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Amyloid β peptide levels and its effects on hippocampal acetylcholine release in aged, cognitively-impaired and -unimpaired rats

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Abstract

Excessive extracellular deposition of amyloid β (Aβ) peptide in neuritic plaques and degeneration of forebrain cholinergic neurones, which innervate the hippocampus and the neocortex, are the invariant characteristic features of Alzheimer's disease (AD). Studies of the pathological changes that characterize AD, together with several other lines of evidence, indicate that Aβ accumulation in vivo may initiate and/or contribute to the process of neurodegeneration observed in the AD brain. However, the underlying mechanisms by which Aβ peptide influences/causes degeneration of the basal forebrain cholinergic neurones in AD brains remain obscure. We reported earlier that nM concentrations of Aβ-related peptides, under acute conditions, can potently inhibit K^+-evoked endogenous acetylcholine (ACh) release from the hippocampus and the cortex but not from striatum in young adult rats (J. Neurosci. 16 (1996) 1034). In the present study, to determine whether the effects of Aβ peptides alter with normal aging and/or cognitive state, we have measured $A\beta_{1-40}$ levels and the effects of exogenous $A\beta_{1-40}$ on hippocampal ACh release in young adult as well as aged cognitively-unimpaired (AU) and -impaired (AI) rats. Endogenous levels of $A\beta_{1-40}$ in the hippocampus are significantly increased in aged rats. Additionally, 10 nM $A\beta_{1-40}$ potently inhibited endogenous ACh release from the hippocampus of the three groups of rats, but the time-course of the effects clearly indicate that the cholinergic neurones of AI rats are more sensitive to Aβ peptides than either AU or young adult rats. These results, together with earlier reports, suggest that the processing of the precursor protein of Aβ peptide alters with normal aging and the response of the cholinergic neurones to the peptide possibly varies with the cognitive status of the animals.

Keywords: Aging; Alzheimer's disease; Amyloid precursor protein; Cognition; Neuromodulation

1. Introduction

Studies of the pathological changes that characterize Alzheimer's disease (AD) and several lines of other evidence indicate that amyloid β (Aβ) peptide accumulation in vivo may trigger degeneration of neurones observed in the AD brain (Seubert et al., 1992; Hardy, 1997; Price and Sisodia, 1998; Selkoe 1999). Of the vulnerable brain regions, the basal forebrain neurones which provide major cholinergic inputs to the hippocampus and the neocortex, are most severely affected in AD (Wurtman 1992; Gentleman et al., 1993; Quirion, 1993; Price and Sisodia, 1998). Given the significance of acetylcholine (ACh) in learning and memory processing, it is suggested that losses of cholinergic innervation, especially in hippocampal and cortical regions, could possibly contribute to the memory deficits associated with the disease (Bartus et al., 1982; Wurtman 1992; Price and Sisodia 1998). However, at present, neither the cause of the preferential decimation of these neurones nor their association to Aβ peptides has been clearly established.

Recently, a plethora of experimental approaches have provided evidence for a functional relationship between Aβ peptides and the cholinergic neurones of the basal forebrain area (Abe et al., 1994; Giovannelli

Abbreviations: Aβ, amyloid beta peptide; ACh, acetylcholine; AD, Alzheimer's disease; AI, Aged-impaired; AU, Aged-unimpaired; APP, amyloid precursor protein; α-APPs, soluble N-terminal APP.

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et al., 1995; Harkany et al., 1995; Kar et al., 1996; Hoshi et al., 1997; Auld et al., 1998; Hellstrom-Lindahl, 2000). For example, it is reported that lesions of basal forebrain cholinergic neurones or the transient blockade of its transmission elevate the synthesis of amyloid precursor protein (APP) in the cerebral cortex (Wallace et al., 1993; Roberson and Harrell, 1997; Lin et al., 1999). Alternatively, single injection or prolonged exposure to Aβ peptides has been shown to induce memory impairment and/or degeneration of the cholinergic neurones (Giovannelli et al., 1995; Harkany et al., 1995; Itoh et al., 1996). Moreover, we have shown that $A\beta_{1-42}$, $A\beta_{1-40}$, $A\beta_{1-28}$ and $A\beta_{25-35}$, at low nM concentrations, can potently inhibit high-affinity choline uptake and endogenous ACh release from the hippocampus and cortex, the regions which are severely affected in AD brains (Kar et al., 1996, 1998). Recently, Wang et al. (1999), apart from confirming our findings, have further indicated that Aβ-mediated inhibition of ACh release is not mediated via a G-protein coupled receptor. Collectively, these results suggest the existence of a functional interrelationships between cholinergic and Aβ-related systems in hippocampal and cortical regions. However, as most of the data have been obtained in young adult animals, it remains to be established whether the effects of Aβ peptides on cholinergic neurons alter with cognitive behavioral deficits and/or normal aging process, two features invariably associated with AD.

