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Impairment of $G_{s\alpha}$ function in human brain cortex of Alzheimer's disease: comparison with normal aging

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Summary. We examined the quantity and quality of G proteins in membrane preparations of post-mortem human brain, i.e. in parietal, temporal and occipital cortical regions, from normal subjects over age (17–89 years old) and with Alzheimer's disease (AD) in comparison with aged-matched controls. In normal aging, the immunoreactivities determined of $G_{i\alpha}$, $G_{q\alpha}$ and G_{β} were inversely correlated with age. The function of G proteins was examined by photoaffinity GTP analogue [azidoanilido GTP (AAGTP)] labelling. AAGTP labelling to $G_{s\alpha}$ and $G_{i/o\alpha}$, and the ratio of $G_{s\alpha}$ to $G_{i/o\alpha}$ AAGTP labelling showed no age-dependent changes. In AD compared to age-matched controls, there were no significant differences in the levels of $G_{sH\alpha}$, $G_{sL\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, $G_{q\alpha}$ and G_{β} subunits. Functional effects of G proteins, however, as measured by AAGTP labelling to $G_{s\alpha}$, but not to $G_{i/o\alpha}$, was significantly decreased in AD compared to controls in the parietal and temporal cortex, but not in the occipital cortex. These results suggest that the disturbances of post-receptor trans-membrane signalling in AD can be attributed to functional changes of $G_{s\alpha}$, and these are independent of alterations in the level for those proteins in normal aging.

Keywords: AD (Alzheimer's Disease), G protein, post mortem brain, immunoblotting, photoaffinity labelling, signal transduction, aging.

Abbreviations

AD Alzheimer's Disease; *DTT* dithiothreitol; *GTP* guanosine triphosphate; guanosine triphosphate binding protein, G protein; *PMSF* phenylmethylsulfonyl fluoride; *SDS* sodium dodecyl sulfate; *AAGTP* P^3(4 azidoanilido)-P^1-5′GTP.

Introduction

Changes in many neurotransmitters, their synthetic enzymes and their receptor binding sites have been demonstrated in the brains of normal human aging and Alzheimer's disease (Fowler et al., 1992; Nordberg, 1992; Greenamyre and Maragos, 1993). However, alterations in presynaptic neurotransmitters or receptor binding are not necessarily linked to disturbances in the cell signalling system such as the coupling interaction of receptor-transducer molecules, effectors that produce the second messenger. Therefore, in recent years there has been a great interest in post-receptor signal transduction relative to aging and neurodegenerative diseases (Fowler et al., 1992). Several lines of research have indicated that disturbances of post-receptor signal transduction exist in Alzheimer's disease. For example, changes of the coupling of muscarinic receptors to GTP binding (G) protein (Warpman et al., 1993), reduction of β adrenergic receptor agonist- or GTP analogue-stimulated adenylyl cyclase (Ohm et al., 1991; Cowburn et al., 1992a, b), and decreased levels of protein kinase in the particulate fraction (Cole et al., 1988) have been reported.

The signal-transduction G proteins are heterotrimeric in structure, consisting of α, β and γ subunits, and the activation of G proteins involves dissociation of their α and βγ subunits. G proteins are key components in the regulation of adenylyl cyclase, cGMP-dependent phosphodiesterase, phospholipase C, phospholipase A2, Ca^2+ channels and K+ channels (Gilman, 1987; Birnbaumer et al., 1990; Simon et al., 1992). Recently the βγ subunit has been also recognized as an active subunit regulating effectors (Clampham and Neer, 1993).

Concerning the amount of G protein in Alzheimer's disease, McLaughlin et al. (1991) reported the robustness of the G protein α subunits G_{i1}, $G_{i2,}$ G_{sH}, G_{sL} and G_o in this disorder by Western blotting analysis. Although lack of changes in the amount of G proteins does not always demonstrate the integrity of there proteins, few studies have attempted to estimate the direct functional aspects of G protein subunits. Furthermore, it has not been established whether similar alterations in G protein mediated signal transduction occurs in human brains in aging or diseases. Therefore, in the present study, we examined the amount of G proteins in more detail by using antisera against the specific G protein subunits $G_{s\alpha}$, $G_{i\alpha}$, $G_{o\alpha}$, $G_{q\alpha}$ and G_β in membrane preparations from parietal, temporal and occipital cortices of post mortem human brains from subjects with normal aging, from patients with dementia of the Alzheimer type (AD) and from controls matched with respect to age and post mortem delay. In addition, we performed in situ photosensitive GTP labelling of the synaptic membrane to investigate whether the functional levels of individual G proteins were changed by this disease.

