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AUTORADIOGRAPHY OF RECEPTOR-ACTIVATED G-PROTEINS IN *POST MORTEM* HUMAN BRAIN

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Abstract—The agonist-stimulated guanosine 5′-(γ-[^{35}S]thio)triphosphate binding assay was used to anatomically localize receptor-activated G-proteins by autoradiography in *post mortem* human brain. The optimal conditions for guanosine 5′-(γ-[^{35}S]thio)triphosphate binding to human brain sections were established in *post mortem* samples of the prefrontal cortex, hippocampus, basal ganglia, brainstem and cerebellar cortex. An excess of GDP (2 mM) was required to decrease basal activity and obtain effective stimulation by specific agonists. guanosine 5′-(γ-[^{35}S]Thio)triphosphate binding was increased after stimulation with specific agonists of different G-protein-coupled receptors. They include cannabinoid (WIN55212-2), μ-opioid ([D-Ala$_2$,*N*-Me-Phe$_4$,Gly$_5$-ol]enkephalin), serotonin-1A [(±)-8-hydroxy-2-(di-n-propylamino)tetralin] and serotonin-1B/1D (sumatriptan), cholinergic muscarinic receptors (carbachol) and α_2-adrenoceptors (UK14304). Such stimulation reached 1458%, 440%, 188%, 219%, 61% and 339%, respectively, over the basal levels. In tissue sections, the use of the above-mentioned agonists (10^{-4} M) showed patterns of anatomical distribution similar to those already described by receptor autoradiography, with high densities over the hippocampus (serotonin-1A receptors), cortex (α_2-adrenoceptors) and striatum (μ-opioid receptors). The highest binding levels were reached with the cannabinoid receptor agonist in most of the analysed brain regions. Carbachol produced only moderate stimulation of those same regions. The blockage of agonist-stimulated guanosine 5′-(γ-[^{35}S]thio)triphosphate binding by selective antagonists verified that the effect was receptor mediated.

This technique provides a method to identify modifications of the receptor-mediated activation of G-proteins in *post mortem* human brain with anatomical resolution. It also provides valuable information on the level of drug efficacy in the human species.

Key words: [^{35}S]GTPγS binding, α_2-adrenoceptors, 5-HT$_1$ receptors, μ-opioid receptors, cholinergic muscarinic receptors, cannabinoid receptors.

Receptor-mediated activation of second messenger-generating effector systems are largely mediated by a family of heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins).[1,14] This type of receptors, known as G-protein-coupled receptors (GPCRs), comprises the majority of receptors for peptide hormones, neurotransmitters, prostanoids, odorants and even light.[1,14] Therefore, GPCRs play an essential role in intercellular signalling and regulation mechanisms.

Nearly all GPCRs share the typical structure in seven membrane-spanning domains. The GPCR alters its conformation when the receptor is activated by specific agonists. This activation promotes the receptor to associate with the α-subunit of the G-protein, increasing the affinity of the α-subunit for GTP and decreasing its affinity for βγ-subunits, causing βγ-subunits to dissociate from α.[1,14] Then, the receptor also dissociates from the α-subunit, allowing the α- and βγ-subunits to interact with effectors such as adenylyl cyclase, phospholipase C or ion channels.[6,34]

The classical methods to measure receptor activation of G-proteins have registered GTPase activity.[3,23,49] However, the GTPase assay measures the inactivation reaction of the G-protein, the GTP hydrolysis, which constitutes an indirect activity that could be altered by other factors independently of the G-protein activation.[50]

The binding of the poorly hydrolysable GTP analogue guanosine 5′-(γ-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) directly reflects the receptor activation, since it measures the exchange of GDP for GTP (or [^{35}S]GTPγS) on the G-protein. The [^{35}S]GTPγS binding assay has been used in reconstituted systems of purified proteins,[5] in membrane homogenates,[20,26,51] in receptor-transfected cells,[13,24,35] immunoprecipitation assays[63] and *in vitro* autoradiography.[10,52] [^{35}S]GTPγS binding assays have currently been performed in tissues from laboratory animals. Interestingly, we have recently demonstrated the applicability of this technique to human membrane homogenates, in spite of the *post mortem* delay period inherent to this tissue.[16]

GPCRs have been localized autoradiographically using radioligand binding techniques, enabling the neuroanatomical identification of many neurotransmitter receptors in experimental animals and *post mortem* human brain. Thus, anatomical alterations of GPCR densities have been described in neurological and psychiatric diseases. Nevertheless, other studies suggest that modifications of G-protein expression and/or function could be implicated in the pathophysiology of these cerebral diseases.[2,7,11,29,38,65]

The quantification of [^{35}S]GTPγS autoradiography in *post mortem* human brain, and its stimulation induced by selective receptor agonists, would represent a powerful technique for a conjoint functional and anatomical study of activated G-proteins in discrete areas of the human brain. The application

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Abbreviations: BSA, bovine serum albumin; DAMGO, [D-Ala$_2$,*N*-Me-Phe$_4$,Gly$_5$-ol]enkephalin; EGTA, ethyleneglycolbis(aminoethyl ether)tetra-acetate; GPCR, G-protein-coupled receptor; [^{35}S]GTPγS, guanosine 5′-(γ-[^{35}S]thio)triphosphate; 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, (±)-8-hydroxy-2-(di-n-propylamino)tetralin; t.e., tissue equivalent.

of this procedure to *post mortem* tissue samples from patients with neurological and psychiatric disorders would be of remarkable interest.

