

Effects of Age, Postmortem Delay and Storage Time on Receptor-mediated Activation of G-proteins in Human Brain

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The influence of age, postmortem delay and freezing storage period on receptor-mediated G-protein activity was quantified in cortical membranes from 34 healthy subjects.

Concentration-response curves of the [³⁵S]GTPγS binding stimulation by agonists for α₂-adrenoceptors (UK14304), μ-opioid (DAMGO), 5-HT_{1A} (8-OH-DPAT), GABA_B (baclofen) and muscarinic (carbachol) receptors were analyzed. Immunoreactivities of G_α-protein subunits were also determined. Basal binding and UK14304, 8-OH-DPAT, and baclofen potency to stimulate [³⁵S]GTPγS binding decreased with aging (1–88 years) without changes of efficacy. DAMGO-mediated stimulation increased both in potency

and efficacy with aging. A negative correlation between age and immunoreactivity was observed for G_{α1/2}, but not for G_{α3}, G_{αo} and G_{αs}-proteins. Neither [³⁵S]GTPγS binding nor G_α-proteins changed with the postmortem delay (8–92 h). Basal [³⁵S]GTPγS binding decreased with the sample storage period (1–85 months). A careful match between cases and controls should be taken into account when designing signal transduction studies in human disorders, specially for variables such as age and storage period.

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Heterotrimeric guanine nucleotide binding proteins (G-proteins) represent a widely used mechanism for signal transduction from membrane receptors to intracellular effectors. Activation of the receptors by their agonists promotes the exchange of GDP by GTP on heterotrimeric G-proteins, and this results in the dissociation of the α

from βγ subunits (Gilman 1987; Birnbaumer et al. 1990; Gudermann et al. 1997). The G-protein α subunit possesses the ability to initiate a variety of effector pathways that involves, among others, cAMP production, phospholipids hydrolysis and changes of ion channel permeability. The subsequent GTP hydrolysis by the G_α-protein subunit facilitates the reassociation of the heterotrimeric protein (α and βγ subunits) which leads G-protein to the inactive GDP-bound state. The heterogeneity of α subunits serves to divide G-proteins into the major classes (G_i, G_o, G_s, G_q, etc.) (Gilman 1987; Simon et al. 1991).

The [³⁵S]GTPγS binding assay has been developed as a functional method to assess the receptor-mediated activation of G-proteins. The binding of the radiolabeled poorly-hydrolyzable GTP analog [³⁵S]GTPγS measures the exchange of GDP by GTP (or [³⁵S]GTPγS) on the G_α-protein subunit and in this way it reflects directly the

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receptor-activation induced by the different agonists. The approach has been used in reconstituted systems of purified proteins (Cerione et al. 1986; Florio and Sternweis 1989), tissue membrane homogenates (Hilf et al. 1989; Olanas and Onali 1996; Elliot and Reynolds 1999; Gessi et al. 1999), lysate of cells transfected with cloned receptors (Lazareno and Birdsall 1993; Newman-Tancredi et al. 1997; Peltonen et al. 1998), tissue autoradiography (Sim et al. 1995; Dupuis et al. 1999) and immunoprecipitation assays (Wang and Friedman 1994). Recently, we have demonstrated the applicability of the [35 S]GTP γ S binding assay to postmortem human brain membrane homogenates and sections (González-Maeso et al. 2000; Meana et al. 2000; Rodríguez-Puertas et al. 2000).

Biochemical and functional alterations of G-protein coupled receptors (GPCRs) have been repeatedly postulated in the pathophysiology of several neurological and psychiatric disorders. Contributions have usually come from *in vitro* or *in vivo* receptor radioligand binding studies. Additional contributions suggest that modifications of G-protein expression and/or function could also be implicated in some of these brain disorders (Manji 1992; Young et al. 1993; Escribá et al. 1994; Wang and Friedman 1994; Cowburn et al. 1996; Friedman and Wang 1996; Pacheco et al. 1996; Spiegel 1996; Jope et al. 1998). The responsiveness to many hormones and neurotransmitters is altered during senescence. Some of these alterations are associated with reduced receptor densities (Dewey et al. 1990; Dillon et al. 1991; Pascual et al. 1991; Nordberg et al. 1992; Arranz et al. 1993; Sastre and García-Sevilla 1994) while others involve age-related alterations in signal transduction elements beyond receptors (Young et al. 1991; Cowburn et al. 1992, 1994; Sastre and García-Sevilla 1994; Greenwood et al. 1995; Busquets et al. 1996; Li et al. 1996).

The use of autopsy tissue represents the only approach that permits an analysis of the pathophysiological conditions of human brain at receptor/G-protein coupling level. However, postmortem studies present several methodological problems including the unavoidable delays between death and tissue dissection (postmortem delay) and the length and temperature of sample storage prior to assay (Perry and Perry 1983). For the most part, postmortem human brain studies have examined receptor agonist/antagonist radioligand binding and immunoreactive levels of the different G-proteins. In contrast, restricted data are available on the functional responses of signal transduction pathways to receptor activation in postmortem human brain, probably due to a substantial loss of receptors and/or G-proteins during postmortem delays and freezing storage periods (Odagaki et al. 1998; Happe et al. 1999).

The aim of the present study was to quantify accurately both receptor-mediated activation of G-proteins and G-proteins themselves in a large number of post-

mortem human brain samples and to investigate their relationship with aging and with limiting parameters such as the postmortem delay and the freezing storage period.