Materials and methods

The procedures used in the Austro-German and Sapporo brain bank system for acquisition, clinical diagnosis, dissection, storage and distribution of brain materials were described in detail by Gsell et al. (1993). The AD patients fulfilled the diagnostic criteria of NINCDS/ADRDA for probable Alzheimer's disease (McKhann et al., 1984). Control patients were hospitalized and died from somatic disorders without a history of either neurological or psychiatric disorders according to their records; causes of death were similar for the AD group. The final diagnosis of AD was

Table 1. Subject characteristics for AD samples and normal controls matched for age and post mortem delay. All values are expressed as group mean ± S.E.M. There were no significant group differences

Cortex region		Parietal	Temporal	Occipital
Sample size [n] and sex distribution (m/f)				
All controls	[22]	19 (10/9)	19 (8/11)	22 (10/12)
matched controls	[15]	10 (5/5)	10 (5/5)	9 (5/4)
AD	[17]	11 (4/7)	9 (3/6)	7 (3/4)
Age [years]				
All controls	(range: 17–89)	59.2 ± 5.1	60.2 ± 5.0	61.8 ± 4.6
matched controls	(range: 68–89)	75.5 ± 2.7	75.5 ± 1.6	74.9 ± 1.7
AD	(range: 73–95)	79.8 ± 1.5	81.0 ± 2.4	78.9 ± 1.8
Post mortem delay period [hours]				
All controls	(range: 3–41)	10.0 ± 2.4	13.5 ± 2.7	11.4 ± 2.1
matched controls	(range: 3–41)	10.2 ± 3.0	14.8 ± 4.1	15.7 ± 4.5
AD	(range: 4–48)	18.9 ± 4.3	10.9 ± 3.3	11.5 ± 4.1

established histologically by the presence of numerous plaques and tangles in the cerebral cortex and pronounced changes in the hippocampus. Cases with moderate or severe vascular changes were excluded. In control brains, macroscopic examination of the brain did not show any pathological changes, and histological examination of the cerebral cortex and the hippocampus did not show more senile plaques and tangles than could be explained by age. All brain regions were not available for every case; thus, the samples consisted of 17 cases with AD and 22 cases of a normal aging group, of which 15 cases were matched to the AD group with respect to age and post mortem delay (Table 1).

Synaptic membrane-enriched fractions were prepared from human cerebral cortex as described by Hatta et al. (1986) and stored at −80°C until use.

Gel electrophoresis and immunoblotting were carried out according to the methods of Ozawa et al. (1993a, b) respectively with minor modifications for post mortem human brain. Human cerebral cortex Membrane (5 μg of membrane protein/lane) was dissolved in 3% Laemmli sample buffer with 50 mM DTT and electrophoresed in 10% SDS/polyacrylamide gels (Laemmli, 1970). Proteins were transferred to nitrocellulose filters by electroelution as described by Towbin et al. (1979). After transfer, filters were blocked by 1 h incubation with 3% BSA in a buffer of 10 mM Tris (pH 7.5), 500 mM NaCI, and 0.1% Tween 20 (TBS-T). The nitrocellulose filters were then incubated with TBS-T containing 0.1% BSA and anti-G protein subunit antibodies (RM/l, AS/7, GC/2, QL and SW/1 specific to $G_{s\alpha}$, $G_{i1 \text{ and } i2\alpha}$, $G_{o\alpha}$, $G_{q \text{ and } i2\alpha}$: and G_{β} subunits, respectively, are commercially available from NEN Dupont [Boston, MA; USA]) at a 1/5000 dilution. After incubation for 14–16 h at room temperature, filters were washed in TBS-T three times and then incubated with TBS-T containing 0.1% BSA and HRP-linked anti-rabbit IgG (F(ab')2) (Amersham) at a 1/5000 dilution for 1 h at room temperature. Filters were washed three times with TBST and immunoreactivity was detected with an enhanced chemoluminescence (ECL) Western blot detection system (Amersham) followed by exposure to ECL HYPER film (Amersham). The developed autoradiographs were analyzed by laser densitometer (Model SLR-2D/1D, Biomed Instrument, Inc.). To standardize the integrated optical density against a known amount of G protein subunit by our immunoblotting methods, purified $G_{o\alpha}$ protein was used as a standard. The individual integrated optical density of G protein was then normalized by comparison against a pooled standard sample of normal rat cerebral cortex membranes containing 397.89 + 4.71 pmol of $G_{o\alpha}$/mg membrane protein, which was run every time.

