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# EFFECTS OF MEMANTINE ON SOLUBLE $A\beta_{25-35}$-INDUCED CHANGES IN PEPTIDERGIC AND GLIAL CELLS IN ALZHEIMER'S DISEASE MODEL RAT BRAIN REGIONS

**M. ARIF,[a,b]* T. CHIKUMA,[c] Md. M. AHMED,[a] M. NAKAZATO,[d] M. A. SMITH[e] AND T. KATO[a]**

[a]*Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236–0027, Japan*

[b]*Department of Biochemistry, Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh*

[c]*Department of Analytical Chemistry, Showa Pharmaceutical University, Machida 194–8543, Japan*

[d]*Department of Internal Medicine, Miyazaki Medical College, Miyazaki 889–1692, Japan*

[e]*Department of Pathology, Case Western Reserve University, Cleveland, OH, USA*

**Abstract—Soluble forms of amyloid-$\beta$ (A$\beta$) have been considered responsible for cognitive dysfunction prior to senile plaque formation in Alzheimer's disease (AD). As its mechanism is not well understood, we examined the effects of repeated i.c.v. infusion of soluble $A\beta_{25-35}$ on peptidergic system and glial cells in the pathogenesis of AD. The present study aims to investigate the protective effects of memantine on $A\beta_{25-35}$-induced changes in peptidergic and glial systems. Infusion of $A\beta_{25-35}$ decreased the level of immunoreactive somatostatin (SS) and substance P (SP) in the hippocampus prior to neuronal loss or caspase activation, which is correlated with the loss of spine density and activation of inducible nitric-oxide synthase (iNOS). Biochemical experiment with peptide-degrading enzymes, prolyl oligopeptidase (POP) and endopeptidase 24.15 (EP 24.15) activities demonstrated a concomitant increase with the activation of glial marker proteins, glial fibrillary acidic protein (GFAP) and CD11b in the A$\beta$-treated hippocampus. Double immunostaining experiments of EP 24.15 and GFAP/CD11b antibodies clearly demonstrated the co-localization of neuro peptidases with astrocytes and microglia. Treatment with memantine, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist significantly attenuated $A\beta_{25-35}$-induced changes of neuropeptides, their metabolizing enzymes, glial marker proteins, and activation of iNOS. Taken together, the data implies that memantine exerts its protective effects by modulating the neuropeptide system as a consequence of suppressing the glial cells and oxidative stress in AD model rat brain regions.**


*Corresponding author. Tel: +9661900-59, Ext: 7643; fax: +880-2-8615583.
E-mail address: marif@univdhaka.edu, marif567@yahoo.com (M. Arif).
*Abbreviations:* AD, Alzheimer's disease; ANOVA, analysis of variance; APP, amyloid-$\beta$ precursor protein; A$\beta$, amyloid-$\beta$; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EP 24.15, endopeptidase EC 24.15; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; iNOS, inducible nitric oxide synthase; LI, like immunoreactivity; LTP, long term potentiation; NMDA, N-methyl-D-aspartate; POP, prolyl oligopeptidase; PS1, presenilin 1; SEM, standard error of the mean; SP, substance P; SS, somatostatin.

**Key words: Alzheimer's disease, memantine, peptidergic system, dendritic spine, glial cells.**

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a progressive memory loss and cognitive decline (Selkoe, 2002). The pathological hallmarks of AD are extracellular plaques containing amyloid-$\beta$ (A$\beta$), dystrophic neurites, activated microglia, reactive astrocytes and synapse loss (Walsh and Selkoe, 2004). Genetic studies and clinical data suggest that A$\beta$ peptide plays the central pathogenic role in AD etiology (Selkoe, 2002, 2004) although this is not definitive (Castellani et al., 2006; Lee et al., 2006). Early evidence for A$\beta$-induced neurotoxicity in cell culture and *in vivo* was associated with insoluble, fibrillar forms of A$\beta$, such as those found in neuritic (amyloid) plaques (Pike et al., 1995; O'Hare et al., 1999). Studies in transgenic mice that over express the A$\beta$ precursor protein (APP) have shown the occurrence of synaptic, electrophysiological and behavioral changes before the onset of amyloid deposition (Hsia et al., 1999; Hartley et al., 1999). Moreover, relatively weak correlations between fibrillar plaque and severity of dementia are found in AD, which is exemplified by the existence of some cognitively normal individuals with high amounts of deposited A$\beta$ (Delaere et al., 1990; Dickson et al., 1995). The missing link between amyloid peptide and cognitive dysfunction appears from the compelling evidence that disruption of synaptic plasticity and memory function *in vivo* and APP transgenic mice were reversed by immunotherapy potentially clearing the soluble oligomers. Therefore, soluble oligomers rather than fibrillar plaque is possibly responsible for neuronal/synaptic dysfunction (Dodart et al., 2002; Klyubin et al., 2005) in AD brain. In support of this possibility, there are reports that synaptotoxic oligomeric A$\beta$ ultra structurally localizes to cell processes in AD brain (Kokubo et al., 2005), inhibits long-term potentiation (LTP) in rat hippocampus (Walsh et al., 2002), and shows strong correlations with the extent of memory impairment (Lue et al., 1999; McLean et al., 1999). However, the molecular basis underlying the progressive decrement of memory and cognitive functions in AD is not clear.