SUBJECTS AND METHODS

Subject Selection and Brain Samples

Human brains were obtained at autopsy in the Basque Institute of Legal Medicine (Bilbao, Spain) and in the Institute of Forensic Medicine (Geneva, Switzerland). In typical conditions, the corpse is usually refrigerated within 3–5 h after death and stored at refrigeration temperature (4°C) until autopsy. Samples from prefrontal cortex (Brodmann's area 9) were dissected at the time of autopsy and immediately stored at –70°C until assay. Collection procedures were performed in accordance with the protocol for postmortem human brain research of the Basque Institute of Legal Medicine. The study was approved by the Research and Ethics Boards of both the Medical School of the Basque Country, Spain and the Department of Psychiatry, University of Geneva, Switzerland.

Samples were obtained (collecting period 1991–1999) from subjects without a known history of neurological or psychiatric disorders and who had mainly died by sudden accidents; other causes of death were myocardial infarction ($n = 2$) and asphyxia ($n = 1$). The samples of subjects with a positive result in the screening for psychotropic drugs were excluded from assays. Ethanol was detected in four subjects (blood concentration, 1.21 ± 0.5 mg/ml). A total number of 34 subjects (20 female, 14 male) that provided a wide range for the variables to be evaluated in the study were used.

Membrane Preparations

Membrane preparations were performed as reported in detail previously (González-Maeso et al. 2000). Briefly, cortical tissues were homogenized in ice-cold Tris-buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1mM EGTA, pH 7.4) supplemented with 1 mM dithiothreitol (DTT) and 0.25 M sucrose. Cellular P₂ fractions were prepared by sequential centrifugations and resuspensions in Tris-buffer. Final aliquots were stored at –70°C for binding and Western blot assays.

[35 S]GTP γ S Binding Assays

[35 S]GTP γ S binding assays were performed as already described (González-Maeso et al. 2000). Briefly, after thawing, 80 μ g/ml of membrane proteins were incubated at 30°C for 120 min in incubation buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM DTT, 1 mM EGTA, 100 mM NaCl and 50 μ M GDP, pH 7.4) containing 0.5 nM [35 S]GTP γ S. Selective agonist drugs for several GPCRs

were included in the assay to evaluate in a concentration-dependent manner (10^{-10} – 10^{-3} M; eight concentrations) the receptor-mediated activation of [35 S]GTP γ S binding to G-proteins. All experiments were performed in triplicate. Nonspecific binding was determined in the presence of 10 μ M unlabeled GTP γ S. The specific [35 S]GTP γ S binding in the absence of any agonist drug was assumed as the basal binding. The reaction was terminated by rapid vacuum filtration through Whatman GF/C glass fiber filters and the remaining bound radioactivity was quantified by liquid scintillation spectrophotometry.

Immunoblot Analysis of G-protein α -Subunits and Quantitation of the Specific Immunoreactivity

Immunoreactive levels of $G_{\alpha 1/2}$, $G_{\alpha 3}$, $G_{\alpha o}$ and $G_{\alpha s}$ were assessed by using specific polyclonal antibodies (Milligan 1988, Escribá et al. 1994). Detection by a monoclonal antibody of the cytoskeletal protein α -tubuline was used as a loading control in each experiment. Membrane preparation aliquots were thawed and resuspended in 500 μ l ice-cold Tris-buffer and diluted up to 1 mg protein/ml (Bradford 1976). Samples were prepared for electrophoresis as described (Escribá et al. 1994) and subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. Proteins were immediately transferred to nitrocellulose membranes and incubated overnight at 4°C in blocking solution containing the primary antibody at 1:7,000 (anti- $G_{\alpha 1/2}$), 1:3,000 (anti- $G_{\alpha 3}$), 1:4,000 (anti- $G_{\alpha o}$) and 1:3,000 (anti- $G_{\alpha s}$) dilutions. The secondary antibody was a horseradish peroxidase-linked donkey anti-rabbit IgG at 1:5,000 dilution. Immunoreactivity was detected with an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK), followed by exposure to AGFA film (RP2 B3324F1) for 1–10 min. Films were scanned in an image analyzer using the NIH-IMAGE software (Bethesda, MD). The nitrocellulose membranes were stripped and α -tubuline was detected using anti- α -tubuline primary antibody (1:3,000) and sheep anti-mouse IgG antiserum (1:5,000) as secondary antibody.

For the quantification of the target proteins, duplicate problem samples were evaluated using standard curves (i.e., total protein loaded vs integrated optical density, IOD) which consisted of at least four points of different protein content (usually 0.25 to 4 μ g of membrane protein, resulting in linear relationships) loaded on the same gel. The standard curves were performed by using a membrane preparation pool of five control subjects not included in the study and who presented a wide range of age, postmortem delay and freezing storage period. Given a known amount of membrane protein (PR, real protein amount) from a problem sample loaded in a gel well, the relative amount of G-protein or α -tubuline was calculated as the ratio between the amount of protein

corresponding to the IOD value of the problem sample interpolated in the control standard curve (PT, the theoretical protein amount) and PR. The relative amount of G-protein was corrected for the relative amount of α -tubuline of the stripped membrane, and given as percentage of immunoreactivity. This quantification procedure was repeated at least twice in different gels, for a total number of four measurements for each subject sample. The mean intra-assay and inter-assay coefficients of variation were $\sim 1\%$ and $\sim 10\%$, respectively.

