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Influence of age, postmortem delay and freezing storage period on cannabinoid receptor density and functionality in human brain

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Abstract

It has been suggested that cannabimimetic drugs could be of interest in the treatment of several nervous disorders. Thus, it is important to analyse the distribution and properties of cannabinoid (CB) receptors directly in human brain. As postmortem human tissue is subjected to the effects of several biological variables, we have analyzed by autoradiography the influence of age, postmortem delay and freezing storage period (at −25 °C) on two parameters corresponding to cannabinoid CB_1 receptors in human frontal cortex: receptor density and degree of activation of G-proteins ([^{35}S]GTPγS assays). A significant decrease in the amount of both receptor density and agonist-stimulated G-protein activity was observed with age, revealing a mean reduction of about 10% per decade. In contrast, no significant correlations were found with postmortem delay either for CB_1 receptors density or functionality. Finally, both parameters (receptor density and [^{35}S]GTPγS response) were significantly reduced with freezing storage period at −25 °C in frontal cortical layers. Non-linear analysis of these data yielded values between 12 and 24 months of storage for a 50% reduction. In conclusion, when studying CB_1 receptor properties in human brain samples, a careful analysis (and matching) for variables such as age and freezing storage period has to be carried out.

Keywords: Autoradiography; Age; Storage; Cannabinoid receptors; G-proteins; Human brain

1. Introduction

Preparations of *Cannabis sativa*, such as marijuana and hashish, have been used for medicinal and recreational purposes for at least 4000 years, and nowadays cannabis derivatives are still among the most commonly used illegal drugs in the US (Adams and Martin, 1996). The major psychoactive constituent of *C. sativa* preparations, Δ^9-tetrahydrocannabinol (Δ^9-THC), as well as other natural and synthetic compounds with cannabimimetic activity are known to bind to and activate at least two G-protein-coupled receptors, named CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993). Most of the central nervous system (CNS) effects of cannabinoid (CB) agonist are mediated by the CB_1 receptor, which is highly expressed in the human brain (Glass et al., 1997). Several studies suggest that biochemical and functional alterations of CB_1 cannabinoid receptor may be implicated in the pathophysiology of distinct neurological and psychiatric disorders, such as Parkinson's disease (Lastres-Becker et al., 2001), Huntington chorea (Richfield and Herkenham, 1994; Lastres-Becker et al., 2002), Alzheimer's disease (Westlake et al., 1994; Fernández-Ruiz et al., 2002), schizophrenia (Dean et al., 2001; Ujike et al., 2002) or major depression (Mato et al., 2001). Thus, the possibility exists for a therapeutic use of CB compounds in these disorders. Furthermore, clinical studies with cannabimimetic drugs have been carried out for the treatment of chronic pain (Campbell et al., 2001), spasticity (Consroe et al., 1997), Tourette's syndrome (Muller-Vahl et al., 2003), migraine (Russo, 1998) and epilepsy (Cunha et al., 1980) among other neurological indications. On the other hand, the possible relationship between the chronic abuse of cannabis and the increased risk for schizophrenic symptoms is a matter of important debate (Zammit et al., 2002).

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Because of this, the detailed knowledge of the properties and distribution of CB receptors in the human brain has become of increasing interest.

In order to demonstrate that a disease process results in modifications in receptor density and/or functionality in human brain, it is necessary to control the possible influence of variables which are thought to alter these parameters. In this context, neurochemical postmortem studies carried out in postmortem tissue present several methodological problems, including the influence of age, the delay between death and tissue dissection and storage (postmortem delay, PD) and several factors related to sample storage prior to assay, such as duration and temperature (freezing storage period, FSP). The responsiveness to many neurotransmitters is known to be modified during senescence, and some of these alterations have been consistently associated with reduced receptor and/or G-protein densities (Dewey et al., 1990; Young et al., 1991; Sastre and García-Sevilla, 1994; Arranz et al., 1993; Li et al., 1996). In contrast, several studies suggest that human brain neurotransmitter receptors are remarkably resistant to long PD at 4 °C (Westlake et al., 1994; Pazos and Palacios, 1989; Rodríguez-Puertas et al., 1996). In the same way, González-Maeso et al. (2002) have recently reported no influence of PD at this temperature on basal or agonist-stimulated [^{35}S]GTPγS binding in human brain. With respect to CB_1 cannabinoid receptors, several studies indicate that the protein and messenger RNA level are disminished in aged rat and human brain (Westlake et al., 1994; Romero et al., 1998). Romero et al. (1998) have also shown a loss of CB agonist-stimulated [^{35}S]GTPγS binding in the brain of aged rats, but this issue has still to be analyzed in postmortem human brain. On the other hand, the influence of PD on the properties of CB receptors in the human species has not yet been analyzed. Finally, long FSP are common in biochemical and morphological studies of neuropsychiatric disorders in postmortem human brain, due to the time necessary to collect a reasonable number of cerebral samples. Furthermore, autoradiographic studies in human brain usually require keeping the tissue blocks stored at −25 °C after the first sectioning process (Kontur et al., 1994; Rodríguez-Puertas et al., 1996), in order to avoid tissue artifacts due to a repeated freezing–thawing process. Nevertheless, data about FSP effects on neurotransmitter receptor levels and functionality are still scarce. Rodríguez-Puertas et al. (1996) have shown a significant decrease of muscarinic receptor density related to the storage of the human brain samples at −25 °C, but there is no information about FSP effects on CB_1 receptor levels and G-protein coupling ability in postmortem human brain.