Therefore, the present study analyses the [^{35}S]GTPγS binding to different representative brain areas in autopsied human brain and the anatomically selective activation of the G-proteins with different agonists of GPCRs of already known autoradiographic localization. A preliminary report of this study was presented at the 1998 Meeting of the Society for Neuroscience.[46]

EXPERIMENTAL PROCEDURES

Materials

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from DuPont NEN (Brussels, Belgium). Atropine, bovine serum albumin (BSA), carbachol, [D-Ala$_2$,*N*-Me-Phe$_4$,Gly$_5$-ol]enkephalin (DAMGO), (±)-8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), DL-dithiothreitol, GDP, GTPγS and naloxone were purchased from Sigma (St Louis, MO, U.S.A.). 5-Bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14304) was obtained from Tocris Cookson (Bristol, U.K.). 2-Methoxyidazoxan (RX821002) was synthesized at Lasa Laboratorios S.A. (Barcelona, Spain). *N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide (WAY100635-A-5) was from Wyeth-Ayerst (Princeton, NJ, U.S.A.). *R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl]-(1-naphthalenyl)methanone monomethanesulphonate (WIN55212-2) was from RBI (Natick, MA, U.S.A.). Sumatriptan was from Glaxo (Greenford, U.K.). *N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A) was from Sanofi Recherche (Montpellier, France). 2′-Methyl-4′-(5-methyl-[1,2,4]oxadiazol-3-yl)biphenyl-4-carboxylic acid[4-methoxy-3-(4-methylpiperazin-1-yl-]phenyl]amide (GR127935) was synthesized and kindly donated by Almirall-Prodesfarma Laboratories (Dr J. M. Palacios, Barcelona, Spain). All other chemicals were obtained from standard sources and were of the highest purity commercially avaiable.

Post mortem *human tissue*

Human brain samples were obtained from four subjects (age 37 ± 9 years, *post mortem* delay 17 ± 4 h, freezing storage period 13 ± 11 months) without any record of neuropsychiatric disease and who died of violent causes. The brains were removed at autopsy at the Instituto Anatómico Forense (Bilbao, Spain) and at the Service of Pathology, University Hospital "Marqués de Valdecilla" (Santander, Spain). The tissue collection was approved by the Ethical Committees of both Forensic Institutes for *post mortem* human studies. The subjects showed a negative test on the toxicological screening for psychotropic drugs and alcohol.

Blocks containing the frontal cortex, hippocampus, cerebellum, striatum or brainstem areas were promptly dissected and immediately stored at −70°C until assay. Consecutive sections (20 μm thick) from every tissue block were cut at −25°C using a microtome-cryostat, mounted on gelatine-coated slides and stored at −25°C until use.

Guanosine 5′-(γ-[^{35}S]*thio)triphosphate binding assay*

[^{35}S]GTPγS binding to human brain slices was carried out with several modifications based on the protocol described by Sim *et al.*[52] for the rat brain. Cortical sections were incubated in a buffer containing 0.2 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM DL-dithiothreitol, 2 mM GDP and 50 mM Tris–HCl at pH 7.7, during 30 min at room temperature. Slides were then incubated in the same buffer in the presence of 0.05 nM [^{35}S]GTPγS for 120 min at room temperature. Non-specific binding of the radioligand was determined by isotope dilution in the presence of 10 μM unlabelled GTPγS. The agonist-stimulated binding was measured under the same conditions in the presence of the specific GPCR agonists (10^{-4} M): WIN55212-2, DAMGO, 8-OH-DPAT, sumatriptan, carbachol and UK14304. The specificity of the receptor-mediated stimulation was verified by the respective antagonists (10^{-5} M): SR141716A, naloxone, WAY100635-A-5, GR127935, atropine and RX821002. A single consecutive 20-μm-thick section was used for each different condition. Preliminary experiments were performed in duplicate consecutive sections, in which we did not observe any remarkable difference between the values measured in both slides. The incubation buffer for the cannabinoid receptor drugs, WIN55212-2 and SR141716, contained 0.5% BSA. After the incubation, the slides were rinsed in cold 50 mM Tris–HCl buffer (pH 7.4, 4°C, 2 × 15 min) and dried on a cold airstream. Sections were then apposed to a β radiation-sensitive film (Hyperfilm β-max, Amersham, U.K.) in a closed cassette with a set of ^{14}C standards (Amersham microscales) at 4°C for 48 h.

[^{3}H]*CP55940 autoradiography*

Autoradiograms showing the distribution of the CB_1 cannabinoid receptor subtype were obtained after incubation of consecutive sections with the cannabinoid agonist radioligand [^{3}H]CP55940 (Dupont/NEN; specific activity 125 Ci/mmol). The incubation procedure was similar to that described by Glass *et al.*,[15] with modifications. Briefly, sections were incubated for 120 min with 3 nM [^{3}H]CP55940 in Tris–HCl buffer (50 mM, pH 7.4) with 5% BSA at 37°C. Non-specific binding was determined with the addition of WIN55212-2 (10 μm). Following the incubation, the slides were rinsed in cold 50 mM Tris–HCl buffer (pH 7.4, 4°C, 2 × 120 min) and dried on a cold airstream. Sections were then apposed to a tritium-sensitive film (^{3}H Hyperfilm, Amersham, U.K.) in a closed cassette at 4°C for two weeks.

Quantitative image analysis of film autoradiograms

The regional densities of [^{35}S]GTPγS binding sites were determined by optical densitometry of film autoradiograms using an image analysis system (NIH-IMAGE program, Bethesda, MA, U.S.A.) after scanning of the films. ^{14}C standards included on each film exposure were also scanned and a standard curve was prepared. The data were corrected for the specific activity of [^{35}S]GTPγS at the calibration date and the decay factor of ^{35}S. The average of duplicate or triplicate different measurements, including the whole extension of the same brain area, were calculated and the densities were converted to nCi/g tissue equivalent (nCi/g t.e.). The slide background and non-specific densities were subtracted. The percentages of stimulation were calculated from the basal and agonist-stimulated [^{35}S]GTPγS binding densities according to the formula: (stimulated − basal) × 100/basal.