Data Analysis

Data are presented as mean \pm S.E.M. values. Concentration-response curves for the agonist-induced stimulation of the [35 S]GTP γ S binding were performed and the pharmacological parameters were calculated by nonlinear regression analysis using the PrismTM software, (GraphPad Inc., San Diego, CA). The theoretical maximal effects fitted by the curves and the concentration of the agonist that determines the half-maximal effect (EC_{50}) were obtained. EC_{50} values were normalized as $\log EC_{50}$ to be analyzed. Agonist-stimulated [35 S]GTP γ S binding is also presented as percentage of the maximal stimulation over basal, ((maximal effect minus basal) \times 100/basal), and as the maximal net stimulation (maximal effect minus basal). Student's 2-tailed *t*-test was used for statistical evaluations. The selection between binding models was made by the extra sum of squares principle (F-test). Linear regressions were calculated by the method of least squares and Pearson's coefficient for simple correlation was calculated to test for possible association among variables. The possible association between G-protein densities and [35 S]GTP γ S binding parameters was evaluated by a partial correlation analysis considering [35 S]GTP γ S binding parameters as dependent variable and both G-protein densities and age, postmortem delay or storage period as independent variables. The level of significance was chosen as $p = .05$.

Materials

[35 S]guanosine 5'-(γ -thio)triphosphate (GTP γ S) (1250 Ci/mmol) was purchased from DuPont NEN (Brussels, Belgium). Aprotinine was purchased from Bio-Rad Laboratories. Bovine serum albumin (BSA), carbachol, [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO), (\pm)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), DL-dithiothreitol (DTT), GDP, GTP γ S, N-ethylmaleimide (NEM), β -mercaptoethanol, sodium dodecylsulphate (SDS), timorolal and Tween-20 were purchased from Sigma (St. Louis, Mo, USA). Baclofen and 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14304) were obtained from Tocris Cookson Inc. (Bristol, UK). Anti- G_{α} -protein polyclonal antibodies raised against specific C-terminal peptides were purchased from Dupont NEN.

Anti- α -tubuline monoclonal antibody (clone DM 1A) was from Sigma. Secondary antibodies and ECL reagents were from Amersham. All other chemicals were obtained from standard sources and were of the highest purity commercially available.

RESULTS

Modulation of Receptor-mediated Stimulation of [35 S]GTP γ S Binding to G-proteins and G_{α} -protein Immunoreactive Levels in Brain during Aging

Basal levels of [35 S]GTP γ S binding to postmortem human brain membranes were negatively correlated

with age (Figure 1, Panel A, Table 1). With this linear decay model, the percentage of decrease of basal [35 S]GTP γ S binding per decade was 4% relative to the estimated binding at the birth time.

The α_2 -adrenoceptor, 5-HT $_{1A}$ -serotonin, and GABA $_B$ receptor agonist-mediated stimulations showed positive correlations between their normalized EC $_{50}$ values and age (i.e., the concentration-response curves were shifted to the right with increasing age) (Figure 1, Panels B and C, Figure 2, Panel A, Table 1). In marked contrast, net stimulations did not correlate significantly with age (Figure 1, Panel D for UK14304, Table 1). The concentration-response curves of [35 S]GTP γ S binding stimulation by 8-OH-DPAT fitted to a biphasic model ($F_{2,569} = 7.05$; $p < .001$) show-

