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Altered Electrophysiological Expression of Synaptic Plasticity and Infrared Spectroscopic Tissue Composition in Long-term β-Amyloid-treated Rats

T.P. HICKS[1], N.V. KRASTENIAKOV[1], M. JACKSON[2], S.M. DONNELLY[2], B. CHAKRAVARTHY[3], L. OHANNESSIAN[1] and J.-I. OKA[4]

[1]Neural Plasticity and Regeneration Group, IBS, [2]Spectroscopy Group, IBD, [3]Cellular Neurobiology Group, IBS, National Research Council of Canada, Ottawa, Ontario, Canada, and [4]Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Meijo University, Nagoya, Japan

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Summary. β-amyloid-filled osmotic minipumps were used for 14-day intracerebroventricular infusion in rats, followed by routine housing for >1 year. Extracellular recording of evoked potentials from cortical slices from β-amyloid-treated animals showed abnormal elicitation of long-term depression. Whereas no statistically significant difference could be found between β-amyloid-treated and control groups in cortical membrane protein kinase C activity, the use of infrared spectroscopy provided some evidence of alterations in the structure of tissue proteins and an increase in the content of acidic amino acids. The results suggest that the combination of β-amyloid infusion and normal aging may result in an animal model possessing certain functional and structural features useful for study, which may help illuminate some of the etiologies relevant to Alzheimer's disease.

Key words—β-amyloid protein, Alzheimer's disease, synaptic plasticity, rat cortex, infrared spectroscopy, protein kinase C.

INTRODUCTION

The brains of Alzheimer's disease (AD) patients are characterized by reduced dendritic densities, loss of synapses and the occurrence of cell death in both cortical and subcortical areas[1,2]. Intracellular neurofibrillary tangles (NFTs) and senile plaques (SPs) are the main neuropathological lesions. Recent evidence suggests that the formation of SPs may precede all other neuropathological features of AD. β-amyloid, the main constituent of the senile plaques, is now believed to have a number of neurotoxic and pathogenic effects on neurones. AD is the most common cause for mental deterioration in the elderly. It is believed that the reduced neocortical synaptic densities are the pathological correlates of mnemonic and cognitive impairment of AD patients[3]. Senile plaques are extracellular aggregates composed of an amyloid core surrounded by collections of abnormal axons and neurites. β-amyloid is comprised largely of non-branching fibrillary proteins that have a characteristic β-pleated sheet conformation. β-amyloid is a 39-43 amino acid peptide derived by proteolitic cleavage from a much larger transmembrane precursor protein, β-APP (β-amyloid precursor protein). An abnormal processing of β-APP results in secretion of the fibrillary, nondiffusable form of β-amyloid which ultimately leads to the formation of plaques[4]. Recent evidence suggests that the formation of amyloid has a central role in the initiation and expression of most pathological features of AD, including massive synaptic loss and NFTs[5,6]. It has been shown that, *in vitro*, β-amyloid can be directly neurotoxic[7,8] and that it can increase neuronal vulnerability to a variety of metabolic or excitotoxic insults[9,10]. Such insults may cause alterations in neuronal signaling and synaptogenesis through mechanisms that are not yet completely understood. Examples of such mechanisms include the loss of Ca^{2+} homeostasis[10,11], and changes in transmitter

Correspondence: T. P. Hicks, Investment, S & T Section, Embassy of Canada, 7-3-38 Akasaka, Minato-ku, Tokyo 107-8503, Japan.

release[12], receptor activity[13], and protein kinase phosphorylation[14,15]. The present experiments were designed to assess the effects of intracerebroventricularly infused β-amyloid on three key markers that we considered potentially useful in attempting to model certain aspects of AD: 1) cortical synaptic plasticity induced by high-frequency stimulation, 2) protein kinase C (PKC) activity, and 3) the molecular integrity of the tissue as assessed through infrared (IR) spectroscopy. The rats involved in the study were all allowed to age for at least one year following the infusion treatment or sham operation.