Descriptive statistical analysis was performed and the mean ± S.E.M. of all the measured cases was calculated for every agonist in all the different brain areas where the autoradiographic density was measured. For some brain areas it was not possible to include samples from the four subjects in the quantification process because of autopsy or technical limitations. Therefore, only areas where it was possible to quantify at least three different human cases were included.

The basal and stimulated binding levels were measured in the frontal cortex (layers I–VI and associated white matter), hippocampal area (dentate gyrus, molecular layer and hilus, CA3 and CA1 regions, subiculum and entorhinal cortex), mesencephalic area (substantia nigra pars compacta and reticulata, ventral tegmental area, periaqueductal gray matter, interpeduncular nucleus, dorsal raphe, red nucleus and superior colliculus), striatal area (caudate, putamen and nucleus accumbens) and cerebellar cortex. Anatomical regions were identified using several human atlases.[8,36]

RESULTS

Basal guanosine 5′-(γ-[^{35}S]*thio)triphosphate binding*

The most appropriate GDP concentration to obtain a measurable basal [^{35}S]GTPγS binding level and a high binding stimulation by specific agonist was determined. A GDP concentration of 2 mM was chosen, which showed basal binding levels ranging from 3 nCi/g t.e. in frontal white matter to 35 nCi/g t.e. in layer III of the frontal cortex area (Table 1, Fig. 1A). In the *post mortem* human brain, the rank order for [^{35}S]GTPγS binding levels (nCi/g t.e., mean ± S.E.M.) was entorhinal cortex (31 ± 17) > frontal cortex (29 ± 3) > striatum (25 ± 3) > hippocampus (21 ± 7) > cerebellum (11 ± 1) > brainstem (7 ± 2) > white matter (3 ± 1) (Tables 1–4, Figs 1A, 2A, 3A, 4A, 5A).

Table 1. Specific guanosine 5′-(γ-[^{35}S]thio)triphosphate binding sites in the frontal cortex area from *post mortem* human brain

Brain area	Basal level	Agonist-stimulated [^{35}S]GTPγS binding (nCi/g t.e.)					
		WIN55212-2	DAMGO	8-OH-DPAT	Sumatriptan	Carbachol	UK14304
Frontal cortex							
Layer I	26 ± 10	328 ± 141	64 ± 14	33 ± 7	52 ± 19	20 ± 2	104 ± 34
Layer II	32 ± 9	371 ± 122	89 ± 9	46 ± 11	64 ± 24	33 ± 7	101 ± 17
Layer III	35 ± 10	369 ± 119	96 ± 28	34 ± 8	43 ± 12	27 + 8	104 ± 16
Layer IV	29 ± 9	377 ± 150	98 ± 28	26 ± 7	34 ± 8	27 ± 7	88 ± 23
Layer V	27 ± 8	339 ± 113	85 ± 26	26 ± 8	32 ± 7	26 ± 8	77 ± 18
Layer VI	26 ± 7	318 ± 77	76 ± 22	26 ± 8	32 ± 7	18 ± 5	77 ± 18
White matter	3 ± 1	5 ± 1	3 ± 2	12 ± 7	3 ± 2	2 ± 1	11 ± 2

Data are mean ± S.E.M. of $n = 3$ cases.

The non-specific binding was determined by isotope dilution in the presence of unlabelled GTPγS at a concentration of 10 μM and represented about 5% of the total [^{35}S]GTPγS basal binding for the brain structures analysed (Figs 1B, 2B, 3B, 4B, 5B).

Agonist-stimulated guanosine 5′-(γ-[^{35}S]thio)triphosphate binding

The addition of GPCR agonists (10^{-4} M) to the incubation buffer increased the binding densities in a specific anatomical pattern. This means that, in the same brain area, for instance layer II of the frontal cortex, the [^{35}S]GTPγS binding was different depending on the agonist. Thus, the ranking order of binding stimulation in layer II of the frontal cortex was WIN55212-2 (1127 ± 358%) > UK14304 (257 ± 104%) > DAMGO (209 ± 49%) > sumatriptan (103 ± 46%) > 8-OH-DPAT (49 ± 17%) > carbachol (9 ± 7%) (Table 1, Fig. 1).

Furthermore, the maximal stimulated binding shown by the different agonists did not correspond with the maximal percentage of stimulation over the basal levels in some regions. Thus, the specific cannabinoid receptor agonist WIN55212-2, which showed the highest stimulation potencies in most of the studied brain areas, reached a density of 423 ± 292 nCi/g t.e. (956 ± 275% stimulation over the basal level) in the subiculum hippocampal region. Nevertheless, the maximal percentage of stimulation was found in the CA3 hippocampal area (284 ± 134 nCi/g t.e., 1458 ± 729% stimulation; Table 2). The rank order of WIN55212-2-stimulated [^{35}S]GTPγS binding was hippocampus > entorhinal cortex > frontal cortex > striatum > cerebellum > brainstem area (Tables 1–4, Figs 1C, 3E, 4G, 5C). However, the capacity of stimulation of the cannabinoid receptor agonist also varied between different nuclei within a brain region. Therefore, for instance, in the substantia nigra pars reticulata, WIN55212-2 showed a 361 ± 253% increase, whereas in the superior colliculus, where the basal levels were similar, the response reached only 30 ± 18% (Table 3, Fig. 3). The CB_1 cannabinoid receptor population was labelled with [^{3}H]CP55940, showing a similar distribution in the striatal area to that observed with [^{35}S]GTPγS binding in the presence of WIN55212-2 (Fig. 6).