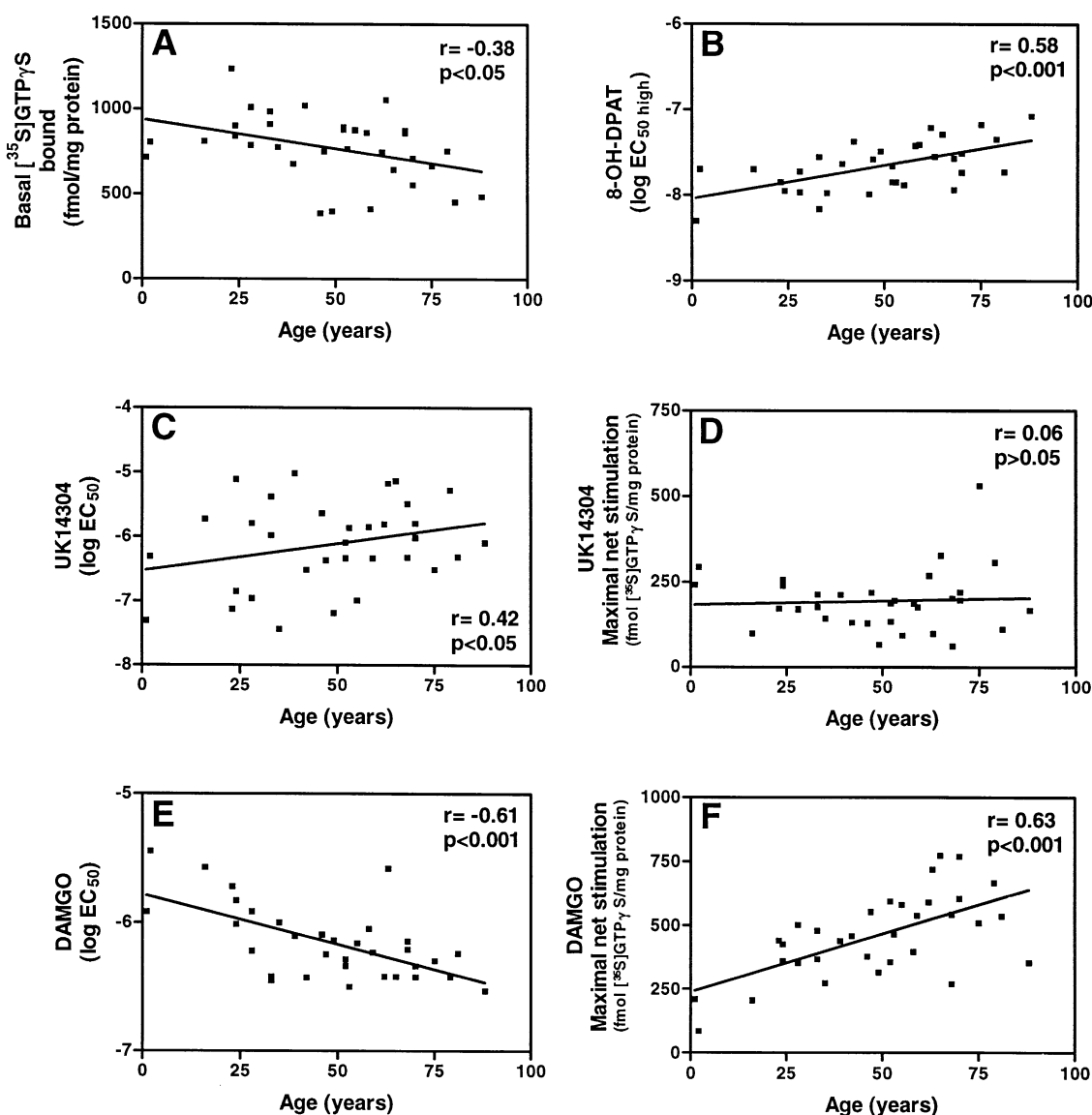


Figure 1. Age-related changes in (A) basal and (B-F) agonist-stimulated [35 S]GTP γ S binding to postmortem human brain cortical membranes. Concentration-response curves to selective agonist drugs were performed to obtain maximal stimulation and EC $_{50}$ values. Maximal net stimulation (stimulated minus basal) and normalized EC $_{50}$ ($\log EC_{50}$) were calculated and expressed in linear relation to the age at death of the subjects. Estimated regression lines are represented.

Table 1. Influence of Age, Postmortem Delay (PMD) and Freezing Storage Period on Agonist-Mediated Stimulation of [³⁵S]GTPγS Binding to Postmortem Human Brain Membranes

	UK14304 (α _{2A} -adrenoceptor)			DAMGO (μ-opioid receptor)			8-OH-DPAT (5-HT _{1A} receptor)			Baclofen (GABA _B receptor)		Carbachol (muscarinic receptor)	
	Basal	NStim	log EC ₅₀	NStim	log EC ₅₀	NStim	log EC _{50H}	log EC _{50L}	%H	NStim	log EC ₅₀	NStim	log EC ₅₀
Age	-.38*	.06	.42*	.63***	-.61***	.10	0.58***	-.006	.18	.005	.50**	-.51**	-.05
PMD	-.03	.32	.06	.005	-.21	.23	.17	.001	.19	.19	-.07	.21	-.14
Storage	-.30	.29	.22	.24	-.15	.29	.24	-.02	.33	.04	.22	.03	.09

Values represent Pearson's correlation coefficients between [³⁵S]GTPγS binding pharmacological parameters and age, postmortem delay (hours) or storage period at -70°C (months). Concentration-response curves to selective agonists were performed by triplicate in 34 (20 female, 14 male) human brain samples as shown in subjects and methods. The influence of age (range 1–88 years), PMD (range 8–92 h) and storage period (range 1–85 months) on basal [³⁵S]GTPγS binding, normalized EC₅₀ (log EC₅₀) and net stimulations (NStim) achieved by the agonists were studied by linear correlation analysis. 8-OH-DPAT showed a biphasic curve stimulating [³⁵S]GTPγS binding (see Results) and correlation coefficients for high-affinity EC₅₀ (EC_{50H}), low-affinity EC₅₀ (EC_{50L}) and high-affinity site fraction (%H) were obtained.

**p* < .05.

***p* < .01.

****p* < .001.

ing a high-affinity EC₅₀ (30.3 ± 1.3 nM; EC_{50 high}) and a low-affinity EC₅₀ (59.7 ± 6.6 μM; EC_{50 low}). Representative individual curves are displayed in Figure 2, Panel A. Only the EC_{50 high} values showed a significant linear correlation with age (Table 1).