Although rodents do not develop AD-like pathology, a subset of the population do develop cholinergic impairments as well as cognitive behavioral deficits with age and may serve as a useful model for studying the role of Aβ related peptides in the regulation of cholinergic function in the normal aging process (Quirion et al., 1995; Anderson et al., 1999). Age-related changes in brain APP mRNA expression and protein immunoreactivity have been reported in rats (Higgins et al., 1990; Beeson et al., 1994; Sugaya et al., 1996). At present, however, it is not known whether the effects of Aβ-related peptides on cholinergic neurones are altered as a function of aging and/or cognitive status of the animals. In the present study, apart from measuring the levels of $A\beta_{1-40}$ peptide (i.e., the predominant Aβ peptide found in normal brains) in the hippocampus, the potential effects of $A\beta_{1-40}$ on hippocampal endogenous ACh release were determined in young adult and well-characterized aged-impaired and -unimpaired rats.

2. Materials and methods

2.1. Animals

Male Long-Evans rats obtained from Charles River (St Constant, Quebec, Canada) at 1 year of age were housed for an additional 12 months according to the guidelines of the McGill University and the Canadian Council for Animal Care. Young controls were obtained at 5 months old and housed for an additional 1 month before the experiments. All animals were maintained on a 12 h light-dark cycle in temperature- and humidity-controlled rooms, and given free access to food and water. The health of the animals was monitored regularly, and any animals with overt signs of chronic respiratory distress, infection or tumors were removed from the study.

2.2. Materials

$A\beta_{1-40}$ was purchased from Bachem (Torrance, CA). Biotinylated monoclonal detection antibody for $A\beta_{1-40}$ (4G8) and assay specific capture antibody R163 were obtained from Senetek (St. Louis, MO) whereas alkaline phosphatase-conjugated streptavidin was from Zymed (San Francisco, CA). [γ-^{32}P]adenosine triphosphate (ATP) was obtained from New England Nuclear (Mississauga, Ontario). ACh chloride, physostigmine sulfate, choline kinase and acetylcholinesterase type V-S were obtained from Sigma (St. Louis, MO) whereas dithiothreitol and ATP were purchased from Boehringer Mannheim (Laval, Quebec). Tetraphenylboron and butyronitrile were from Aldrich (Milwaukee, WI) and AG 1-X8 Resin was from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased either from Sigma or Fisher Scientific (Montreal, Quebec).

2.3. Behavioral screening

Aged (24–25 months old) and young (6 months old) Long–Evans rats were screened for spatial memory deficits using the established Morris Water Maze task as described in detail elsewhere (Aubert et al., 1995; Quirion et al., 1995). In brief, the animals were required to find a submerged (2 cm) platform in a pool (1.6 m diameter) of opaque water using only distal, spatial cues available in the testing room. Animals were given three trials per day over five consecutive days with the platform submerged. The cognitive status of the aged rats was defined on the basis of their performance (latency), relative to the young adult animals, in the Morris Water Maze task on days 2–5 of testing. At the end of the testing period, all animals were run on probe trials with elevated platform to ensure that impairments were not related to either visual or motor defects.