The [^{35}S]GTPγS binding stimulation by the μ-opioid receptor agonist DAMGO reached the highest densities over the nucleus accumbens (156 ± 16 nCi/g t.e.), which corresponded to the highest percentage of stimulation (440 ± 65%; Table 4, Fig. 4). The rank order of DAMGO-stimulated [^{35}S]GTPγS binding for the different areas was striatum > frontal cortex > hippocampal area > cerebellum > brainstem (Tables 1–4, Figs 1D, 3G, 4C, 5E).

The serotonin-1A ($5\text{-}HT_{1A}$) receptor agonist 8-OH-DPAT stimulated the [^{35}S]GTPγS binding up to 60 ± 38 nCi/g t.e. in the subiculum and reached the maximal percentage of stimulation over the CA1 hippocampal region (188 ± 28%; Table 2, Fig. 2C). This percentage duplicates that observed in the subiculum (80 ± 54%). Both frontal and entorhinal cortices showed intermediate densities (26–46 nCi/g t.e.; Tables 1, 2, Figs 1E, 2C). The striatal area levels ranged from 23 ± 1 nCi/g t.e. for the caudate nucleus to 28 ± 2 nCi/g t.e. for the putamen and accumbens (Table 4). The lowest densities were measured over the brainstem nuclei (3–12 nCi/g t.e.; Table 3, Fig. 3C) and cerebellar cortex (7 ± 1 nCi/g t.e.; Table 4).

The [^{35}S]GTPγS binding stimulated by the $5\text{-}HT_{1B/1D}$ receptor agonist sumatriptan showed a similar anatomical distribution to that observed for 8-OH-DPAT stimulation. The subiculum also showed the maximal [^{35}S]GTPγS autoradiographic density (95 ± 68 nCi/g t.e.), and both frontal (32–64 nCi/g t.e.) and entorhinal (51 ± 33 nCi/g t.e.) cortices had higher binding levels with sumatriptan than with 8-OH-DPAT stimulaton (Tables 1, 2, Fig. 1E, F). The maximal percentage of stimulation was also quantified over the CA1 hippocampal region (219 ± 29%; Table 2, Fig. 2C, E).

The cholinergic muscarinic receptor agonist carbachol produced weak stimulation when compared with the other assayed GPCR agonists. The maximal binding levels were measured in the putamen and accumbens nuclei (41 ± 8 and 41 ± 2 nCi/g t.e., respectively; Table 4, Fig. 4E). The rank order of the binding densities was: striatum > frontal cortex > entorhinal cortex > hippocampus > cerebellum > brainstem. The highest percentage of stimulation over the basal level was reached in the dorsal raphe nucleus (62 ± 46%), but similar percentages were reached in the hilus of the hippocampal dentate gyrus (58 ± 43%), the interpeduncular nucleus (51 ± 30%) and the caudate nucleus (51 ± 21%).

UK14304, a selective agonist of the α_2-adrenoceptor, exhibited a high stimulation of [^{35}S]GTPγS binding in the different layers of the frontal cortex (from 213 ± 40% in layer VI to 324 ± 71% in layer I; Table 1, Fig. 1H). Nevertheless, the highest stimulation was calculated for the molecular layer of the dentate gyrus (339 ± 86%). Regarding the autoradiographic densities, the rank order of stimulation was: subiculum (163 ± 118 nCi/g t.e.) > frontal cortex > entorhinal cortex > hippocampus (dentate gyrus) > striatum > brainstem > cerebellar cortex (Tables 2–4, Figs 1H, 2G).