[^{32}P]-P^3(4-Azidoanilido)-P^1-5′GTP (AAGTP) was synthesized by the method of Pfeuffer (1977). AAGTP binding experiments were performed as described by Ozawa et al. (1993a, b). Human cerebral cortex membrane was washed and resuspended in 2 mM HEPES (pH 7.4)/1 mM $MgCl_2$. Membrane suspensions (1–1.5 mg of protein/ml) were incubated 0.1 mM [^{32}P]-AAGTP for 5 min at 23°C, and the reaction was terminated by dilution with ice-cold buffer followed by centrifugation at 15,000 g for 10 min to remove unbound [^{32}p]-AAGTP. Membranes were washed again and resuspended in the same buffer followed by 5 min of UV photolysis with a Spectroline UV lamp (254 nm, 9W) an ice-cold 2 mM HEPES (pH 7.4)/1 mM $MgC1_2$/4 mM DTT, then centrifuged at 15,000 g for 10 min. Membrane pellets were dissolved in 3% SDS Laemmli sample buffer with 50 mM DTT and electrophoresed in 10% SDS/polyacrylamide gels by the procedure of Laemmli (1970). After electrophoresis, gels were stained with Coomassie Blue, then dried and autoradiographed with Kodak XAR-5 film. Radioactive bands corresponding to $G_{s\alpha}$ and $G_{i/o\alpha}$ were excised from the dried gel, immersed in 4 ml of scintillation cocktail, and counted using a Beckman LS 5801 scintillation counter.

Membrane protein content was determined by the Coomassie Blue binding method (Bradford, 1976) with bovine serum albumin as a standard.

Data of G protein immunoreactivity from the control and AD groups were analyzed for significant differences using a two-tailed Student's t-test. Pearson's linear regression analysis was performed for normalized G protein immunoreactivity versus age or post mortem delay time and the data from blots processed with each antiserum (StatView, Abacus Concepts, Inc.). Level of significance was set at $p < 0.05$, uncorrected for multiple comparisons.

Results

Effects of age and post mortem delay an immunoreactivity of G proteins

The immunoreactivity results on aging and post mortem delay are shown in Table 2. There was a significant negative correlation with age by linear regression analysis in immunoreactivity for $G_{i\alpha}$ in the parietal ($p = 0.048$), temporal ($p = 0.018$) and occipital ($p = 0.077$) cortices, for $G_{q\alpha}$ in the parietal ($p = 0.013$), temporal ($p = 0.033$) and occipital ($p = 0.098$) cortices and the G_β in the temporal ($p = 0.019$) and occipital ($p = 0.050$) cortices. No significant correlation with age was observed for immunoreactivity to the other G protein subtypes in any regions examined. Immunoreactivity for any G protein subtypes did not correlate with post mortem delay.

Effects of age and post mortem delay on photoaffinity guanine nucleotide labelling of G proteins

To elucidate the functions of G proteins, we introduced the hydrolysis-resistant photoaffinity GTP analogue, AAGTP. This compound has been used to identify adenylyl cyclase activation associated with G proteins in synaptic membranes. We have used this compound as a functional probe as well as a photoaffinity compound (Hatta et al., 1986; Ozawa and Rasenick, 1991) for G protein and the adenylyl cyclase system. As reported previously for rat brain (Hatta et al., 1986; Ozawa and Rasenick, 1991), we were able to identify both $G_{s\alpha}$ and $G_{i/o\alpha}$ ($G_{i\alpha}$ plus $G_{o\alpha}$), in human brain membranes by AAGTP labelling in the present study and in previous reports (Ozawa et al., 1993a, b). Linear regression analysis revealed no significant relationship between AAGTP labelling ($G_{s\alpha}$ and $G_{i/o\alpha}$) and age in parietal, temporal and occipital cortices (Table 2). Additionally, both