The purpose of this study was to examine the influence of aging, PD and FSP on CB_1 receptor density and functionality in a large number of postmortem human samples from patients who had died without evidence of neuropsychiatric disorders. In order to gain a high level of anatomical resolution, the study was carried out by means of autoradiographic techniques.

2. Materials and methods

2.1. Subject selection and brain samples

Human brains were obtained from 31 subjects (18 men and 13 female, age = 22–74 years; PD at 4 °C = 0–66 h; FSP at −25 °C for [^{3}H]CP55940 autoradiography = 2–91 months; FSP at −25 °C for [^{35}S]GTPγS autoradiography = 0–89 months) without any record of neurological or psychiatric disorders and who had mainly died by sudden accidents (n= 18). Other causes of death included neoplasia (n= 10), myocardial infarction (n= 2) and asphyxia (n= 1). These subjects showed a negative test on the toxicological screening for psychotropic drugs and alcohol.

The brains were obtained and removed at the Department of Pathology, University Hospital "Marqués de Valdecilla". The procedures for obtention and handling were approved by the Ethical Research Committee of this Institution. Blocks containing the frontal cortex (Brodmann area 9) were promptly dissected and stored at −25 °C. Consecutive tissue sections were cut at −25 °C using a microtome-cryostat, mounted on gelatinized slides, and stored at −25 °C until assayed.

2.2. Cannabinoid receptor autoradiography

CB receptor autoradiography in postmortem human brain was carried out by the incubation of consecutive 15 μm-thick sections in the presence of the CB agonist [^{3}H]CP55940 (Dupont/NEN; specific activity 125 Ci/mmol). The incubation procedure was based on the method described by Glass et al. (1997) with modifications (Mato et al., 2001). Sections were incubated for 2 h at 37 °C with 3 nM [^{3}H]CP55940 in a 50 mM Tris–HCl buffer (pH 7.4) containing 5% BSA. Non-specific binding was determined in the presence of 10 μM WIN55212-2 (RBI, Natick, MA, USA). Following the incubation, the sections were washed twice for 2 h at 4 °C each, in a 50 mM Tris–HCl buffer (pH 7.4) with 1% BSA, and dipped briefly in distilled water. Finally, the sections were dried on a cold air-stream.

Autoradiograms were generated by apposing the labelled tissues to tritium-sensitive films ([^{3}H]-Hyperfilm, Amersham, Buckinghamshire, UK) together with ^{3}H polymer standards (Amersham microscales). The films were developed after a 15 day exposure at 4 °C. After the scanning of the films, the autoradiograms were analyzed as described by Unnerstall et al. (1982),

using a computerized image analysis system (NIH-IMAGE program, Bethesda, MA, USA).

2.3. [^{35}S]GTPγS autoradiography

[^{35}S]GTPγS (Dupont/NEN; specific activity 125 Ci/mmol) binding to human brain slices was performed according to the protocol described by Sim et al. (1996), with several modifications (Rodríguez-Puertas et al., 2000). Twenty micrometer-thick sections were preincubated for 30 min at 25 °C in a buffer containing 50 mM Tris–HCl, 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 2 mM GDP, 0.5% BSA (pH 7.7) and then incubated for 120 min in the same buffer containing 0.04 nM [^{35}S]GTPγS. Non-specific binding of the radioligand was determined by isotope dilution in the presence of 10 μM GTPγS. The CB agonist-stimulated binding was measured under the same conditions in the presence of 100 μM WIN55212-2. The specificity of the CB_1 receptor-mediated stimulation was verified by coincubation with 10 μM SR141716A (kindly supplied by Sanofi Reserche, Montpellier, France). After the incubation, the slides were washed twice for 15 min at 4 °C each in cold 50 mM Tris–HCl buffer (pH = 7.4), and dried on cold airstream. The sections were then exposed to β radiation-sensitive films (Hyperfilm β-max, Amersham, UK) together with ^{14}C polymer standards (Amersham microscales) for 48 h at 4 °C.