Synaptic plasticity may be affected through all of the above mechanisms, but similar evidence coming from *in vivo* studies is insufficient. It has been demonstrated that neurodegeneration can be induced by injecting synthetic β-amyloid directly into the rat cerebral cortex[16]. In addition, β-APP transgenic mice display deficits in learning and the maintenance of long-term potentiation (LTP) with age, while the induction and expression of long-term depression (LTD) remain intact[17]. Negative studies, however, showing no detectable pathological reactions to β-amyloid treatment, have also been reported[18,19]. Moreover, one laboratory has found that β-APP antibodies injected intracerebroventricularly impair memory in rats[20]. Thus far, convincing evidence regarding the pathological influences of β-amyloid on normal synaptic function in the intact rodent brain is lacking. We therefore sought to determine whether the expression form of synaptic plasticity, as elicited from brain slices maintained *in vitro*, might provide some indication of a deficit produced by treatment with (β-amyloid that could shed some light on the responsible mechanism for aberrant information processing, e. g., memory impairment.

PKC is known to be elevated under conditions of enhanced synaptic functioning and in situations where glutamate levels are elevated, e. g., in ischaemia and during the induction of LTP[21-23]. However, long-term changes in PKC as a consequence of altered signal processing due to events occurring as long as one year previously have not been documented. We therefore sought to determine if alterations in synaptic activity in tissues treated with β-amyloid might also be correlated with relative changes in this marker of synaptic activity.

IR spectroscopy, the study of how materials interact with infrared light, is a powerful tool for determining the molecular composition of biological samples[24]. The wavelengths of IR light absorbed by a sample depend upon the atoms involved in the bonds, the type of bonds, and factors such as hydrogen bonding. The wavelengths of IR light absorbed by a sample therefore provide a molecular fingerprint of the sample. This technique has been used previously to examine autopsy tissue derived from Alzheimer's patients[25]. Neuritic plaques could be clearly identified in tissue by the presence of an absorption band at around 1628 cm^{-1}, a frequency characteristic of the presence of aggregated protein. Thus, deposits of β-amyloid *in situ* posses a unique IR spectral signature. We therefore applied IR microscopy to 10 μm sections of cortex from control and β-amyloid-treated rats.

MATERIALS AND METHODS

Animal care and slice preparation

Seven to 10-week-old male Wistar rats were treated and housed in compliance with the Canadian Council for Animal Care guidelines and in accordance with the EU Communities Council Directive of 24 November 1986 (86/609/EEC). The animals were prepared similarly to procedures described in detail elsewhere[26-28]. Briefly, rats were anaesthetized by sodium pentobarbital (50 mg/kg, i. p.) and held in a stereotactic apparatus (David Kopf Instruments). A midsaggital incision exposed the skull for cannular placement in the lateral ventricle (-0.8 mm AP; 1.4 mm ML) with reference to Bregma, and its tip was positioned 3.5 mm below the dural surface according to the atlas of Paxinos and Watson[29]. Osmotic minipumps (2002, ALZA Corp.) having reservoir volumes of 200 μl and pumping rates of ~0.5-μl/h were housed in a subcutaneous pocket near the animal's back; these were filled with either: 1) a solution containing β-amyloid$_{1-40}$ (300 pmol/day, Sigma Chemical Co) plus vehicle, or 2) vehicle solution alone. The vehicle solution comprised 35% acetonitrile and 0.1% trifluoroacetic acid made up in distilled water. The outlet of the minipump was attached to the implanted cannula, referred to above. During surgery, $AlCl_3$ (0.5 μg as aluminum in 5 μl) was injected (2 μl/min) into the contralateral ventricle of both groups of pump-implanted animals, following procedures introduced by Oka et al.[28] The implantation sites were covered with dental cement and the wound sutured closed. Rats were treated postsurgically with benzylpenicillin potassium (2000 units intramuscularly and 4000 units around the wound suture) immediately, as well as a second treatment delivered 48 h later. After surgical recovery, all animals including one sham-operated rat were housed for more than a year in the IBS animal facility.