2.4. Determination of $A\beta_{1-40}$ in the hippocampus

The levels of $A\beta_{1-40}$ in the homogenates of hippocampus from young ($n = 8$), aged-unimpaired (AU; $n = 8$) and aged-impaired (AI; $n = 8$) rats were

determined using sandwich ELISAs with a biotinylated monoclonal detection antibody (4G8) as described earlier (Beffert et al., 1999). This antibody has been used previously to detect Aβ peptide in rat hippocampal neurons (Brewer, 1997). Assay-specific capture antibody R163 (2 μg/ml) which recognizes $A\beta_{1-40}$ was used to coat 96-well microtiter plates. Capture antibody was incubated overnight at 4°C in 10 mM sodium carbonate (pH 9.6). Non-specific binding sites were blocked with the addition of 0.1% bovine serum albumin for 2 h. The plates were washed, and 50 μl of either sample or standard were applied to the plates for 2 h at room temperature with gentle shaking. Following several washes, the biotinylated detection antibody was applied for 1 h. Alkaline phosphatase-conjugated streptavidin was used to detect the biotinylated antibody. The amount of enzyme bound was determined using Attophos substrate according to the manufacture's instructions. Fluorescence reader (Bio-Tek Instruments) at ex = 450 nm/20 nm and em = 560 nm/20 nm. The sensitivity of the assay was approximately 100 pg/ml.

2.5. Determination of in vitro ACh release

Hippocampal slices from young ($n = 18$), AU ($n = 18$) and AI ($n = 15$) rats were prepared and superfused as described earlier (Kar et al., 1996). Briefly, rats were decapitated, the hippocampi from brains were dissected out and sliced at 400 μm with a McIlwain tissue chopper. The tissue slices were then superfused with oxygenated Krebs buffer [(in mM) NaCl 120, KCl 4.6, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.2, D-glucose 9.9, $NaHCO_3$ 25, adjusted pH to 7.4] at a rate of 0.5 ml/min at 37°C using Brandel Superfusion apparatus (Brandel Instruments, Gaithersburg, MD). Following a 45 min stabilization period, samples were collected every 20 min for 1 h to establish the basal efflux. The tissues were then stimulated with submaximal concentration of high K^+ Krebs buffer (25 mM) for 1 h either in the presence or absence of 10^{-8} M $A\beta_{1-40}$. Due to the limited number of AI and AU rats available, a single concentration of $A\beta_{1-40}$ (i.e. 10^{-8} M) which was found to potently inhibit K^+-evoked ACh release from rat brain slices (Kar et al., 1996, 1998) was selected for the present study. At the end of the experiment, tissue slices were removed and protein content was measured (Lowry et al. 1952). The superfusates collected every 20 min all through the experiment were spun (15 000 × *g*, 5 min 4°C) and 1.5 ml of the supernatant was then processed using radioenzymatic assay as described earlier (Kar et al., 1996). To monitor the recovery, standard amounts of ACh were processed along with each experiment. Evoked transmitter release was calculated by subtracting the basal efflux from the total release and is expressed as pmol ACh/min per mg protein.

2.6. Data analysis

The data from Aβ peptide and ACh release which are presented mean ± S.E.M. were analyzed using one-way ANOVA followed by Fisher's post-hoc test with level of significance set at $P < 0.05$.

3. Results

3.1. Behavioral screening

Compared to the performance of young adult rats in the Morris Water Maze task, the aged rats were grouped into AU and AI rats. The AI group was comprised of animals whose mean latency in finding the submerged platform differed significantly ($P < 0.05$) between days 2–5 from that of the young adult and AU rats. Animals were considered AU if their average latency was not significantly altered from young controls at any point during testing (Fig. 1A). In the visually-cued trial, the latency in finding the platform was similar among young adult, AI and AU rats, indicating that altered ability of AI animals to locate the submerged platform was not due to motor deficits (Fig. 1B).

3.2. Levels of $A\beta_{1-40}$ in the hippocampus of young, AU and AI rats

To assess whether endogenous levels of $A\beta_{1-40}$ are altered with aging or cognitive performance, we use a sensitive ELISA technique to determine hippocampal $A\beta_{1-40}$ levels in young, AU and AI rats. In young adult rat hippocampus, $A\beta_{1-40}$ level was found to be 4.9 ± 0.2 ng/mg protein (Fig. 2). Compared to young rats, a significant ($P < 0.05$) increase in the levels of $A\beta_{1-40}$ was apparent in both AU (8.7 ± 0.8 ng/mg protein) and AI (7.2 ± 0.7 ng/mg protein) rats. Although, $A\beta_{1-40}$ level in the hippocampus of AU rats was higher than AI rats, it did not exhibit statistically significant difference between the two groups (Fig. 2).