2.4. Data analysis

Autoradiographic data correspond to the mean of duplicate different measures for each case. CB receptor autoradiographic densities were corrected for the specific activity of [^{3}H]CP55940 at the calibration date, and presented as *B* (binding density) in fmol/mg tissue equivalent (fmol/mg t.e.). [^{35}S]GTPγS binding data were also corrected for the specific activity of the radioligand at the calibration date, and the decay factor of ^{35}S. Basal values are presented in nCi/g tissue equivalent (nCi/g t.e.) and CB agonist-stimulation data are presented as percentage of WIN55212-2 effect over basal values ([agonist − basal] × 100/basal).

The possible association between CB receptor density and age, postmortem delay or FSP was evaluated by partial correlation analysis (SPSS 4.0®), and Pearson's coefficients (*r*) were obtained. Linear and non-linear (one phase exponential decay) regressions were calculated (GraphPad Prism 3.0 for Windows, GraphPad Software, San Diego, CA, USA) between [^{3}H]CP55940 autoradiographic densities and age or FSP by the method of least squares, and Pearson's coefficients for simple correlation were obtained. The FSP values associated with a 50% decline in CB receptor density (FSP_{50}) were calculated by both analysis methods. The comparison between analysis models was made by the extra sum of squares principle (Snedecor *F*-test).

In the same way, partial correlation analysis was performed to evaluate the possible association between basal or WIN55212-2-stimulated [^{35}S]GTPγS autoradiographic data, and age, PD or FSP. Lineal and non-linear regressions between basal or CB agonist-stimulation percentages and age or FSP were calculated and FSP_{50} values obtained. The level of significance was chosen as $p \leq 0.05$.

3. Results

3.1. Age

Partial correlation analysis showed a significant decrease of CB receptor density in human frontal cortex with age ($p < 0.01$) (Table 1). Nevertheless, linear regression analysis including all the subjects resulted in no significant variations of [^{3}H]CP55940 autoradiographic densities related to age (Fig. 1A). In contrast, when the same analysis was carried out including only the subjects with FSP below 40 months ($n = 18$), significant regression lines were obtained for all the layers of the human frontal cortex ($r = -0.49$ to -0.59; $p < 0.05$). With this linear decay model, 8–10% decreases of CB receptor density per decade relative to the stimulated binding at birth time were calculated.

On the other hand, basal [^{35}S]GTPγS binding levels in postmortem human cortex were negatively and significantly correlated with age ($p < 0.01$) (Table 1, Fig. 1B). With the linear decay model, the percentage of decrease of basal [^{35}S]GTPγS binding values per decade was 9–11% relative to the stimulated binding at birth time. In contrast, through the range of age analyzed in this study, no significant variations were found in the percentages of WIN55212-2-induced-stimulation [^{35}S]GTPγS binding (Table 1, Fig. 1C).

3.2. Postmortem delay

CB receptor density in human cortex did not significantly correlate with postmortem delay (Table 1). In the same way, neither basal nor WIN55212-2-stimulated [^{35}S]GTPγS binding levels showed a correlation with postmortem delay within the range of values analyzed in the present study (Table 1).

3.3. Freezing storage period

CB receptor densities decreased significantly with FSP in all the layers of the human frontal cortex ($p < 0.0001$) (Table 1, Fig. 2). FSP_{50} values of 32–34 months were obtained with the linear regression model. In a marked contrast, the fitting of the data to a one

Table 1
Influence of age, postmortem delay and FSP on cannabinoid receptor density, and basal or WIN55-212-2-stimulated [^{35}S]GTPγS autoradiographic levels in human frontal cortex