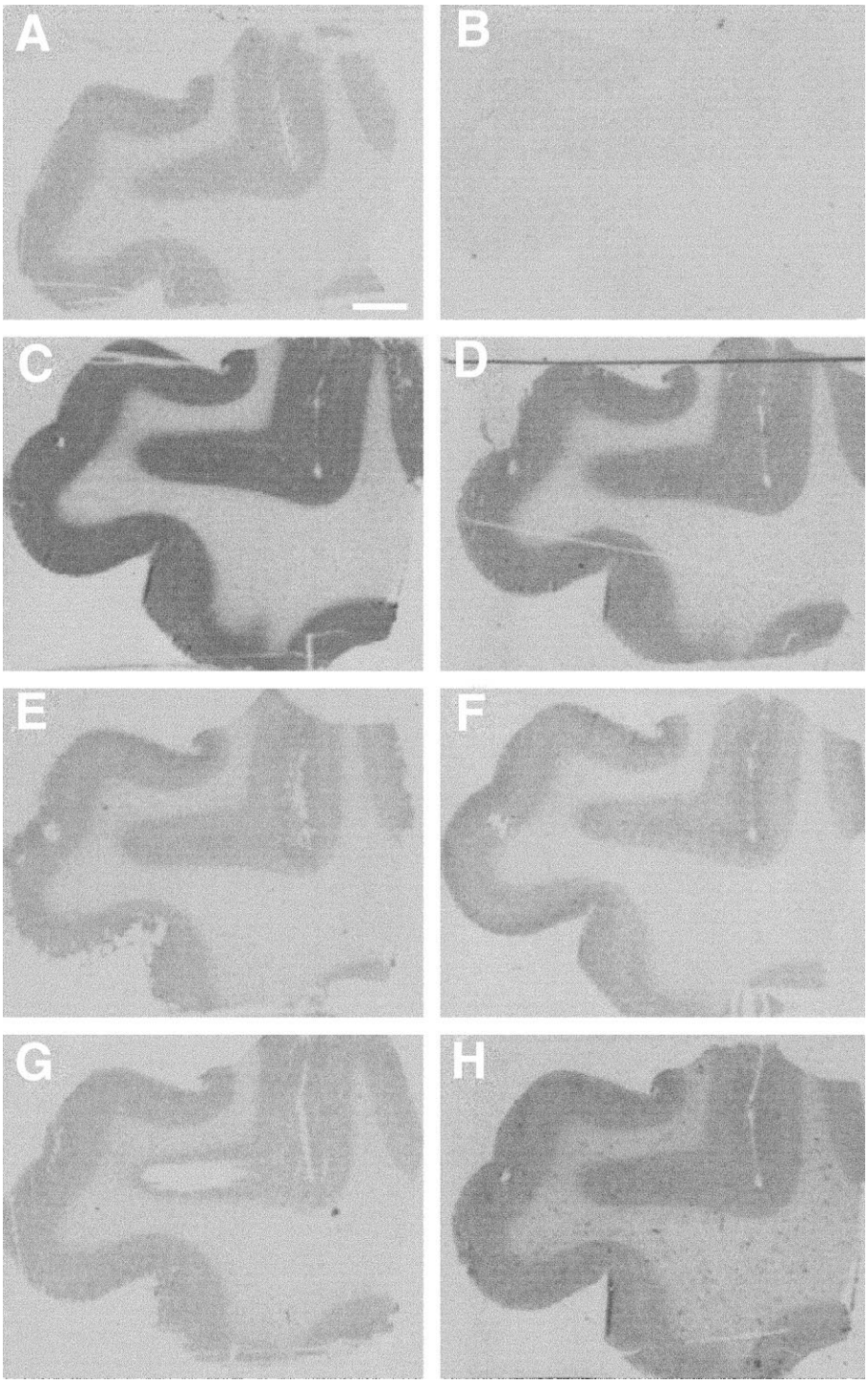

Fig. 1. Autoradiographic images of consecutive tissue sections from human frontal cortex, showing the basal (A), non-specific (GTPγS at 10 μM; B) and agonist (100 μM)-stimulated [^{35}S]GTPγS (0.05 nM) binding (C–H) with the cannabinoid receptor agonist WIN55212-2 (C), the μ-opioid agonist DAMGO (D), the 5-HT$_{1A}$ agonist 8-OH-DPAT (E), the 5-HT$_{1B–D}$/5-HT$_{1F}$ agonist sumatriptan (F), the cholinergic muscarinic agonist carbachol (G) and the α_2-adrenoceptor agonist UK14304 (H). Note the different anatomical pattern of distribution and binding densities in the presence of the different agonists. Scale bar = 5 mm.

The white matter was quantified in the frontal cortex region and showed a very low basal [^{35}S]GTPγS binding (3 ± 1 nCi/g t.e.), which was uniquely increased by 8-OH-DPAT (12 ± 7 nCi/g t.e., 212 ± 162%) and UK14304 (11 ± 2 nCi/g t.e., 216 ± 25%) (Table 1, Fig. 1).

The incubation of consecutive slices in the presence of the different agonists (10^{-4} M), together with their respective antagonists (10^{-5} M), produced an inhibition of the agonist-stimulated [^{35}S]GTPγS binding to levels similar to those observed under basal conditions (Figs 2–5). Nevertheless, a certain degree of stimulation of the binding remained on some regions for several of the antagonists used. Thus, incubation in the presence of the cannabinoid agonist and the specific antagonist SR141716 still resulted in a stimulation, from 25 ± 13% in the mesencephaplic red nucleus to 248 ± 64% in the caudate nucleus (Figs 3F, 4H). However,

the [^{35}S]GTPγS binding in the presence of the 5-HT$_{1A}$ agonist and the specific antagonist, WAY100635-A-5, was even lower than the basal [^{35}S]GTPγS binding in some areas, such as the hippocampal CA3 area ($-12 \pm 14\%$) and frontal cortex (layer V, $-16 \pm 26\%$).