3.3. Effects of $A\beta_{1-40}$ on hippocampal ACh release in young, AU and AI rats

In keeping with our earlier results (Kar et al., 1996), 10^{-8} M $A\beta_{1-40}$ significantly ($P < 0.05$) inhibited K^+-evoked endogenous ACh release from young rat hippocampal slices (Fig. 3A). The time-dependency of the effects revealed a potent inhibition of ACh release during the final 20 min of stimulation (Fig. 3A). To assess whether this response alters as a function of aging and/or cognitive status of animals, hippocampal slices from AU and AI rats were superfused under similar conditions, in either presence or the absence of

10^{-8}M $A\beta_{1-40}$. The results clearly show that $A\beta_{1-40}$ induced a potent inhibition of hippocampal ACh release from both AU as well as AI rats (Fig. 3B, C). The effects, as in young animals, were apparent primarily during the final 20 min of stimulation in AU rats (Fig. 3B), whereas in AI rats, a significant decrease ($P < 0.05$) in ACh was evident during the final 40 min of evoked release (Fig. 3C). AI rats showed a greater percentage of ACh release inhibition in presence of $A\beta_{1-40}$ peptide but this failed to reach significance (Fig. 4).

4. Discussion

The present study shows that aged rats have increased levels of $A\beta_{1-40}$ in the hippocampus and exposure of hippocampal slices to $A\beta_{1-40}$ significantly reduced K^+-evoked ACh release in young, AU and AI rats. These results, together with earlier data, indicate that processing of APP is possibly altered as a function of normal aging and Aβ peptides may play a role in the modulation of cholinergic function in young as well as aged rats.

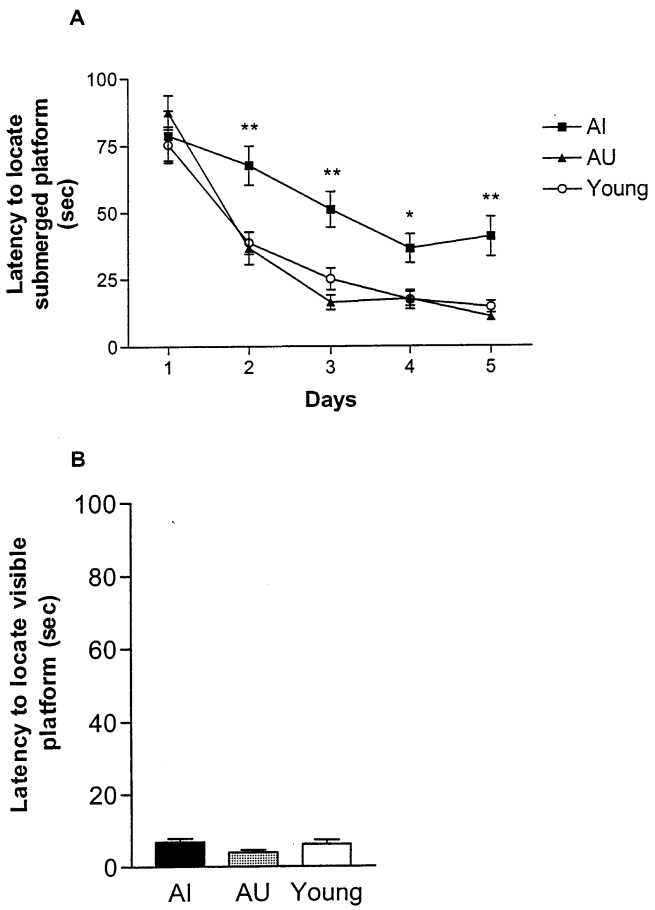

Fig. 1. The performance of young (young) and aged Long-Evans rats was evaluated using the Morris Water Maze task. Results are expressed as the mean ± S.E.M. of the latency to find the submerged (A) or visible (B) platform. A — Statistical differences ($P < 0.05$) in the latency to find the platform were observed between the aged-impaired (AI) vs aged-unimpaired (AU) and young adults from test days 2–5. B — in the visually cued condition evaluated at the end of day 5, all animal groups performed similarly.