	CB_1 receptor density (fmol/mg t.e.)			Basal [^{35}S]GTPγS binding (nCi/g t.e.)			WIN55,212-2-stimulated [^{35}S]GTPγS (percentage of stimulation)		
	Age	PD	FSP	Age	PD	FSP	Age	PD	FSP
Layer I	−0.53*	−0.09	−0.87***	−0.55*	−0.19	−0.62**	−0.03	−0.08	−0.58*
Layer II–III	−0.58*	−0.11	−0.86***	−0.58*	−0.19	−0.52*	−0.01	−0.07	−0.58*
Layer IV	−0.58*	−0.01	−0.87***	−0.59*	−0.19	−0.52*	−0.02	−0.05	−0.63**
Layer V	−0.58*	−0.05	−0.83***	−0.49*	−0.17	−0.52*	−0.11	−0.04	−0.57*
Layer VI	−0.59*	−0.22	−0.86***	−0.52*	−0.16	−0.53*	−0.23	−0.12	−0.51*

Values represent Pearson's correlation coefficients between CB_1 receptor density, basal or WIN55212-2-stimulated [^{35}S]GTPγS binding values in human frontal cortex and age (range 22–73 years), postmortem delay (PD, range 0–66 h) and FSP at −25 °C (range 2–91 months for [^{3}H]CP55,940 autoradiography; 0–82 months for [^{35}S]GTPγS autoradiography), determined by partial correlation analysis.

* $p < 0.01$.
** $p < 0.001$.
*** $p < 0.0001$.

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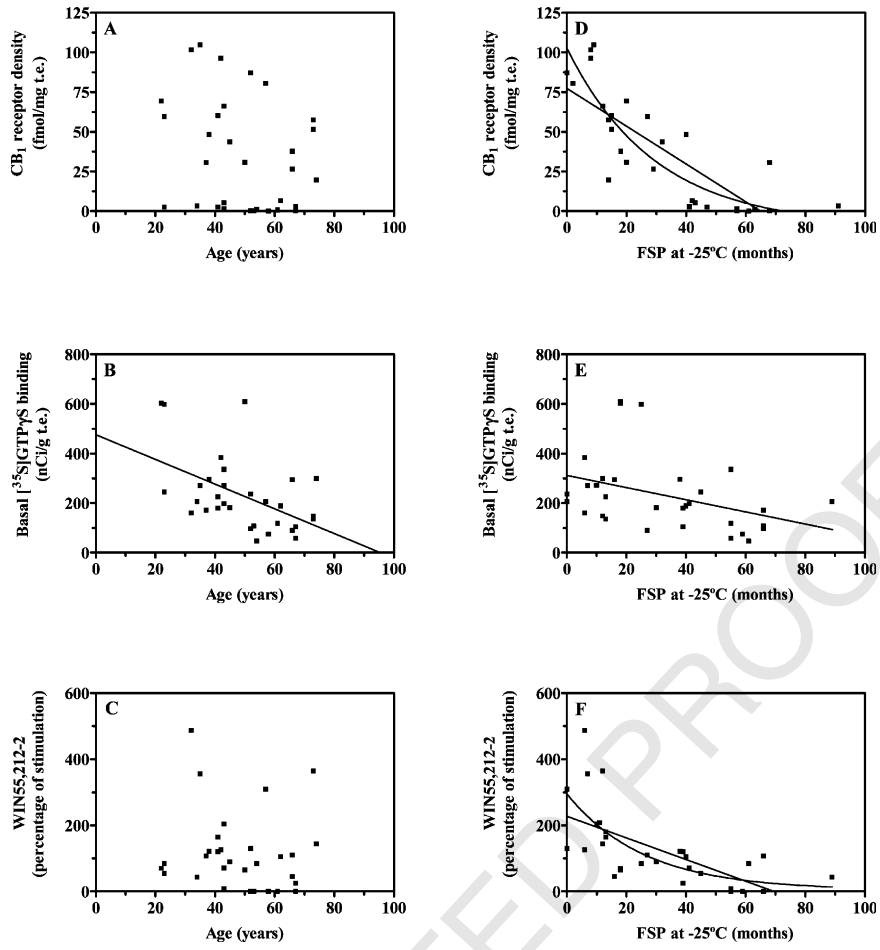

Fig. 1. Effect of age (range 22–73 years), left, and FSP at −25 °C (range 2–91 months for [^{3}H]CP55,940 autoradiography and 0–89 months for [^{35}S]GTPγS autoradiography), right, on cannabinoid receptor density (A, D) and basal (B, E) or WIN55212-2-stimulated [^{35}S]GTPγS autoradiographic levels (C, F) in the layer IV of human frontal cortex. Individual values (■) and significant regression lines are shown. The comparison between the linear and non-linear regression models resulted in a better fitting of CB_1 receptor density and WIN55,212-2-stimulation values to FSP through the one phase exponential decay model.

phase exponential decay model yielded relative lower FSP_{50} values, ranging from 14 to 17 months of storage at −25 °C (Table 2, Fig. 1D). The comparison between both analysis methods resulted in a better fitting of CB receptor density–FSP values relationship to the non-linear regression model ($p< 0.001$).