Coronal brain slices (~400 μm) were prepared using standard procedures. Rats were anaesthetised with halothane followed by surgical decapitation and subsequent rapid brain removal. A block of tissue containing the entire left or right hemisphere was trimmed in ice-cold (about 4℃) artificial cerebrospinal fluid (aCSF). The standard aCSF contained (in mM): NaCl 118.0, KCl 3, $MgSO_4$ 0.8, NaH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 20.0, and D-glucose 10.0, equilibrated to pH 7.4 with 95: 5 O_2/CO_2. Slices were prepared using a D. S. K. Rotoslicer and quickly transferred to an incubation chamber containing O_2-enriched aCSF and maintained at room temperature. The duration of slice preparation was carefully monitored and never exceeded 6.5 min (from decapitation to incubation).

Electrophysiology

The selected brain slice was placed on a nylon mesh in the recording chamber and perfused with warmed aCSF (32-33℃)at a rate of 2.5 ml/min. Extracellular field potentials were recorded from layer III of the parietal cortex using glass micropipettes filled with 4M NaCl and having tip resistances of 2-4 MΩ. Bipolar stimulating electrodes insulated except at the tips were positioned ventral to the recording sites in cortical layer IV. The stimulus was generated by a Grass-88 unit fed through a constant-current stimulus-isolation unit. Control responses elicited by single shocks (100 μS duration, 0.1 Hz) were recorded for at least 10 min, after which repetitive stimuli were applied to the same afferents. The LTP-induction protocol consisted of a high-frequency pulse train (4 pulses at 100 Hz) given at the θ-rhythm (5 Hz) for 2 seconds, and this was repeated five times at 10-second intervals (theta-burst stimulation). All recordings and data acquisitions were filtered and amplified by a Dagan 2400 amplifier, digitized by an A/D converter, and analyzed on- and off-line using a commercial software package (Datawave Technologies) that interfaced with a PC (IBM Pentium 130).

Tissue samples from each of the brains that were studied electrophysiologically were removed from regions of the brain that were not damaged by the slice preparation. These chunks of tissue were then divided into two groups, quick-frozen on chunks of dry ice, and kept at −80℃ until used for the analysis and measurement of PKC activity and IR spectroscopic analysis.

Membrane-associated PKC measurement

For the measurement of PKC, tissue samples were homogenized in a hypotonic medium (1 mM $NaHCO_3$, 5 mM $MgCl_2$ and 100 μl phenylmethyl sulfonylfluoride) using a Potter-Elvehjem homogenizer and membranes isolated as described by Chakravarthy et al.[30] Isolated membranes were suspended in PKC assay buffer [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 μM $CaCl_2$, 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 100 μM phenylmethylsulfonyl fluoride] at 300 μg/ml of which 8-10 μg was used in the PKC assays. Membrane-associated PKC activity was measured by the direct PKC assay in which enzymatic activity present in isolated intact membranes was determined by the level of ^{32}P incorporation into the PKC-selective peptide substrate, Ac-FKKSFKL-NH_2, essentially as described by Chakravarthy et al.[30]

IR spectroscopy

Frozen tissue blocks were mounted onto a cryostat chuck with a small drop of optimal cutting medium (OCT; Tissue-Tek), ensuring that the OCT did not contaminate the region of tissue to be analysed, and thin frozen sections (10 μm) obtained. Tissue sections were mounted on CaF_2 windows and air-dried. Spectra were acquired using a Bruker IR Scope II infrared microscope interfaced to a Bruker Equinox 55 Fourier transform infrared spectrometer. The microscope aperture was set to 100 μm x 100 μm. A grid encompassing the entire section was defined, with measurement points set every 100 μm in both the x and y directions. In this way, measurement points covering the entire tissue section were defined. During data acquisition, 32 interferograms were collected from each point sequentially, signal-averaged and Fourier-transformed to generate spectra with a nominal resolution of 4 cm^{-1}.