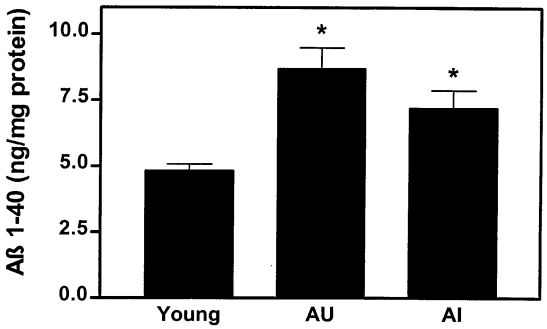

Fig. 2. Hippocampal $A\beta_{1-40}$ levels, as determined by ELISA, in young adult (Young, $n = 8$), aged-unimpaired (AU, $n = 8$) and aged-impaired rats (AI, $n = 8$). A significant increase in $A\beta_{1-40}$ levels was observed in AU and AI rats compared to young rats. The level of $A\beta_{1-40}$ in AU rats was not significantly different than AI rats. The data are expressed as mean ± S.E.M.

A variety of experimental approaches have shown that APP, the precursor of Aβ peptides, is proteolytically processed by two alternative pathways, (i) a non-amyloidogenic pathway mediated via α-secretase which precludes the formation of Aβ peptide and generates soluble N-terminal APP (α-APPs) and a membrane associated C-terminal fragment, (ii) an amyloidogenic pathway mediated via β- and γ-secretases which generates Aβ related peptides (Nitsch and Growdon, 1994; Farber et al., 1995; Price and Sisodia, 1998; Selkoe, 1999; Vassar et al., 1999). The selective processing of APP can be regulated by the activation of a variety of neurotransmitter receptors including ACh, serotonin, glutamate, vasopressin and bradykinin (Buxbaum et al., 1992; Nitsch and Growdon, 1994; Farber et al., 1995). There is also evidence that normal aging is able to modulate both the expression of APP mRNA as well as its processing. It was reported that mRNA for APP_{695}, the predominant isoform expressed in the brain, is elevated whereas its protein level is decreased in the hippocampus of aged rats (Sugaya et al., 1996). The level of α-APPs was also found to be decreased in the cerebrospinal fluid of aged rats (Anderson et al., 1999). These results together with the present data which showed a significant increase in $A\beta_{1-40}$ levels in the aged rat hippocampus suggest that normal aging increases the expression and possibly favours the amyloidogenic processing of APP in the brain.

Apart from aging, impaired cognitive status and hypofunctioning of the cholinergic system have been shown to increase expression of APP mRNA and also influence the processing of the precursor protein via the amyloidogenic pathway (Sugaya et al., 1996; Lin et al.,

1999). It was reported that stimulation of muscarinic M1 and M3 receptors elevates whereas that of M2 receptors most likely reduces α-APPs formation (Nitsch and Growdon 1994; Farber et al., 1995; Roberson and Harrell, 1997). Given the evidence that aged impaired rats exhibit relatively low levels of endogenous ACh along with a significant increase in the density of negative muscarinic M2 autoreceptors (Aubert et al., 1995; Quirion et al., 1995; Vannucchi et al., 1997), it was expected that levels of Aβ peptides should be elevated in these rats compared to age-matched unimpaired rats. Since there was no significant difference in hippocampal $A\beta_{1-40}$ levels between the two groups, it suggests that processing and/or metabolism of Aβ peptides is possibly altered differentially in aged-impaired rats. Whether this encompasses changes in the rate of secretion, enzymatic degradation and/or clearance of Aβ peptides need clarification from future studies.