On the other hand, basal [^{35}S]GTPγS levels showed a negative correlation with FSP (Table 1, Fig. 3). Fitting of the data to the linear regression model yielded FSP_{50} values of 56–68 months (Table 2, Fig. 1E). In the same way, FSP significantly decreased WIN55,212-2-induced stimulation of [^{35}S]GTPγS binding in human frontal cortex (Table 1, Fig. 3), FSP_{50} values of 35–47 months being obtained (Table 2). In the latter case, the comparison between linear and one phase exponential decay analysis models resulted in a better fitting to the exponential decay model in the layers IV–VI of the frontal cortex ($p< 0.05$). Again, the non-linear regression model yielded relative lower FSP_{50} values for these layers (12–18 months) (Table 2, Fig. 1F).

4. Discussion

Cannabinoid CB_1 receptors have been implicated in the pathophysiology of several neurological and psychiatric syndromes, including spasticity, pain, epilepsy and pychiatric disorders (see Introduction), suggesting a possible role for cannabimimetic drugs in their treatment (Consroe et al., 1997; Russo, 1998; Campbell et al., 2001; Croxford, 2003). However, most of the information on the characteristics of brain CB receptors comes from studies performed on animal models. In this regard, studies carried out on human tissue samples would be of special interest from both the clinical and the pharmacological point of view. Nevertheless, the results of these studies are subjected to the influence of a series of variables, such as age, PD and

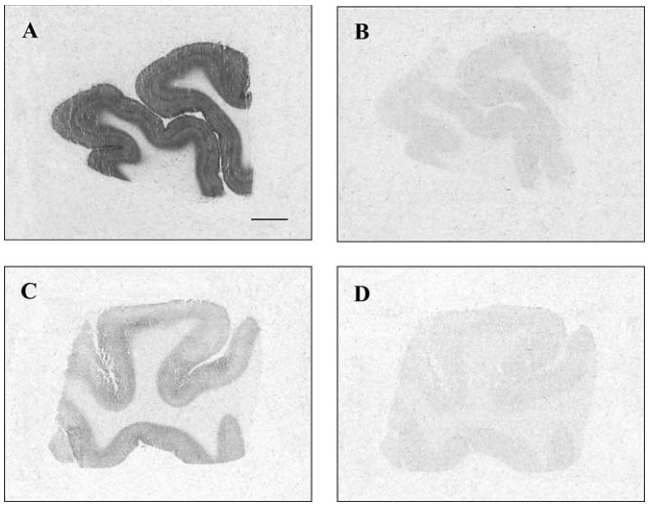

Fig. 2. Autoradiographic images corresponding to cannabinoid receptors in postmortem human frontal cortex. (A, B) case with a short FSP at −25 °C (2 months); (C, D) case with a long FSP at −25 °C (41 months). (A, C) total [^{3}H]CP55940 binding; (B, D) [^{3}H]CP55940 binding in the presence of 100 μM WIN55,212-2. Note the clear decrease in [^{3}H]CP55940 labelling with increasing FSP. Scale bar = 5 mm.

the time in which the tissue remains stored. In this context, to our knowledge, this is the first autoradiographic study assessing in detail the influence of age, PD and FSP at −25 °C on CB receptor expression and functional coupling to G-proteins in the human frontal cortex.

Our partial correlation analysis suggests a loss of CB receptors in human frontal cortex with normal aging. This observation confirms and extends previous findings, indicating reduced densities of these receptors in the brain of aged rats (Berrendero et al., 1998; Romero et al., 1998) and humans (Westlake et al., 1994). This negative influence of aging on central receptors has been previously shown for several neurotransmitter receptors in animal models (Nyakas et al., 1997; Wenk and Barnes, 2000) as well as in human brain (Meana et al., 1992; Sastre and García-Sevilla, 1994; Wang et al., 1998), and probably indicates that physiological senescence results in a general loss of brain receptor proteins. Age-related CB_1 receptor reduction could theoretically be explained as a consequence of neuronal degeneration, as it has been also proposed for the decline in CB_1 receptors in the basal ganglia of aged rats (Mann et al., 1983; Romero et al., 1998).