Numerous studies have documented that neuropeptides play an important role in the CNS as neurotransmitters, and deficiencies of the peptides have been linked to a variety of behavioral abnormalities and decreased cognitive abilities (Gottfries et al., 1995). Substantial deficits in several neuropeptides, such as somatostatin (SS), substance P (SP), and alterations of their degradating en-

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zymes, such as prolyl endopeptidase, endopeptidase 24.15 (EP 24.15), are characteristic of brains from patients with AD (Gottfries et al., 1995; Gabriel et al., 1996; Waters and Davis, 1995, 1997; Terwel et al., 1998), indicating that clinical symptoms could at least in part be attributable to changes in chemical communication processes. Since brain peptides particularly, SP and SS, have been shown to facilitate the generation of LTP in hippocampal slices (Matsuoka et al., 1991; Kato and Yoshimura, 1993; Langosch et al., 2005) and also have significant contributions in neural plasticity processes associated with learning and memory, their possible role in soluble A$\beta$-induced cognitive dysfunction remains to be clarified *in vivo*.

There is extensive evidence that A$\beta$ provokes the glial-mediated inflammatory response that contributes significantly to the cognitive decline and oxidative stress-dependent neurodegeneration (Akiyama et al., 2000; Auld and Robitaille, 2003). In addition, glial cells have important contribution to synaptic transmission (Piet et al., 2004) and hence it might have some regulatory role in peptidergic transmission as well since many of the peptide degrading enzymes are derived from astrocytes and microglia in the brain (Arif et al., 2007). On the other hand, glutamate, an excitatory neurotransmitter, leads to the over activation of N-methyl-D-aspartate (NMDA) mediated excitotoxicity in AD (Rogawaski and Wenk, 2003) and treatment with non-competitive low-to moderate affinity NMDA receptor antagonist, memantine showed protective effect in both *in vivo* and *in vitro* studies (Miguel-Hidalgo et al., 2002; Song et al., 2008). Recent MRI study showed that memantine leads to behavioral improvement and amyloid reduction in APP/PS1 (presenilin 1) transgenic mice (Scholtzova et al., 2008). We previously demonstrated that memantine recovered the changes on the levels of glial cells, neuropeptides and peptide-degrading enzymes in ibotenic acid-treated rat brain (Ahmed et al., 2004). In the present study, we assess the effect of repeated i.c.v. infusion of soluble? $A\beta_{25\text{-}35}$ and co-administration of memantine on neuropeptides and their degrading enzymes as well as their possible correlation with glial cells in rat brain regions.