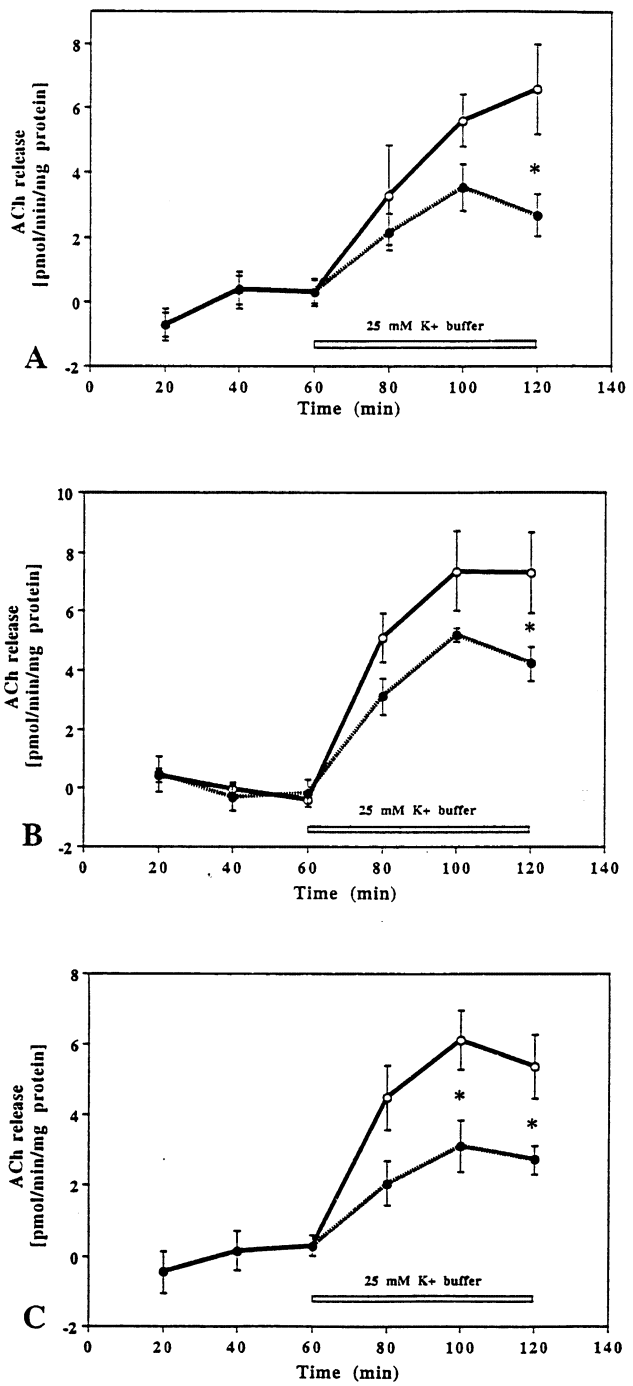

Fig. 3. Comparative effects of $A\beta_{1-40}$ on evoked hippocampal ACh release from young adult ($n = 18$) (A), aged-unimpaired ($n = 18$) (B) and aged-impaired ($n = 15$) (C) rats. Hippocampal slices from three different groups of rats were depolarized with 25 mM K^+ buffer in the presence (dotted line) or absence (solid line) of 10 nM $A\beta_{1-40}$. Endogenous ACh release was inhibited potently in the presence of $A\beta_{1-40}$ during later periods of stimulation. Data are expressed as mean ± S.E.M.

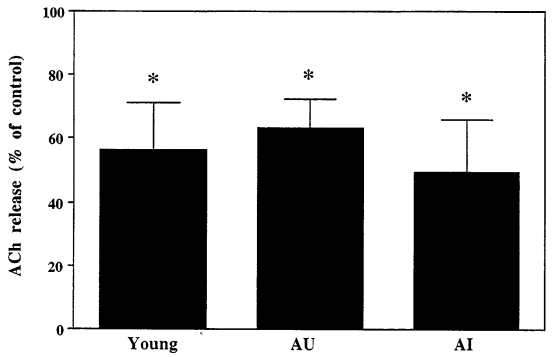

Fig. 4. Comparative effects of 10 nM $A\beta_{1-40}$ on K^+-evoked ACh release from hippocampal slices of young adult (Young, $n = 18$), aged-unimpaired (AU, $n = 18$) and aged-impaired (AI, $n = 15$) rats showing total ACh release as percentage of control over 1 h period. Evoked ACh release was found to be decreased more in AI rats than AU or young adult rats but was not significant from either group. * represents significant difference in $A\beta$ mediated ACh release in young adult, aged-unimpaired (AU) and aged-impaired (AI) rats compared to the control of the respective groups.