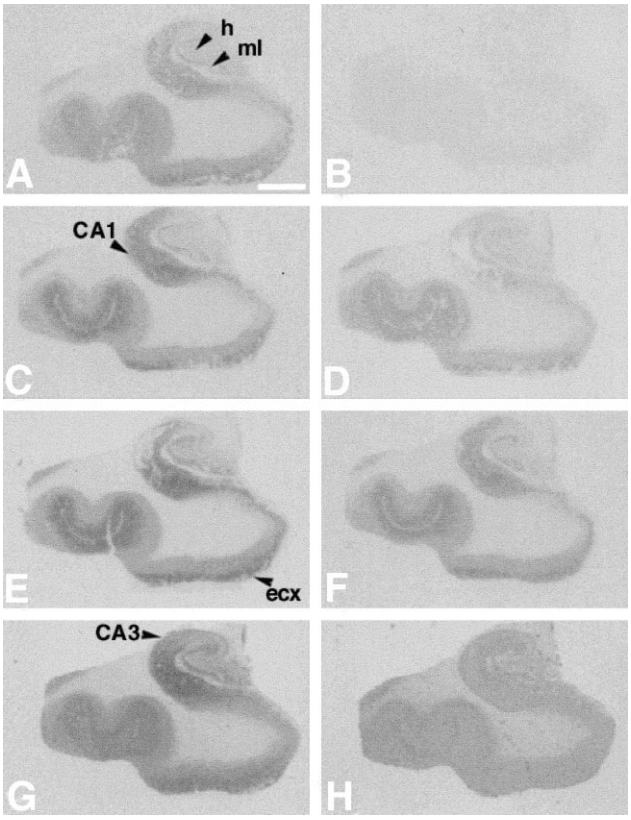

Fig. 2. Autoradiographic images of consecutive tissue sections from the human hippocampal area of a representative human case showing the basal (A), non-specific (GTPγS at 10 μM; B), agonist (100 μM)-stimulated [^{35}S]GTPγS (0.05 nM) binding (C, E, G) and the antagonism of the stimulation by respective antagonists (10 μM; D, F, H). C corresponds to the binding in the presence of the 5-HT$_{1A}$ agonist 8-OH-DPAT, and D to the binding in the presence of both 8-OH-DPAT and the antagonist WAY100635-A-5. E shows the stimulated binding with the 5-HT$_{1B-D}$/5-HT$_{1F}$ agonist sumatriptan, and F the binding in the presence of sumatriptan and the antagonist GR127935. G shows the stimulated binding by the α_2-adrenoceptor agonist UK14304, and H the binding in the presence of UK14304 and the antagonist RX821002. The inhibition of the agonist-stimulated [^{35}S]GTPγS binding by the respective antagonist follows an anatomical selectivity and decreases almost to the [^{35}S]GTPγS binding basal levels. CA1, CA1 area; CA3, CA3 area; e cx, entorhinal cortex; h, hilus; ml, molecular layer of the dentate gyrus. Scale bar = 5 mm.

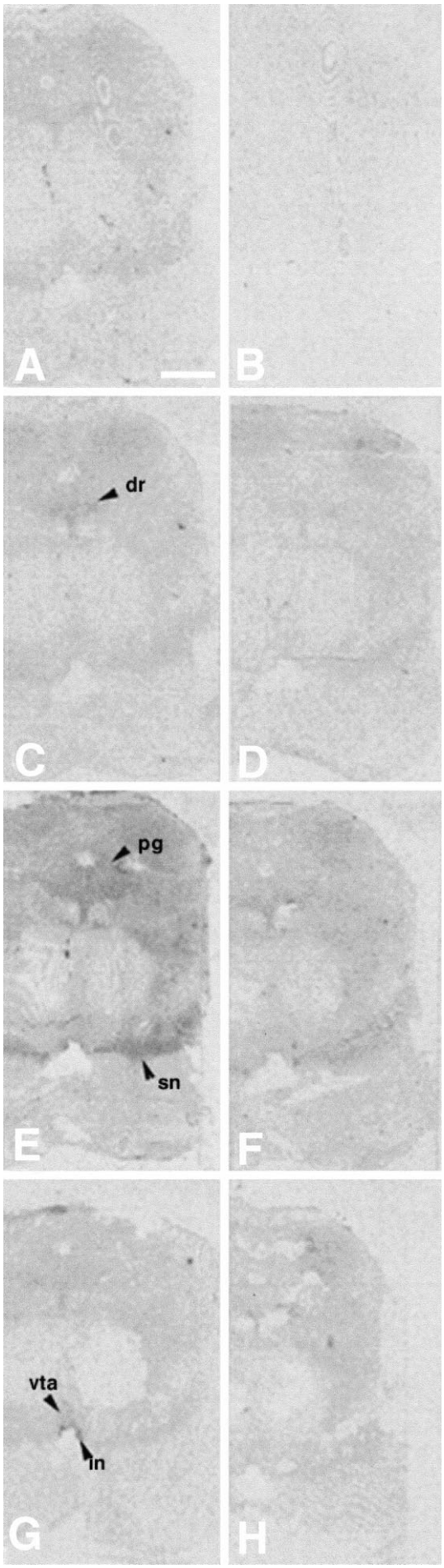

Fig. 3. Autoradiographic images of consecutive tissue sections from the human brainstem area, showing the basal (A), non-specific (GTPγS at 10 μM; B), agonist (100 μM)-stimulated [^{35}S]GTPγS binding (C, E, G) and the antagonism of the binding by respective antagonists (10 μM; D, F, H). [^{35}S]GTPγS binding in the presence of the 5-HT$_{1A}$ agonist 8-OH-DPAT is shown in C. D shows the binding in the presence of 8-OH-DPAT and WAY100635-A-5. Note the highest autoradiographic density in the dorsal raphe nucleus and the antagonism of the binding in the presence of the antagonist. E and F show the binding in the presence of the cannabinoid receptor agonist WIN55212-2, and with this drug and the antagonist SR141716, respectively. The highest densities are located in the substantia nigra and the periaqueductal gray matter. G shows the stimulated [^{35}S]GTPγS binding by the μ-opioid agonist DAMGO. H shows the blockade of the binding by the antagonist naloxone. Note the specific DAMGO stimulation in the interpeduncular nucleus. dr, dorsal raphe; in, interpeduncular nucleus; pg, periaqueductal gray matter; sn, substantia nigra; vta, ventral tegmental area. Scale bar = 5 mm.

DISCUSSION

The present study demonstrates the applicability of the [^{35}S]GTPγS binding technique to identify receptor-activated G-proteins by autoradiography in tissue slices from *post mortem* human brain. The optimal conditions for the assay were defined on the basis of previous studies carried out on rodent tissue slices[52] and results from our own group on human membrane homogenates.[16] The GDP concentration in the incubation buffer was found to be critical, as described previously by Sim *et al*.[52] for rat tissue. Therefore, in previous assays, different GDP concentrations were tested in human tissue sections, the results showing that concentrations between 1 and 2 mM were necessary to minimize the agonist-independent [^{35}S]GTPγS binding without lowering the agonist-induced binding. The results were further improved by previously incubating the tissue slices for 30 min in a buffer containing 2 mM GDP.