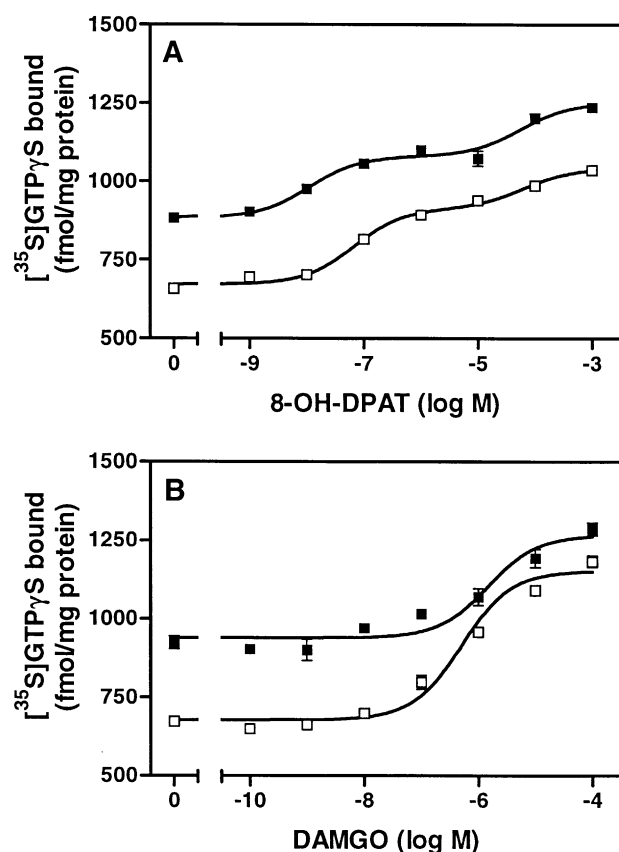


Figure 2. Representative concentration-response curves of the stimulation of [³⁵S]GTPγS binding to postmortem human brain cortical membranes by 8-OH-DPAT (A) and DAMGO (B). Data correspond to a 24-year-old male (■) and a 75-year-old female (□).

In relation to age at death, the [³⁵S]GTPγS binding stimulation by the μ-opioid receptor agonist DAMGO displayed a negative correlation for normalized EC₅₀ values and a positive correlation for net stimulation values (Figure 1, Panel E and F, Table 1). Therefore, the concentration-response curves for DAMGO were shifted to the left and showed enhanced efficacy with increasing age (Figure 2, Panel B). According to this linear model, the average increase per decade for the DAMGO-induced net stimulation was 19% relative to the estimated value of net stimulation at the birth time.

Net stimulations reached by carbachol in [³⁵S]GTPγS binding to postmortem human brain membranes were negatively correlated with age (Table 1). On the contrary, no significant correlation was found between normalized EC₅₀ values for carbachol and age. The average decrease of the carbachol-induced net stimulation per decade was 6% relative to the estimated value of net stimulation at the birth time.

In the prefrontal cortex, the immunoreactive densities of G_{αi1/2}- and G_{αo}-proteins negatively correlated with age, although in the case of G_{αo}-proteins the correlation did not reach the level of statistical significance (Figure 3, Table 2). There was no significant correlation between age at death and the levels of G_{αi3}- or G_{αs}- (52 kDa isoform) proteins (Table 2). With this linear model, the average decrease per decade for the G_{αi1/2}-protein density was 4% relative to the estimated value at the birth time.

Relation between G_α-protein Immunoreactivity and [³⁵S]GTPγS Binding

Functional receptor parameters of [³⁵S]GTPγS binding were correlated with G_α-protein densities. Only basal [³⁵S]GTPγS binding to cortical membranes displayed a positive simple correlation with G_{αi1/2}-protein immunoreactive levels (*r* = 0.39; *p* < .05). As reported above, both basal [³⁵S]GTPγS binding and G_{αi1/2}-protein levels were

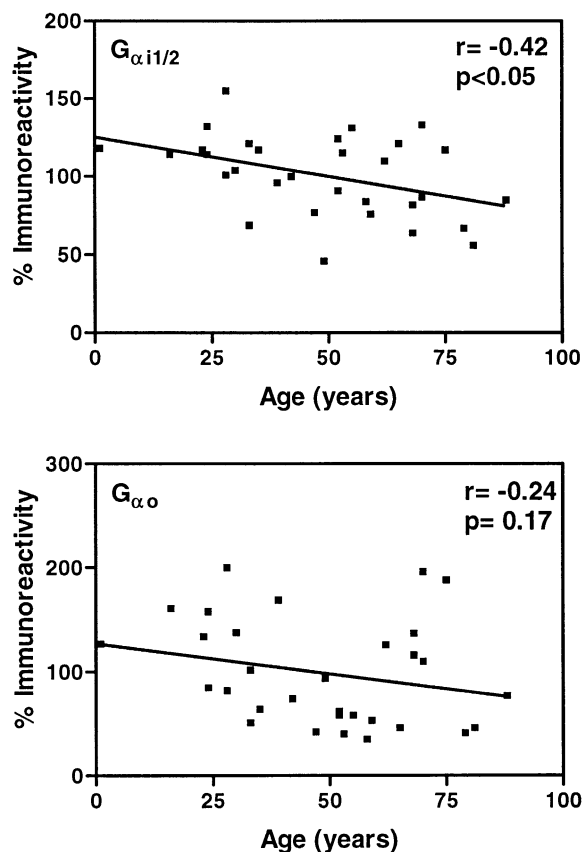


Figure 3. Age-related changes in the immunoreactive levels of $G_{\alpha i1/2}$ and $G_{\alpha o}$ -proteins in cortical membranes. The levels of G_{α} -proteins were quantified by immunoblotting detection (Western blot) and extrapolation from standard curves. Estimated regression lines are represented.

negatively correlated with age (Figure 1, Panel A, and Figure 3). Therefore, further statistical analyses were performed in order to control for possible cross correlations between these three parameters (i.e., age, basal [35 S]GTP γ S binding, and $G_{\alpha i1/2}$ -protein levels). Partial correlation analysis between basal [35 S]GTP γ S binding (dependent variable), and age and $G_{\alpha i1/2}$ -protein levels (independent variables) showed that basal [35 S]GTP γ S binding was not correlated with $G_{\alpha i1/2}$ -protein density ($r = 0.23$; $p > .05$) but only negatively with age ($r = -0.39$; $p < .05$). Therefore basal [35 S]GTP γ S binding does not seem to be a direct reflect from $G_{\alpha i1/2}$ -protein density.