We have reported earlier that Aβ related peptides, likely acting directly on the cholinergic terminals, inhibit K^+- as well as veratridine-evoked endogenous ACh release from young rat hippocampal slices (Kar et al., 1996, 1998). The present results extend the evidence by demonstrating that suppression of ACh release by a Aβ peptide can be detected not only in young but also in aged rats. However, the observation that $A\beta$-mediated inhibition of ACh release was apparent at much later time in young as well as AU rats compared to AI sub-group, indicates that cholinergic neurones of AI rats may possibly be more sensitive to Aβ than the other two groups. This result is pertinent in context of the evidence that AI rats, despite low amounts of endogenous Aβ peptide, exhibit significantly decrease levels of basal ACh than AU rats which may, in part, contribute to the poor cognitive performance observed in these animals (Quirion et al., 1995; Vannucchi et al., 1997). Subtoxic concentrations of Aβ, apart from inhibiting ACh release, have recently been shown to modulate uptake of choline, reduce carbachol-induced

GTPase activity and activity of pyruvate dehydrogenase, which generates acetyl-CoA from pyruvate during cellular metabolism (Kelly et al., 1996; Hoshi et al., 1997; Kar et al., 1998). These results, together with the evidence that Aβ peptides are produced constitutively by normal brain cells (Seubert et al., 1992; Selkoe, 1999), suggest a role for these peptides in the regulation of normal cholinergic functions both in young as well as aged rats.

A variety of experimental approaches suggest that normal cholinergic innervation ensures nonamyloidogenic maturation of APP whereas surgical/pharmacological lesions or blockade of basal forebrain cholinergic transmission increase the level of APP and possibly favour amyloidogenic maturation of the precursor (Wallace et al., 1993; Nitsch and Growdon, 1994; Roberson and Harrell, 1997; Auld et al., 1998; Lin et al., 1999; Hellstrom-Lindahl, 2000). Aβ related peptides, on the other hand, appear to be involved in the synthesis/regulation of ACh release from selected regions of the brain (Kar et al., 1996; Pedersen et al., 1996; Hoshi et al., 1997; Auld et al., 1998; Wang et al., 1999). It is likely that increased hippocampal levels of $A\beta_{1-40}$ observed in aged rat may participate, at least in part, in maintaining lower extracellular levels of ACh in these animals compared to young adult rats. These results, taken together, suggest the existence of a reciprocal control mechanism between Aβ related peptides and the functioning of cholinergic neurones. At present, however, it is not clear how the modulatory role of Aβ peptides is being compromised with age leading to degeneration of neurones and/or development of cognitive deficit observed in AD patients. Although chronic infusion and/or in vivo overexpression of Aβ peptide can induce cognitive deficit with age, the underlying mechanism by which Aβ can cause cognitive impairment characteristic of AD remains unclear (Hsiao et al., 1996; Itoh et al., 1996; Nalbantoglu et al., 1997). Additionally, it is not known whether the deficit observed in these animals are mediated by a concurrent decrease in the levels of endogenous ACh. It is also of interest to note that apart from Aβ peptides several other factors that could modulate ACh release, such as growth factors and other neurotransmitters/modulators, may contribute to the cognitive impairment associated with aging (Roberson and Harrell, 1997; Vannucchi et al., 1997). As for degeneration of neurones, it is likely that, in addition to increased levels of Aβ peptide, an alteration in the microenvironment which could trigger aggregation of Aβ peptide, inflammatory response and/or depletion of specific protective/survival factor which may render neurones susceptible to Aβ related toxicity. This is supported, in part, by the evidence that (i) some but not all APP transgenic mice overexpressing Aβ peptide exhibit loss of neurones (Irizarry et al., 1997; Colhoun et al., 1998), (ii) exposure to fibrillar Aβ peptide induces selective loss of cholinergic neurones both under in vitro and in vivo conditions (Giovannelli et al., 1995; Harkany et al., 1995; Olesen et al., 1998) and (iii) aging renders neurones more vulnerable to Aβ-induced inflammatory as well as toxic insults (Geula et al., 1998). Although the factors involved in mediating AD related pathology remain to be fully established, the present study clearly showed that normal aging elevates the level of endogenous $A\beta_{1-40}$ and the cholinergic neurones in cognitively impaired sub-group of aged animals are more sensitive to Aβ peptide than either young or aged-unimpaired rats.

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