Table 2. Effects of age and post mortem delay time (PMDT) on G protein immunoreactivity and AAGTP photoaffinity labelling, respectively, in cerebral cortex membrane preparations from a normal aging group

Cortex region	Parietal		Temporal		Occipital	
	age	PMDT	age	PMDT	age	PMDT
immunoreactivity for the various G protein subunits						
$G_{sH\alpha}$	−0.10	0.04	−0.32	0.24	−0.06	−0.10
$G_{sL\alpha}$	−0.05	−0.21	−0.11	0.28	−0.07	−0.14
$G_{i\alpha}$	−0.46*	0.33	−0.55*	0.46	−0.38(*)	0.21
$G_{o\alpha}$	−0.25	−0.30	−0.21	0.27	−0.27	0.09
$G_{q\alpha}$	−0.56*	0.31	−0.50*	0.16	−0.36(*)	0.27
G_{β}	−0.10	−0.17	−0.55*	0.16	−0.42(*)	0.25
AAGTP photoaffinity labelling to various G protein α subunits						
G_s	−0.21	0.29	−0.18	−0.08	0.14	−0.04
$G_{i/o}$	−0.27	0.12	0.01	0.23	0.13	0.35
Ratio G_s to $G_{i/o}$	−0.06	0.26	−0.20	−0.36	0.08	0.02

Tissue samples from the three different brain regions were not available from all brains. Thus, the data are listed for each region separately. Data were calculated as normalized values (band peak height for subject divided by corresponding band peak height for the reference pooled rat brain sample run in all blots) versus age or PMDT, respectively, and presented as Pearson's linear correlation coefficients, * = $p < 0.05$, (*) = $0.05 < p < 0.05$. A significant inverse correlation is evident for $G_{i\alpha}$, $G_{q\alpha}$ and G_{β} subtypes with age, whereas no significant correlations were found for the other G protein subtypes. There is no significant correlation between AAGTP labelling to any G protein and age or PMDT, respectively

AAGTP labelling to $G_{s\alpha}$ and $G_{i/o\alpha}$ did not correlate with post mortem delay (Table 2).

Immunoreactivity of G proteins in control and AD subjects

Figure 1 shows representative immunoblots obtained in the assay of G protein α and β subunits from membrane preparations of temporal cortex from AD and age-matched control brains. The summarized data on the quantitative changes in the assays of G protein subunits in parietal, temporal and occipital cortical areas from control and AD subjects are shown in Table 3. There were no significant differences in immunoreactivities of any G protein subtypes in any cortical regions between AD and control subjects.

Photoaffinity guanine nucleotide labelling for G proteins in control and AD subjects

Figure 2 shows representative autoradiographs with [^{32}P]AAGTP photoaffinity labelling in temporal and occipital cortex membranes from controls (C) and AD subjects (SD). The mean values (±S.D.) of AAGTP labelling of $G_{s\alpha}$ for the control and AD subjects, respectively, were 362.9 ± 34.3 and 253.3 ± 35.9 in the parietal cortex, 321.1 ± 30.6 and 199.1 ± 13.2 in the temporal cortex and 356.4 ± 31.9 and 343.9 ± 46.1 in the occipital cortex. Thus, AAGTP binding to

