfunction, our work supports the earlier findings that lysosomal dysfunction may perhaps be among the early markers of cognitive decline in AD. Lysosomal dysfunction may not play a direct role in glutamate receptor modulation. However, a synergetic effect of lysosomal disruption and glutamate receptor dysfunction leading to cognitive decline can not be ruled out. Thus, restoring or enhancing lysosomal function should be considered in the future as a possible therapeutic target for treating AD.

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