RESULTS

Phenotypically, the treated animals did not show any overt signs of abnormality compared with non-treated ones in terms of motor function, social interaction, or cognitive performance up to the one year point after their implantation and delivery of β-amyloid. However, we did not conduct a detailed behavioural assessment of the animals used in this study. No body weight differences were found between treated and untreated animals. For the

electrophysiological experiments, 13 animals (14-16 months old) were included: five β-amyloid-treated, seven vehicle-treated, and one sham-operated age-matched control. The data obtained from the sham-operated rat was included and analyzed together with the data obtained from the vehicle control group. A single slice was selected for study from each animal. The criteria for slice selection included the size and stability of the evoked response, its amplitude, and the latency. The sizes and shapes of the field potentials recorded in cortical layer IV induced by white matter stimulation were very similar to the evoked potentials in layer III induced by stimulation of layer IV afferents. In coronal slices taken from the rat parietal cortex, the field potential displayed characteristic properties such as short, monosynaptic latencies and a sharp, negative potential that was due to the post-synaptic current sink. Despite all these similarities, however, high-frequency shocks applied to the white matter, which normally result in a robust layer IV LTP in young rats, failed to induce any long-lasting post-synaptic changes in our aged animals. For that reason, in the present study we have compared the properties of synaptic plasticity in layer III cells in response to the stimulation of layer IV.

The field potential shape and amplitude in response to single shocks did not differ between β-amyloid-treated and control animals. There were no differences between the groups in the stimulus intensities required for the evoked potential induction, nor were there any differences in the response latencies (stimulus to peak amplitude). As shown in Figs. 1 and 2, theta-burst stimulation (TBS) of the same afferents resulted in LTP of the evoked response amplitude (from pretetanic controls) in all control animals (30 min after TBS: potentiation by 16.8 ± 4.7%, mean ± s.e.m., n=8). The magnitude and duration of LTP seen here was not different from that observed in young rats, as determined from other data collected in this laboratory previously. This finding suggests that, in 14-16 month-old rats, there is no age-related decay in the ability of the tissue to express synaptic modification. TBS delivered to the afferents of the β-amyloid-treated animals resulted in an almost immediate depression of the field potential amplitude (30 min after TBS: depression by 13.3 ± 3.5%, n=5; $p<0.01$, vs the control group, two-tailed Dunnett test) that proved to be long-lasting (Figs. 1 and 2). These findings suggest that the tissue's ability to produce synaptic strengthening and modification is lost or altered in the β-amyloid-treated animals.

The results of PKC measurements are summarised in Fig. 3. Whereas PKC activity initially appeared to be relatively higher in the β-amyloid-treated group of animals, statistical analysis of the data indicated that

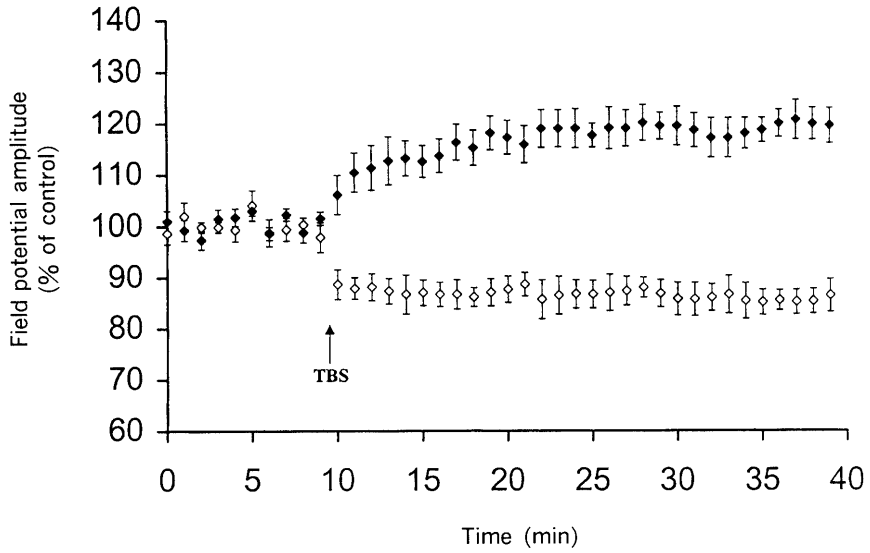

Fig. 1. Activity-dependent synaptic modifications in slices from the rat parietal cortex in control (filled symbols; n=8) and β-amyloid-treated (empty symbols; n=5) aged rats. Each point is a time-matched mean value normalized first to each slice pretetanic baseline and then across all rats, with s.e.m. indicated. TBS, theta-burst stimulation.