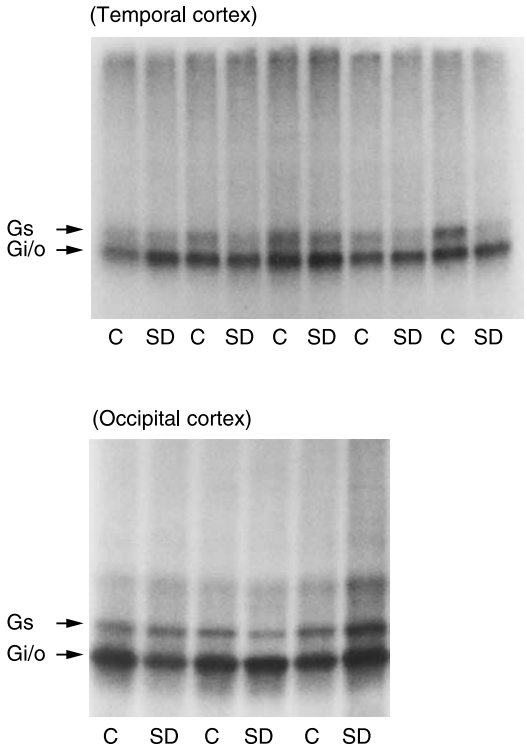

Fig. 1. Representative immunoblots for specific G protein subunits using polyclonal rabbit antiserum against G protein subtypes (RM/1, AS/7, GC/2, QL and SW/1 specific to α subunits of G_s, $G_{i1\ and\ i2}$, G_o, $G_{q\ and\ i2}$ and G_β subunits) in temporal cortex membranes from control (C) and AD (SD) subjects. Detailed methods are explained in the text. The exposure time to ECL HYPER films was 30 sec – 1 minute except for $G_{s\alpha}$ which exposed for 3–10 minutes

Table 3. Quantitative estimation of the various G protein immunoreactivities in cerebral cortex membranes from controls and AD subjects

Cortex region	Parietal	Temporal	Occipital
$G_{sH\alpha}$			
Controls	0.95 ± 0.08	0.94 ± 0.08	1.04 ± 0.14
AD	0.89 ± 0.08	1.08 ± 0.10	1.00 ± 0.10
$G_{sL\alpha}$			
Controls	0.93 ± 0.08	0.92 ± 0.15	0.97 ± 0.19
AD	0.97 ± 0.09	1.03 ± 0.09	0.99 ± 0.11
$G_{i\alpha}$			
Controls	0.96 ± 0.06	0.92 ± 0.05	0.73 ± 0.07
AD	1.00 ± 0.07	0.91 ± 0.05	0.82 ± 0.07
$G_{o\alpha}$			
Controls	0.96 ± 0.06	1.09 ± 0.07	0.88 ± 0.06
AD	1.08 ± 0.06	1.01 ± 0.06	0.96 ± 0.08

(*continued*)

Table 3 (*continued*)

Cortex region	Parietal	Temporal	Occipital
$G_{q\alpha}$			
Controls	0.76 ± 0.06	0.89 ± 0.06	0.67 ± 0.05
AD	0.92 ± 0.09	0.82 ± 0.08	0.73 ± 0.06
G_{β}			
Controls	1.06 ± 0.05	1.07 ± 0.05	0.79 ± 0.05
AD	1.05 ± 0.04	1.12 ± 0.05	0.88 ± 0.06

Data are expressed as group means ± SEM. The integrated optical density was standardized against purified $G_{o\alpha}$ protein, and the individual integrated optical density of G protein was normalized to a pooled standard sample of normal rat cerebral cortex membranes, which was run on every gel. Values are expressed as means ± S.E.M. of normalized immunoreactivity. There are no significant changes in any G protein immunoreactivity between AD samples and controls, which were matched for age and post mortem delay time

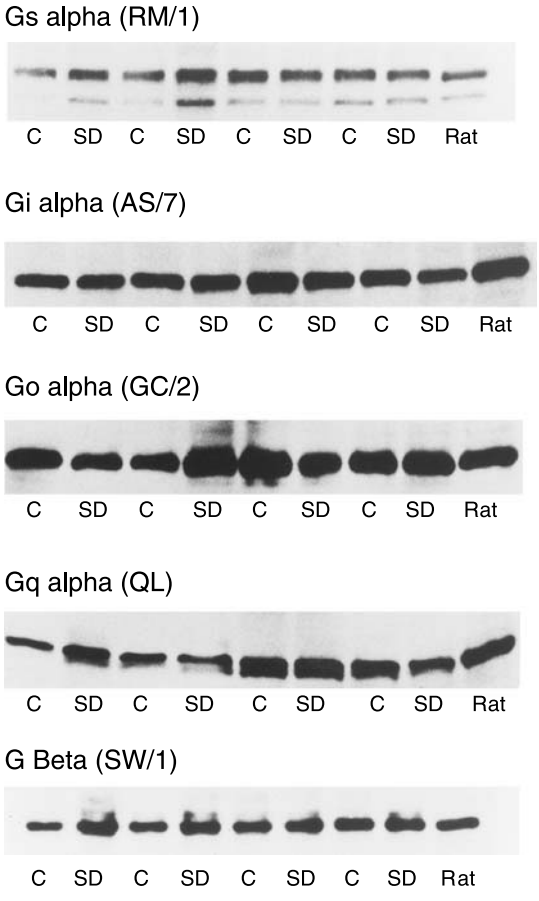

Fig. 2. [^{32}P]AAGTP photoaffinity labelling in temporal and occipital cortex membranes from controls (C) and AD subjects (SD). Detailed methods are explained in the text