Effect of Postmortem Delay on Receptor-Mediated Stimulation of [35 S]GTP γ S Binding to G-proteins and G_{α} -protein Immunoreactive Levels in Brain

Basal levels of [35 S]GTP γ S binding to human brain membranes did not correlate significantly with the postmortem delay (Table 1). Moreover, none of the functional receptor parameters for the [35 S]GTP γ S binding stimulations induced by the different agonists showed a correlation with

Table 2. Influence of Age, Postmortem Delay (PMD) and Freezing Storage Period on G_{α} -protein Immunoreactive Levels in Postmortem Human Brain Membranes.

	$G_{\alpha i1/2}$	$G_{\alpha i3}$	$G_{\alpha o}$	$G_{\alpha s}$
Age	-.41*	-.0002	-.24	.18
PMD	-.28	-.2	-.11	-.23
Storage	.13	-.08	.13	.25

Values represent Pearson's correlation coefficients between G_{α} -protein immunoreactive levels and age, postmortem delay (hours) or storage period at -70°C (months). The density of $G_{\alpha i1/2}$, $G_{\alpha i3}$, $G_{\alpha o}$, and $G_{\alpha s}$ (52 kDa)-proteins were measured in duplicate by immunoblot analysis in 34 postmortem human brain samples (20 female, 14 male) as shown in subjects and methods. The influence of age (range 1–88 years), PMD (range 8–92 h) and freezing storage period (range 1–85 months) on G_{α} -protein immunoreactivities were studied by correlation analysis. * $p < .05$.

the postmortem delay within the range (8–92 h) of values analyzed in the present study (Table 1).

No significant linear correlation was observed between immunoreactive levels of any of the G_{α} -proteins and the postmortem delay, although the regressions displayed negative slopes for all the G -protein α subunits evaluated (Table 2).

Effect of Freezing Storage Period on Receptor-Mediated Stimulation of [35 S]GTP γ S Binding to G-proteins and G_{α} -protein Immunoreactive Levels in Brain

In the absence of agonist, basal [35 S]GTP γ S binding to cortical brain membranes showed a weak negative correlation ($r = -0.30$; $p = .08$) with the freezing storage period of the samples (1–85 months) at -70°C (Figure 4, Panel A, Table 1). With this linear model, the average decrease of basal [35 S]GTP γ S binding per year of storage at -70°C for the human brain samples was 3% relative to the estimated binding at the moment of storage. However, the normalized EC_{50} values and the net stimulation values elicited by the different agonists did not show a significant correlation with the storage time (Figure 4, Panel B for baclofen, Table 1). Therefore, the rate of stimulation (unmodified parameter) over basal levels (tendency to be decreased) presented significant correlations with freezing storage period for all the studied agonists (see Figure 4, Panel C, for baclofen).

The time of tissue storage (1–85 months) at -70°C did not affect significantly the immunoreactivity of any of the analyzed G_{α} -proteins (Table 2).

DISCUSSION

The decline in cognitive and motor capabilities that often accompanies aging might represent the functional consequence of a decrease in the activities of signal transduction processes at neuronal level. Furthermore, age-related ef-