In a similar way, our data indicate that basal G-protein activity in human frontal cortex, measured as [^{35}S]GTPγS autoradiographic levels in the absence of agonist, progressively decreases with the age of the subject. Similar results have recently been reported in membranes by González-Maeso et al. (2002). Taking into account that inhibition of adenylyl cyclase activity is one of the main mechanisms of cellular transduction for the endocannabinoid system, our results could be in line with previous observations related to an age-related decline in the basal levels of this enzyme in human brain (Cowburn et al., 1992; Ozawa et al., 1999). With respect to G-protein subunits, significant reductions of $G_{\alpha i1/2}$- and $G_{\alpha i3}$-proteins, as well as non-significant reductions in $G_{\alpha o}$-proteins, have been reported to occur in human frontal cortex with senescence (Sastre and García-Sevilla, 1994; Ozawa et al., 1999; Sastre et al., 2001; González-Maeso et al., 2002). On the contrary, other authors have reported no age-related changes on $G_{\alpha i1/2}$ (Li et al., 1996) or $G_{\alpha i3}$-proteins (Young et al., 1991) in the same region. These discrepancies might reflect differences in the type of antibody used, the distribution and range of the age of the subjects included, or even the possible influence of uncontrolled variables, as may be the case of FSP. In any case, taken together these data seem to indicate a negative influence of brain aging on $G_{\alpha i/o}$-protein expression. As basal [^{35}S]GTPγS binding in postmortem human brain samples mainly involves the activity of the $G_{\alpha i/o}$ subtype (González-Maeso et al., 2000), an age-related decrease of $G_{\alpha i/o}$-protein levels might very well contribute to the described decline in basal [^{35}S]GTPγS in human frontal cortex.

In contrast, we have found no significant modifications of CB agonist-stimulated [^{35}S]GTPγS binding in postmortem human frontal cortex with increasing age. González-Maeso et al. (2002) have recently reported that the effects of aging on the response of agonists of different systems in this functional assay depend on the receptor analyzed. They have shown a decrease in the potencies of the α_2-adrenoceptor agonist UK14304 and the 5-HT_{1A} agonist 8-OH-DPAT to activate G-proteins in the frontal cortex of aged subjects, without changes in the efficacies of both agonists. On the contrary, an increase in the potency and efficacy of the μ-opioid receptor agonist DAMGO to stimulate [^{35}S]GTPγS binding has been reported in the same study (González-Maeso et al., 2002). The absence of a significant influence of age on the CB agonist WIN55,212-2 efficacy to activate G-proteins does not correlate with the decline in CB_1 receptor expression observed in the same subjects. Moreover, previous reports have shown a decrease of WIN55,212-2 ability to stimulate [^{35}S]GTPγS binding in rat brain sections with increasing age (Romero et al., 1998). In this regard, it should be taken into account that, due to the limited availability of tissue, our studies are restricted to cortical membranes, while it has been suggested that the age-dependent decline in CB_1 functionality could be a region-selective process (Wang et al., 2003). In addition, the apparent lack of concordance between our data and those obtained in the rat may be due to several facts. First of all, the age-related decline in CB agonist efficacy reported by Romero et al. (1998) only resulted statistically significant in those brain areas

Table 2
Influence of FSP on cannabinoid receptor density, and basal or WIN55-212-2-stimulated [^{35}S]GTPγS autoradiographic levels in human frontal cortex. Values represent FSP_{50} (FSP value corresponding to a 50% decrease) in months. Comparison between linear (LR) and non-linear (one phase exponential decay, OPED) regression models

	CB_1 receptor density (fmol/mg t.e.)		Basal [^{35}S]GTPγS binding (nCi/g t.e.)		WIN55,212-2-stimulated [^{35}S]GTPγS (percentage of stimulation)	
	LR	OPED	LR	OPED	LR	OPED
Layer I	32.2	16.4***	55.7	NS	35.7	23.4
Layer II–III	32.1	16.2***	59.1	NS	34.9	24.3
Layer IV	32.4	16.5***	62.2	NS	34.8	17.9*
Layer V	32.2	14.2***	67.3	NS	38.4	16.1*
Layer VI	33.1	16.7***	65.6	NS	47.1	12.6*

Only significant regression data are shown. Both analysis models were compared through a Snedecor *F*-test. NS, not significant; LR, linear regression model; OPED, one phase exponential decay model.

** $p < 0.001$.

* $p < 0.01$.