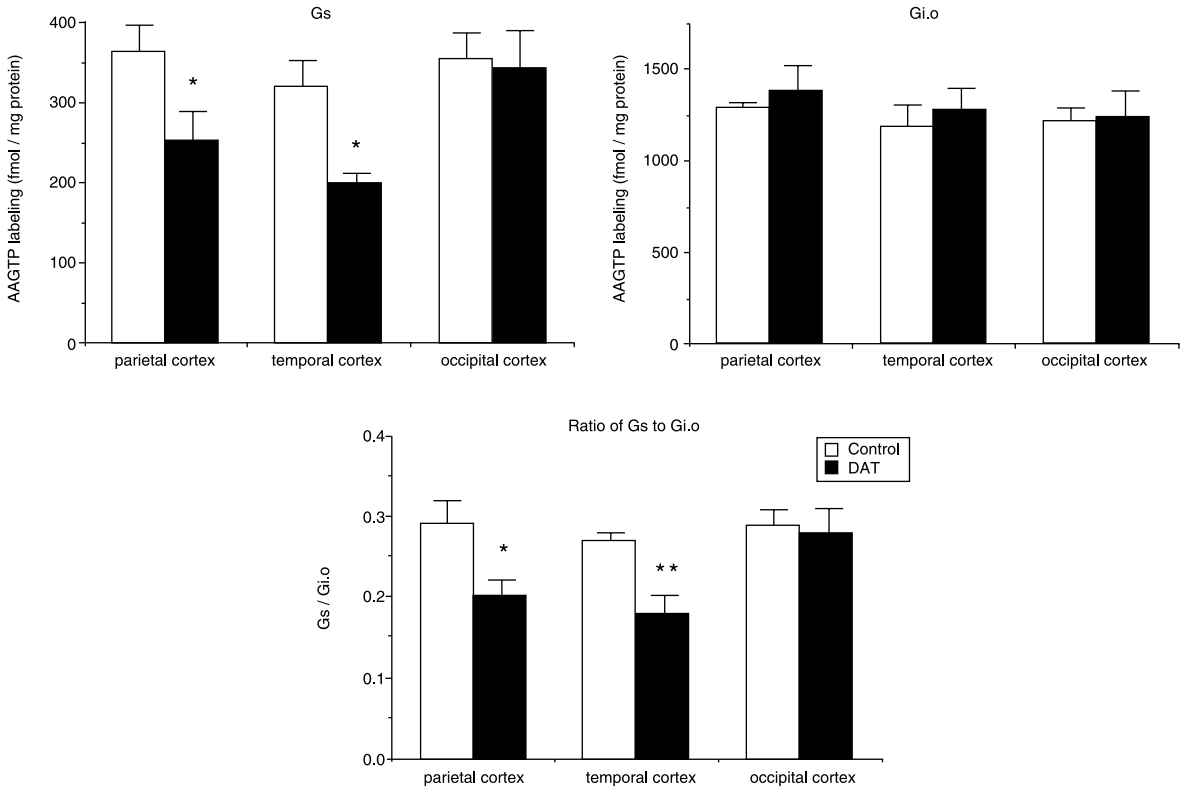

Fig. 3. Quantitative estimation of the [^{32}P]AAGTP labelling to G proteins in cerebral cortex membranes from controls and AD subjects. Data are expressed as mean ± SEM. $^{*}p<0.05$ and $^{**}p<0.01$ indicate significant differences compared with the corresponding control

$G_{s\alpha}$ showed a significant decrease in AD in the parietal and temporal cortices but not in occipital cortex, while that of $G_{i/o\alpha}$ showed no significant difference between AD and control subjects in any cortical regions (Fig. 3). Moreover, the ratio of $G_{s\alpha}$ to $G_{i/o\alpha}$ in AD was significantly lower in both parietal and temporal cortices (Fig. 3).