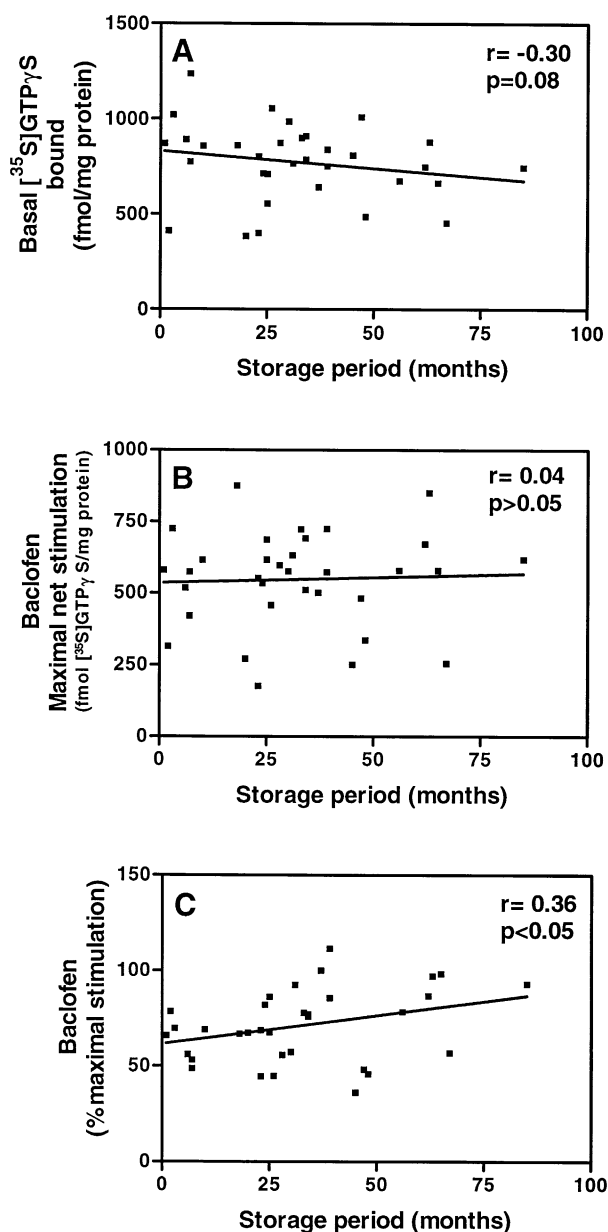


Figure 4. Storage-related changes in (A) basal [³⁵S]GTPγS binding to postmortem human brain cortical membranes, (B) maximal net stimulation of [³⁵S]GTPγS binding induced by the GABA_B receptor agonist baclofen, and (C) maximal stimulation induced by baclofen expressed as percentage over basal values. Concentration-response curves to selective agonist drugs were performed to obtain maximal stimulation and EC₅₀ values. Maximal net stimulation (maximal stimulation minus basal) and percentage of maximal stimulation over basal ((maximal stimulation – basal) × 100/basal) were calculated and expressed in linear relation to the period of storage of the samples at –70°C. Estimated regression lines are represented.

fects on G-protein density and/or activity have been suggested to be an important factor that interplay with other pathophysiological conditions in the manifestations of neuropsychiatric disorders whose incidence increase with advancing age (Scherman et al. 1989; Meana et al. 1992; Calne 1994; Cutler et al. 1994; Fülöp and Seres 1994; Greenwood et al. 1995; Li et al. 1996; Cowburn et al. 1996; Spiegel 1996). In this context, the present study has evaluated by means of both functional [³⁵S]GTPγS binding and Western blot techniques the influence of aging on the G-protein signal transduction system. The influence of important variables in postmortem human brain studies such as the postmortem delay and the freezing storage period were also analyzed.

Basal G-protein activity (i.e., [³⁵S]GTPγS binding in the absence of agonist) was found to progressively decrease with the age of the subject at death. This observation extends previous findings indicating that the basal activity of G-protein/adenylyl cyclase signal transduction pathway declines with aging (Cowburn et al. 1992; Ozawa et al. 1999). A similar inverse relationship was found between the immunoreactivity of G_{ai1/2}-proteins and age, while the immunoreactivities of other G-protein α subunits were not significantly changed with age. The finding should not be simply interpreted as basal [³⁵S]GTPγS binding representing functional activity of G_{ai1/2}-proteins. In fact, when functional binding and immunoreactive values for G_α-proteins were controlled for the confounding effect of age, the significant relationship between basal [³⁵S]GTPγS binding and G_{ai1/2}-protein levels was not observed. Therefore, it is likely that basal [³⁵S]GTPγS binding in postmortem human frontal cortex involves basal activity of multiple G_α-proteins, and particularly of the pertussis toxin-sensitive G_{ai/o}-proteins (González-Maeso et al. 2000). Declines of G_{ai}-protein (Young et al. 1991; Cowburn et al. 1994), and G_{ai1/2}-protein (Sastre and García-Sevilla 1994; Ozawa et al. 1999; Sastre et al. 2001) immunoreactivity have been previously shown in human brain. The immunoreactive levels of G_{ai3}-proteins have also been found to decrease with aging (range 1 month–89 years, n = 21) (Sastre et al. 2001). On the contrary, Li et al., (1996) have reported no age-related changes on G_{ai1/2}-proteins in prefrontal cortex (range 19–100 years, n = 13). The levels of G_{ao}-proteins presented a non-significant trend to negatively correlate with age in a similar way to previous findings in the same brain area (Li et al. 1996; Ozawa et al. 1999; Sastre et al. 2001). In contrast, in parietal cortex, no age-related changes of G_{ao}-proteins levels have been shown (range 3 days to 92 years, n = 18) (Young et al. 1991). G_{as}-proteins (52 kDa isoform) immunoreactive levels presented a slight but not significant increase with aging. Similar results have been reported for G_{as}-proteins in prefrontal cortex (Li et al. 1996) with significant positive correlation in one study (Cowburn et al. 1994). On the other hand, an in-

verse relationship between $G_{\alpha s}$ -protein densities and age has also been reported in frontal (Ozawa et al. 1999; Sastre et al. 2001), and parietal (Young et al. 1991) brain cortex. The discrepancies between studies might be a consequence of the brain area analyzed, the type of antibody used for the immunodetection, the number of subjects included in the analysis or the distribution and range of their age at death. In the present work, a larger number of individuals ($n = 34$) presenting well spread ages (1–88 years) were included in order to improve the discrimination power of the statistical analysis.

The human brain contains the three subtypes of α_2 -adrenoceptors (α_{2A} , α_{2B} , α_{2C}) although the predominant one, especially in the prefrontal cortex, is the α_{2A} -adrenoceptor subtype (Grijalba et al. 1996). The present results show a decrease in the potency of the α_2 -adrenoceptor agonist UK14304 to stimulate G-proteins with increasing age (i.e., increase of the EC_{50} values), whereas [^{35}S]GTP γ S binding net stimulation reached by the agonist did not show any correlation. Age-related decreases in the density of α_2 -adrenoceptors without changes in affinity have been reported by using radiolabeled agonists (Pascual et al. 1991; Meana et al. 1992) and antagonists (Sastre and García-Sevilla 1994). These results taken together with those herein described imply that a reduction in the high-affinity conformation of the receptor originates a decrease in the potency, but not in the efficacy, of the α_{2A} -adrenoceptor agonist UK14304 to promote signal transduction mechanisms. Because of the predominant presynaptic location of α_{2A} -adrenoceptors, the age-related decreases of the UK14304 potency to stimulate [^{35}S]GTP γ S binding and the previously reported declines of receptor density (Pascual et al. 1991; Meana et al. 1992) could be related to the loss of noradrenergic nerve terminals arising from the *locus coeruleus*, which are known to degenerate with aging (Vijayashankar and Brody 1979; Mann et al. 1983).