The levels of [^{35}S]GTPγS basal binding, obtained in the absence of agonist, varied depending on the different brain areas. Thus, the highest basal levels were observed over the frontal and entorhinal cortices, the densities being intermediate in the striatal and hippocampal areas and lower in other brain regions, such as the cerebellum and brainstem. This pattern agrees quite well with both the autoradiographic distribution of the GTP analogue [^{3}H]Gpp(NH)p binding in rat brain[12] and the mRNA localization of G-proteins by *in situ* hybridization in *post mortem* human brain.[32] In addition, the pattern of $G_i\alpha$-subunit immunoreactivity reported in human brain by Young *et al*.[65] is also consistent with the regional distribution of [^{35}S]GTPγS binding observed in the present study.

The stimulated [^{35}S]GTPγS binding was measured after incubation with the different agonists at a concentration of 100 μM in order to obtain the maximal percentage of stimulation. This concentration was chosen according to previous experiments performed by our own group in membrane homogenates of the frontal cortex from human *post mortem* brain samples,[16] where most of the agonists used showed EC_{50} values in the micromolar range. In this regard, it is noteworthy that previous autoradiographic studies analysing the agonist-stimulated [^{35}S]GTPγS binding in rodent brain tissue have shown maximal effects around 10 μM: this is the case for the μ-opioid receptor agonist DAMGO[52,54] and the cannabinoid receptor agonist WIN55212-2.[53]

The autoradiographic localization pattern shown by the different GPCR agonists used in this assay revealed, in general terms, an anatomical distribution rather similar to that obtained by classical radioligand autoradiography of receptors in the human brain. Thus, the incubation with the cannabinoid receptor agonist WIN55212-2 showed a high [^{35}S]GTPγS binding in the hippocampus, entorhinal and

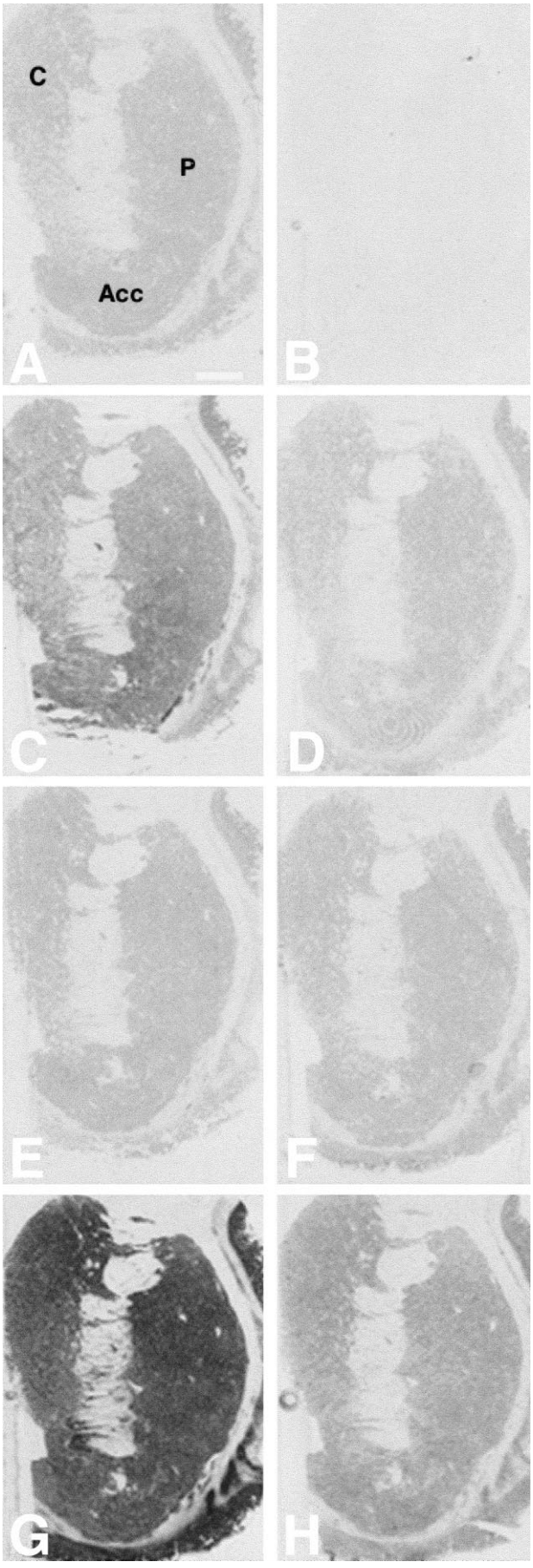

Fig. 4. Autoradiographic images of consecutive tissue sections from the human striatal area, showing the basal (A), non-specific (GTPγS at 10 μM; B), agonist (100 μM)-stimulated [^{35}S]GTPγS binding (C, E, G) and the antagonism of the binding by respective antagonists (10 μM; D, F, H). C and D show the binding in the presence of the μ-opioid agonist DAMGO and the antagonism by the antagonist naloxone, respectively. E shows the stimulated [^{35}S]GTPγS binding in the presence of the cholinergic muscarinic agonist carbachol. F shows the [^{35}S]GTPγS binding in the presence of carbachol and atropine. G shows the binding in the presence of the cannabinoid receptor agonist WIN55212-2, and H the blockade of the binding by its respective antagonist SR141716. Acc, nucleus accumbens; C, caudate nucleus; P, putamen nucleus. Scale bar = 5 mm.