## EXPERIMENTAL PROCEDURES

### Materials

Male Wistar rats (10–11 weeks) weighing 300–320 g were obtained from Sankyo Laboratory (Tokyo, Japan). Memantine, dithiothreitol, and Tris base were purchased from Sigma, Chemical Co. (Tokyo). $A\beta_{25\text{-}35}$, 7-amino-4-methylcoumarin (AMC) and 7-(succinyl–Gly–Pro)-4-methylcoumarinamide (Suc–Gly–Pro-MCA), 7-methoxycoumarin-4-acetyl-Pro–Leu–Gly–Leu-$A_2$pr(Dnp)-Ala–Arg-$NH_2$ (QFS), MCA-Pro–Leu–Gly, anti-rabbit SS were from Peptide Institute Inc. (Osaka, Japan). Ac-Asp–Glu–Val–Asp-AMC and the specific inhibitor of EP 24.16 (Pro–Ile) were purchased from Bachem Bioscience Inc. (CA, USA). Nitrocellulose membrane was from Schleicher and Schuell Bioscience (Dassel, Germany). Mouse anti-rat CD11b monoclonal antibody was from BD Bioscience Pharmingen (CA, USA). Anti-mouse glial fibrillary acidic protein (GFAP) and NeuN were purchased from Chemicon International (CA, USA), mouse anti-rat iNOS antibody was from Transduction Laboratories (Lexington, KY, USA) and rabbit anti-rat $\beta$-Tubulin antibody was obtained from Cell Signaling Technology Inc. (Tokyo, Japan). Anti-rat EP 24.15 rabbit serum (raised against the C-terminus 521–537 amino acids of EP 24.15) was prepared from female white rabbit as reported previously (Yamamoto et al., 2003). Anti-SP antibody was prepared in the same way. The specificities of both EP24.15 and SP antibody have been described previously (Ahmed et al., 2004; Arif et al., 2006). Cy2-conjugated anti-rabbit IgG and Cy3-labeled anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories Inc. (PA, USA) whereas Alexa Fluor® 488 mouse IgG was purchased from Invitrogen (OR, USA). Fluoromount G™ was purchased from Electron Microscopy Technique (PA, USA). Other chemicals were of analytical reagent grades.

### Animal surgery and drug administration

Male Wistar rats were housed under standard environmental conditions (12 h light/dark cycle, 22 °C) and allowed to free access to food and water for 2 weeks prior to the treatment. All animal experiments were carried out according to the Japanese Government Law of Animal Experiments Guidelines (1980), and were approved by the Animal Experiment Committee of Yokohama City University Graduate School of Integrated Science (YCU20409). The number of animals was the minimum required for statistical analysis and all precautions were taken to minimize animal suffering.

$A\beta_{25\text{-}35}$ was dissolved initially in autoclaved Milli-Q water and finally at a concentration of 3.5 mM with PBS solution (vehicle). After being anaesthetized with sodium pentobarbital (50 mg/kg i.p.), the rats were set in stereotaxic instrument. Guide cannula (BAS, Tokyo, Japan) was implanted into both ventricles under aseptic conditions through holes drilled in the skull at the following coordinates: anterior–posterior = −0.3, medial–lateral = +1.1, dorsal–ventral = 3.6 mm from bregma according to the atlas of Paxinos and Watson (1986). Two days following cannula implantation, 5 $\mu$l of $A\beta_{25\text{-}35}$ was injected in both ventricles sequentially over 5 min by 5 $\mu$l Hamilton syringe through cannula tubing for consecutive 3 days with light ether anesthesia. The needle was left in place for 5 min after the injection and was withdrawn gradually for 5 min. Control rats were infused with 5 $\mu$l PBS using the same procedure. At post-operative hour three and continuing for 5 days thereafter, rats were injected once a day with an i.p. injection of either memantine (5 mg/kg per day) or saline (0.9% NaCl). The dose of $A\beta_{25\text{-}35}$ as well as memantine was based on previous studies, respectively (Cullen et al., 1996; Anderson et al., 2004).

### Sample preparation

Animals were decapitated 2 days after last A$\beta$ infusion and both right and left parts of the frontal cortex, hippocampus, and striatum were collected rapidly on ice and stored at −80 °C until use. Each brain region was homogenized in nine volumes of 20 mM Tris-HCl buffer (pH 7.4) and centrifuged at 100,000×*g* for 60 min at 4 °C. The resultant supernatant was used for enzyme activities measurement and immunoblot analysis. For enzyme immunoassay (EIA), another group of animals were prepared and brain regions were homogenized in 500 $\mu$l of 2 M acetic acid solution and then centrifuged for 60 min at 100,000×*g*. The resultant supernatant was lyophilized followed by resolubilization in EIA buffer (10 mM PBS, pH 7.4, containing 0.1% bovine serum albumin (BSA), 0.05% Tween-20, and 200 kIU/ml aprotinin). The sample was again centrifuged at 15,000×*g* for 15 min at 4 °C and the supernatant was used for EIA analysis.

### EIA procedures

EIA was performed for the measurement of SS and SP followed by double antibody method (Takeda et al., 1989). In brief, the total assay volume of 0.3 ml contained 0.1 ml of the diluted primary antiserum (anti-SS, 1/1000 and anti-SP, 1/8000 in EIA buffer), 0.1 ml of enzyme-conjugated solution (SS-horseradish peroxidase