An increase in the efficacy of the μ -opioid receptor agonist DAMGO to activate G-proteins ([^{35}S]GTP γ S net stimulation) is presented with aging; furthermore, the potency of DAMGO to promote guanine nucleotide exchange on G_{α} -protein subunits is increased with advancing age. Previous studies have also demonstrated an age-related increase of μ -opioid receptor density in human brain by using [3H]DAMGO binding (Gabilondo et al. 1995), and of μ -opioid receptor binding potential (B_{max}/K_D) by in vivo positron emission tomography (PET) (Zubieta et al. 1999). Therefore, the possibility arises that enhanced μ -opioid receptor-mediated activity of G-protein is related to an overexpression of μ -opioid receptors with increased functional coupling to G-proteins.

The concentration-response curves to the 5-HT $_{1A}$ -serotonin receptor agonist 8-OH-DPAT showed a biphasic pattern stimulating [^{35}S]GTP γ S binding in human prefrontal cortex and only the potency of the high-affin-

ity component displayed a decrease with aging. Further work is needed to clarify the identity of the low-affinity component of 8-OH-DPAT-mediated stimulation of [^{35}S]GTP γ S binding in human prefrontal cortex. These results are concordant with those reported previously showing a decrease of 5-HT $_{1A}$ -serotonin receptor density in the human brain cortex (Dillon et al. 1991; Burnet et al. 1994). However, [^{35}S]GTP γ S binding net stimulation induced by 8-OH-DPAT did not correlate with age, which suggest that the decline of the agonist activity is related more to the disappearance of receptors expressed by neurons degenerating progressively with aging than to a loss of their functional coupling to G-proteins.

Similar behavior to that presented by UK14304 and 8-OH-DPAT (i.e., decreased potency as age increases without changes in [^{35}S]GTP γ S binding net stimulation) was shown by the GABA $_B$ receptor agonist baclofen. Although no previous information is available about human GABA $_B$ receptor evolution with aging, a decrease between postnatal day 28 and adulthood has been described in rat brain (Turgeon and Albin 1994; Happe et al. 1999). In contrast to the above mentioned receptors, the acetylcholine muscarinic agonist carbachol displayed a negative correlation between net stimulation of [^{35}S]GTP γ S binding and age at death without changes in EC_{50} values. The finding likely represents the activation of G-proteins by different muscarinic M receptors which could be differently regulated and expressed during aging. Non-selective decreases of the in vitro muscarinic receptor density (Rinne 1987; Giacobini et al. 1989; Giacobini 1990; Nordberg et al. 1992) and in vivo (PET) binding potential (Dewey et al. 1990; Lee et al. 1996) have been reported with increasing age in human brain cortex. Since muscarinic M receptors are coupled to pertussis toxin-sensitive ($G_{\alpha i/o}$) and pertussis toxin-insensitive ($G_{\alpha q/11}$) G-proteins, further studies with selective agonists are needed to assess the biochemical and functional regulation of the different M receptor subtypes during the process of aging.

Neuropsychiatric disorder studies in postmortem human brain tissue are performed usually by matching cases and controls by sex and age of the subjects. Inherent variables such as the postmortem delay or the freezing storage period may produce changes that could alter the result of the analysis (Perry and Perry 1983; Whitehouse et al. 1984; Burke and Greenbaum 1987; Rodríguez-Puertas et al. 1996; Paul et al. 1997). In this context, the present data show that the postmortem delay did not affect either the receptor-mediated activity or the immunoreactive levels of G-proteins within the range analyzed (8–92 h). It seems that within these time limitations, no important effects of postmortem delay on receptor densities and activities are induced when the corpse is stored early at refrigeration temperature ($\sim 4^\circ C$) (Whitehouse et al. 1984; Rodríguez-Puertas et al.

1996; Paul et al. 1997; Palego et al. 1999). Immunoreactive levels of G_{α} -proteins have been shown not to be affected by the postmortem delay at 4°C (Young et al. 1991; Escribá et al. 1994; Sastre and García-Sevilla 1994; Dowlatshahi et al. 1999) but some groups have reported decreases of $G_{\alpha o}$ - (Li et al. 1996), and increases of $G_{\alpha i}$ -protein (Cowburn et al. 1994) immunoreactivities.

The effect of storage time of the human brain samples at -70°C was analyzed and only basal [^{35}S]GTP γ S binding showed a weak negative correlation with storage period without being affected EC_{50} or net stimulation values achieved by the different agonist drugs. The immunoreactivity of G_{α} -proteins was not altered by storage periods. In general, it is assumed that most biochemical activities are stable for several years when tissue is stored at very low temperature (-70°C or less) (Perry and Perry 1983) whereas decreases of the receptor density have been clearly demonstrated when postmortem human brain samples are stored at -25°C (Rodríguez-Puertas et al. 1996).

To our knowledge, this is the first study evaluating the influence of age, postmortem delay and freezing storage time on functional [^{35}S]GTP γ S binding to postmortem human brain. The present findings further demonstrate the influence of aging on the G-protein signal transduction system. The data also confirm the importance of examining potential artifacts that may alter receptor-G-protein coupling and, thus, impair a clear interpretation of the specific role of transduction mechanisms in different neuropsychiatric diseases.

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