*** $p < 0.0001$.

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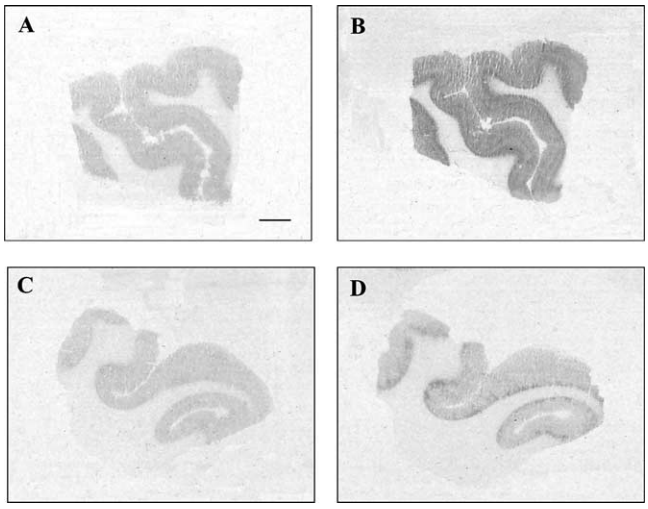

Fig. 3. Autoradiographic images corresponding to [^{35}S]GTPγS binding postmortem human frontal cortex. (A, B) case with a short FSP at −25 °C (<1 month); (C, D) case with a long FSP at −25 °C (39 months). (A, C) basal [^{35}S]GTPγS binding; (B, D) WIN55,212-2 (100 μM)-stimulated [^{35}S]GTPγS binding. Note the slight reduction in basal [^{35}S]GTPγS binding and the clear decrease in WIN55,212-2-stimulated [^{35}S]GTPγS binding with increasing FSP. Scale bar = 5 mm.

with a marked loss of CB receptors, such as the substantia nigra. On the other hand, we have not assessed the possible modifications of G-protein levels in our brain tissues as a function of age. Therefore, it is possible that the decrease in the density of CB receptors in the human cortex with increasing is not marked enough to induce a significant reduction of the CB agonist efficacy in the [^{35}S]GTPγS assay. It has been also suggested that CB receptors of the CB_1 subtype can sequester $G_{i/o}$-proteins from a common pool, preventing other $G_{i/o}$-coupled receptors from transducing their biological signals (Vásquez and Lewis, 1999). As a result, CB agonist ability to activate G-proteins might be less sensitive to a possible age-related decline in the receptor and/or in the $G_{i/o}$-protein levels than that corresponding to other neurotransmitter receptors in the human brain. It is noteworthy that WIN55,212-2 has been reported to bind to a non-CB_1 receptor in rodents (Breivogel et al., 2001; Hájos et al., 2001). The possibility exists that the stimulation of G-protein activity induced by this compound could be partially dependent on this component. However, the existence and properties of this atypical site in human brain remain to be clarified.

On the other hand, autolysis of the tissue due to the postmortem delay and to the freezing storage process should be expected to alter neurotransmitter receptors, affecting agonist binding and G-protein activation (Perry and Perry, 1983; Whitehouse et al., 1984; Paul et al., 1997; Palego et al., 1998). Both the temperature and the duration of the tissue storage period before and after the freezing of the samples are considered key factors in the tissue degradation processes (Whitehouse et al., 1984; Rodríguez-Puertas et al., 1996). Our results indicate that the PD within the range analyzed in this study (0–66 h at 4 °C) does not significantly affect either CB receptor density or CB agonist activation of G-proteins in human frontal cortex. In a similar way, we have found no correlation between basal G-protein activity and the PD of the human brain samples. This lack of influence of long PDs at 4 °C on neurotransmitter receptor labelling in human brain tissue stored at 4 °C has been previously reported for other receptors (Rodríguez-Puertas et al., 1996). In fact, using a rat model of human autopsy process, Whitehouse et al. (1984) only found a slight but statistically significant PD-related reduction in muscarinic receptor agonist binding when the tissue was stored at room temperature (22 °C). No alterations were observed when samples were kept at refrigeration temperature (4 °C). The absence of correlation between basal G-protein activity and PD at 4 °C has been suggested in previous studies in membranes (Palego et al., 1999; González-Maeso et al., 2002), and it is consistent with the lack of significant effects of this parameter on both neurotransmitter receptor densities and G_α-proteins immunoreactivity (Young et al., 1991; Escribá et al., 1994; Sastre and García-Sevilla, 1994; Dowlasatsi et al., 1999). A possible limitation of this study is due to the fact that brain endocannabinoid levels rapidly increase with postmortem delay (Felder et al. 1996; Sugiura et al., 2001). This could result in changes in CB1 receptor occupancy by endogenous ligands, which in turn might influence receptor density measurements. Although this possibility cannot be totally ruled out, it is minimized by the evidence of a rapid inactivation in the brain by reuptake and enzymatic hydrolysis (Giuffrida et al., 2001). Finally, the absence of PD-related alterations on CB agonist-induced activation of G-proteins reported in this study is in good agreement with the results obtained in membranes for other systems (González-Maeso et al., 2002).