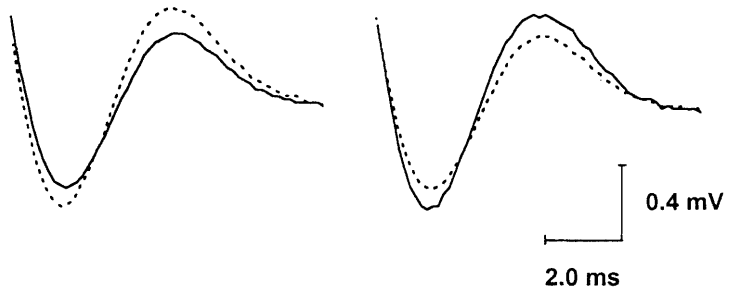

Fig. 2. Examples from single recordings of evoked field potential amplitude changes in response to the same stimulus in control (*left sample*) and β-amyloid-treated (*right sample*) rats. Stimulating and recording sites were in cortical layer IV and layer III, respectively, for each tracing. *Solid lines*, Control (pretetanic) response; *dotted lines*, result obtained 30 min after tetanus.

the apparent difference did not reach statistical significance.

In addition to electrophysiological and PKC measurements, a set of tissue samples from control and β-amyloid treated animals was also analysed by IR spectroscopy. A representative spectrum of the cortex of control rats is shown in Fig. 4A. The major absorption band is the so-called amide I absorption band and 1654 cm^{-1}, which arises from stretching vibrations of protein C=O groups. The frequency of this absorption band is characteristic of proteins with predominantly α-helical secondary structures. A shoulder at 1635 cm^{-1} indicates the presence of some (β-sheet secondary structures.) Other prominent absorption bands are attributed to the N-H bending (1550 cm^{-1}), COO-symmetric stretching (1400 cm^{-1}), and PO_2^- asymmetric and symmetric stretching (1240 and 1080 cm^{-1} respectively) vibrations.

Spectra acquired throughout the cortex of control animals exhibited the general features described above. However, in one section from a treated animal, a significantly different spectrum was acquired. This "abnormal" spectrum is shown in Fig. 4B. In particular, increased absorption intensity was seen at 1635, 1585 and 1400 cm^{-1}, and a series of new absorption bands was apparent between 1000-1200 cm^{-1}.

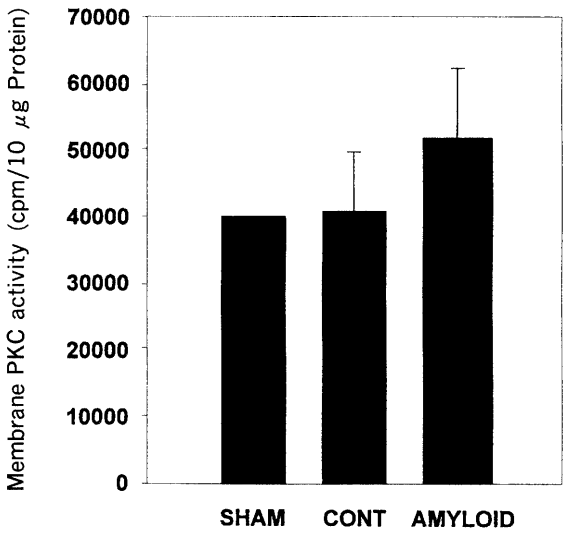

Fig. 3. Membrane PKC activity from three sets of animals used in the present study: sham-treated age-matched (n=1, in duplicate), vehicle-treated controls (n=5) and β-amyloid-treated animals (n=4). Each column except for the sham-treated group indicates mean±s.e.m. No statistically significant difference was found between control and β-amyloid-treated groups (two-tailed Student's t-test, $p>0.05$).