Discussion

Our previous studies and those of others using animals have indicated that receptor-effector coupling via G proteins in the brain changes with aging (Nomura et al., 1986, 1987; Ozawa et al., 1989; Duman et al., 1989; Ikeda and Saito, 1989a, b; Yamagami et al., 1992, Cowburn et al., 1992a). The first part of the present study used immunoblotting with polyclonal G protein antibodies showed significant negative correlations ($P<0.05$) with age in immunoreactivities for $G_{i\alpha}$ and $G_{q\alpha}$ in the parietal and temporal cortex, and for G_{β} in the temporal and occipital cortex, independent of post mortem delay time. Our data are partially consistent with those from Young at al. (1991) concerning $G_{i\alpha}$, but not concerning $G_{s\alpha}$, which was unchanged in our study. Young et al. (1991) have also studied the effects of early childhood development on G protein levels, which was not addressed by our study. In this study, we demon-

strate for the first time a significant inverse correlation of $G_{q\alpha}$ with age in the parietal and temporal cortex, which is of particular relevance because $G_{q\alpha}$ is involved in receptor-coupled regulation of phosphoinositidase C.

Although there are changes in the concentrations or biosynthesis of certain G protein subtypes at the gene-expression level in psychiatric diseases, including Alzheimer's disease, a change in the amount of protein or mRNA does not always imply disturbed protein function, but rather often appears to reflect compensatory consequences. Actually, an increase of $G_{s\alpha}$ mRNA was reported in Alzheimer's disease (Harrison et al., 1991), but no change in the quantities of G protein subunits in AD brain (McLaughlin et al., 1991; Young et al., 1993, present data).

Therefore, to study the function of G proteins, we utilized the hydrolysis-resistant photoaffinity GTP analogue AAGTP as a functional probe to investigate the binding of GTP to the two G protein alpha subunits, G_s and $G_{i/o}$. In contrast to the immunoblotting study, AAGTP labelling experiments showed no significant dependency on age. This discrepancy between immunoblotting and photoaffinity labelling findings might indicate that adaptive alterations in G protein function occur to compensate for the reduction of G protein levels during aging, in accord with the general concept of adaptive mechanisms with aging (Adelman, 1979).

In AD compared to age-matched controls, our functional studies showed a significantly decreased AAGTP labelling to $G_{s\alpha}$, but not to $G_{i/o\alpha}$, in parietal and temporal, but not in occipital cortex in AD. No significant differences were found on the protein level, e.g. in immunoreactivities of $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$, in keeping with data by McLaughlin et al. (1991). In addition, the present study also revealed the quantitative integrity of $G_{q\alpha}$ and G_{β} in AD. These data together suggest a selective and topographically distinct impairment in $G_{s\alpha}$ function in AD brain and argue against unspecific changes due to neuronal loss. Ohm et al. (1991) have suggested that a reduction in adenylyl cyclase activity may be associated with Alzheimer-related histopathological changes and be due to a decreased functional level of the catalytic unit of adenylyl cyclase. Moreover, Cowburn et al. (1992a, b) have reported that GTP analogue- or fluoride-stimulated adenylyl cyclase is impaired in the Alzheimer brain membrane without any changes of the inhibition of adenylyl cyclase activity by GppNHp, suggesting a functional alteration of the G protein. Our data for the first time directly demonstrate selective alterations in the function of G proteins in AD brain.

The most interesting explanation for the impaired $G_{s\alpha}$ function in AD implies an interaction with the disturbances of cytoskeletal architecture in AD, e.g. the accumulation of neurofibrillary tangles (Bancher et al., 1989). Our group and others have demonstrated that microtubule elements interact with components of the cellular signal transduction process (Rasenick et al., 1989; Hatta et al., 1994, 1995). Thus, the pathological alteration of microtubules in AD could induce refractoriness of G protein function via disturbances of tubulin – G protein. However, further studies are required to confirm this hypothesis.

In summary, the present results show a specifically impaired $G_{s\alpha}$ protein function in AD, independent of the normal ageing process. Our observations are consistent with other results from human post mortem brain tissues (Ohm et al.,

1991; Cowburn et al., 1992a, b) and peripheral tissues (Huang and Gibson, 1993) indicating an impaired receptor-Gs-mediated adenylyl cyclase activity and c-AMP accumulation in Alzheimer's disease. Thus, it appears important to examine the function of post-receptor signal transduction through G proteins when seeking and developing novel candidate agents to treat dementia patients.

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