Taking into account that the collection of human postmortem samples demands very long periods of time, it is necessary to assay sections from the same tissue block in different autoradiographic experiments with long intervals in between. Although brain samples are first kept at −70 °C, they are brought to −25 °C when being sectioned. Tissue sections (and blocks) are then kept at −25 °C, as re-storage at −70 °C could result in artifacts linked to dramatic changes in temperature, which would impair the histological quality of the tissue. Our results indicate a marked influence of the storage of brain samples at −25 °C on CB receptor density and G-protein coupling ability, as well as on basal G-protein activity in human frontal cortex. This negative effect of FSP on neurotransmitter receptor expression has been previously reported for muscarinic

receptors in the human brain (Rodríguez-Puertas et al., 1996). One of the interesting findings in this study is that the rate of CB1 receptor density loss as a function of FSP results notably higher when calculated from an exponential model. In fact, whereas Rodríguez-Puertas et al. (1996) reported $FSP5_0$ values about 50–60 months for muscarinic receptors expression decrease in postmortem human frontal cortex, our results demonstrate that the storage of the brain samples at −25 °C for 17 months already induces a 50% decline of CB receptor labelling in the same brain area. As there is no evidence suggesting that CB1 receptors are especially sensitive to the effects of tissue storage, these data indicate that the negative influence of this parameter on neurotransmitter receptor density in human brain could be even more marked than what it has been proposed (Rodríguez-Puertas et al., 1996). In a similar way, the FSP-related decrease of CB agonist ability to activate G-proteins in cortical sections from the same subjects also tends to fit better to a one phase exponential decay model. Furthermore, the fact that the $FSP5_0$ values obtained for the decline of CB1 receptors, with the one phase exponential decay model, are quite close to those calculated with the same model for the decrease in CB-induced activation of G-proteins, strongly suggests that the FSP-related loss of CB1 functionality reflects the negative effect of this parameter on CB1 receptor expression. However, the possible influence of other regulatory proteins, including RGSs and RAMPs (Sexton et al., 2001; Zhong and Neubig, 2001), on CB1 receptor coupling cannot be fully discarded.

The decrease in basal G-protein activity associated to FSP fits better to a linear regression decay model, which yields higher $FSP5_0$ values than those calculated for the CB1 receptor labelling and G-protein coupling (14–20 vs 55–65 months of storage at −25 °C). These results suggest that basal activity of $G_{i/o}$-proteins in postmortem human brain is less sensitive to FSP negative effects than neurotransmitter receptors themselves. This is not surprising, as the real significance of this parameter is not fully clarified at the present time. Studies carried out in several GPCR-transfected cell lines strongly suggest that the majority of this binding component corresponds to G-proteins activated by precoupled receptors (Newman-Tancredi et al., 1997; Audinot et al., 2001). Thus, the constitutive activity of the family of $G_{i/o}$-dependent receptors would be the main responsible for the basal G-protein activity. Therefore, the degree of FSP-dependent decline in the basal activity of G-proteins would depend on the relative influence of multiple types of receptors. In any case, the information about the consequences derived from the maintenance of the brain samples at −25 °C for long storage periods of time is still scarce. Although it could be assumed that the negative effect of sample storage on neurotransmitter receptor labelling in human brain (Rodríguez-Puertas et al., 1996) is only evident at −25 °C and is not relevant when the tissue is stored at lower temperatures (Perry and Perry, 1983), it must be taken into account that González-Maeso et al. (2002) have recently shown a weak negative correlation between basal [^{35}S]GTPγS binding values in human frontal cortex membranes and FSP, in samples stored at −70 °C.

In conclusion, our results indicate that factors such as the age and the storage period should be taken into account when analyzing CB receptor properties in human brain samples. They also reinforce the importance of a thorough matching of pathological and control cases in terms of these variables in order to allow a correct interpretation of the possible role of CB neurotransmission in different neuropsychiatric disorders.

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