DISCUSSION

The main conclusion of this study is that chronic β-amyloid infusion in the intact rat brain may lead to alterations in the ability of the nervous system to express modifiable neurone-to-neurone communication. Previous work has shown that *in vitro* β-amyloid can be neurotoxic[7,8], and that it can facilitate many pathological events such as biochemical abnormalities, metabolic disorders, and excitotoxic insults[9,10]. Most of those studies, however, have used concentrations of β-amyloid that are much higher than the concentrations usually found in the cerebrospinal fluid of AD patients. In addition, neurotoxicity experiments have been performed with soluble forms of β-amyloid that may or may not aggregate

into β-pleated sheet conformations, considered to be the β-amyloid neurotoxic form. It is conceivable that high concentrations of the soluble β-amyloid may affect neurones through mechanisms different from those that occur naturally in the brain. Finally, the synthetic peptides used in the neurotoxicity studies may be metabolised differently from those β-amyloids that are generated by the intact brain.

All of the above problems apply also to experiments designed to assess β-amyloid effects on the brain *in vivo*. In fact, evidence from *in vivo* work has been thus far contradictory. Some studies have demonstrated that β-amyloid can cause neurodegeneration, behavioural, and electrophysiological abnormalities[16,17,27,28]. Other groups have found no significant neurotoxic reactions in response to β-amyloid treatments[18,19]. For example, previous work from one of our labs (Oka, J.-I., 1999; unpublished results) has shown that one year following the infusion of β-amyloid, dense immunoreactivity can be seen in many brain areas, and in those same animals, impairment of learning as well as of voluntary movement is manifested. No such studies were performed on the animals in this set of experiments.

IR spectroscopy

A number of differences between the normal spectra and the unusual region from the β-amyloid-treated tissue are immediately apparent. For example, the abnormal spectrum exhibited a significant increase in intensity at 1585 cm^{-1}, a spectral region typically dominated by absorptions arising from the asymmetric stretching vibrations of COO^- groups of acidic amino acids, suggesting an increased proportion of negatively charged amino acids in tissue proteins. Additionally, increased intensity was seen at 1635 cm^{-1} in the abnormal spectrum, indicating an increase in proportion of β-sheet secondary structures (Inset, Fig. 4). This clearly demonstrates that the proteins in regions of tissue giving rise to this abnormal spectrum are altered. However, while evidence exists to suggest an alteration in the structure of tissue proteins or the expression of new tissue proteins, no spectroscopic features characteristic of aggregated proteins (i. e. amyloid deposition) were detected.

The most noticeable changes in spectra occurred in the region 1000–1150 cm^{-1}, a region populated by

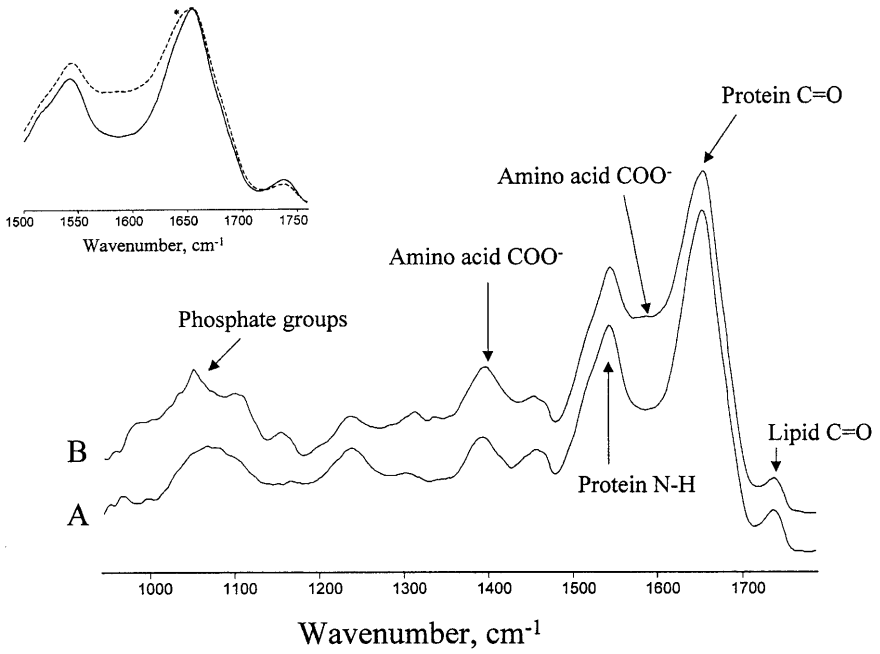

Fig. 4. Infrared spectra of control (**A**) and β-amyloid-treated (**B**) rat cortical tissue. Spectra were acquired from 100 × 100 μm regions of tissue using an infrared microscope (see text for more details). The assignments of major absorption bands are highlighted on the figure. Inset: superimposed spectra of control (*solid*) and β-amyloid-treated (*broken line*) cortical tissue showing increased intensity at 1635 cm^{-1} in cortical tissue from β-amyloid-treated rats (*).

absorption from phosphate groups. In control tissue, this region is dominated by a broad absorption at 1080 cm^{-1}, arising from symmetric stretching vibration of PO_2^- groups. However, a sharp new absorption appeared at 1050 cm^{-1}, with a weaker absorption apparent at 1100 cm^{-1}. Whereas the position of these new features strongly suggested that they arose from some type of phosphate species[24], the narrow width of the absorption bands and lack of any counterparts in the region 1200-1300 cm^{-1} implies that such is not the case. Thus, if these absorption bands arise from phosphate moieties, they must come from either PO_3^{2-} or PO_4^{2-} groups. As PO_4^{2-} groups usually are present only in mineralised tissues like bone or enamel, the most logical assignment for the new absorption bands is vibration from PO_3^{2-} groups. If so, then the question arises as to the nature of these PO_3^{2-} groups. Potential sources include AMP, ADP, ATP, and other phosphorylated nucleotides and phosphorylated proteins. However, the concentrations of phosphorylated nucleotides present within tissues is too low to be detected by IR spectroscopy. The most likely source of the new PO_3^{2-} groups therefore appears to be phosphorylated proteins. In this respect, it is interesting to note that a major pathological hallmark of Alzheimer's disease is the accumulation of hyperphosphorylated tau protein within neurones.

The present study does not directly address the mechanisms of β-amyloid mediated neuropathology; instead it provides evidence for the neuronal dysfunction associated with β-amyloid neurotoxicity. The loss of LTP in the β-amyloid-treated animals suggests abnormalities in the mechanisms involved in synaptic plasticity. Both LTP and LTD are forms of activity-dependent synaptic plasticity that to a large extent depend on transient changes in intracellular Ca^{2+} levels[31]. Under normal conditions, cytoplasmic Ca^{2+} is carefully regulated through a number of mechanisms such as Ca^{2+}-permeable ion channels, plasma membrane Ca^{2+} pumps and exchangers, and pumps on intracellular membranes of Ca^{2+} stores. It has been demonstrated that β-amyloid can disrupt Ca^{2+} homeostasis by interfering with membrane associated Ca^{2+} transport mechanisms[10,11]. Disruption of Ca^{2+} homeostasis may render neurones vulnerable to excitotoxic insults. Recent studies have shown that β-amyloid may increase the activity of NMDA receptors[13] or it may cause abnormalities in transmitter release[12]. Alterations in Ca^{2+} levels, glutamate receptor activity, and transmitter release, singly or in combination, may disrupt normal synaptic communication and may account for the loss of LTP and the appearance of LTD in our β-amyloid-treated animals. The locus of action thus could be both presynaptic and postsynaptic.

Taken together, the results presented in this study show that chronic β-amyloid treatment can cause abnormalities in cortical synaptic plasticity, and this falls nicely in line with other evidence from behavioural studies indicating that similarly-treated animals suffer from compromised learning and memory function[26-28]. β-amyloid may most likely cause such abnormalities through the disruption of Ca^{2+} homeostasis, normal receptor activity, and (or) alterations in the normal processes of transmitter release. Indeed, the mini-pump model of β-amyloid infusion has already been well-accepted elsewhere as providing a good model for amnesia[26-28], and it has been reported further that neuropathological changes accompany this delayed amnesia. Oka et al.,[28] reported that the reproducibility and reliability of amyloid-induced amnesia is greatly enhanced if the animals are pretreated with a single administration of $AlCl_3$. It remains to be seen how many features common to this model and the human disease can be found through multidisciplinary approaches such as those performed and reported here.

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