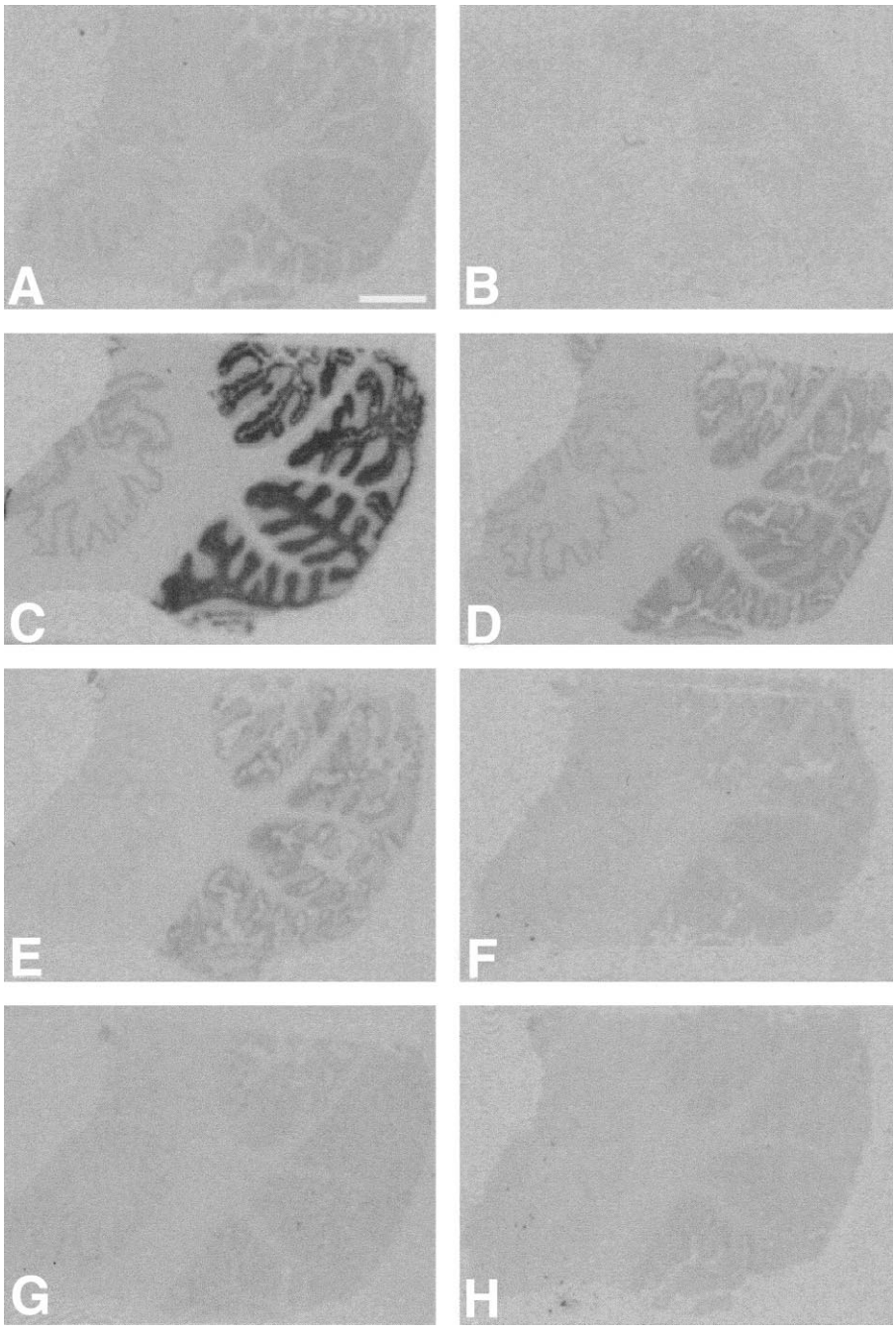

Fig. 5. Autoradiographic images of consecutive tissue sections from the human cerebellum, showing the basal (A), non-specific (GTPγS at 10 μM; B), agonist (100 μM)-stimulated [35S]GTPγS binding (C, E, G) and the antagonism of the binding by respective antagonists (10 μM; D, F, H). C and D correspond to the cannabinoid receptor agonist WIN55212-2 binding stimulation and to the inhibition by the antagonist SR141716, respectively. E shows the stimulated [35S]GTPγS binding by the μ-opioid receptor agonist DAMGO. F shows the binding in the presence of DAMGO and naloxone. G shows the binding in the presence of the 5-HT_{1B-D}/5-HT_{1F} agonist sumatriptan. H shows the binding in the presence of sumatriptan and GR127935. Scale bar = 5 mm.

frontal cortices, and striatal area, moderate levels in the cerebellar cortex and substantia nigra pars reticulata, and lower labelling in the brainstem and white matter. This distribution is similar to that described previously with the potent cannabinoid receptor radioligand [3H]CP55940 in human brain[19,64] and also similar to the cannabinoid receptor mRNA localization.[64] However, some discrepancies exist in the relative densities obtained. Thus, the highest stimulation of [35S]GTPγS binding with WIN55212-2 was observed in the subiculum of the hippocampus, while autoradiography of the receptor described the highest cannabinoid receptor densities in the substantia nigra pars reticulata.[27,28] In this regard, it must be taken into consideration that this technique is labelling the α-subunit of the G-proteins, not the receptor recognition site. Regarding the stimulation of [35S]GTPγS binding with the μ-opioid receptor agonist DAMGO, this showed a characteristic pattern with high densities in cortical and mesolimbic areas. Moderate levels were observed in the hippocampal area and low densities in the cerebellum and brainstem, where the ventral tegmental area showed the highest binding. This distribution also agrees with that described previously for [3H]DAMGO

Table 2. Specific guanosine 5′-(γ-[^{35}S]thio)triphosphate binding sites in the hippocampal area and entorhinal cortex from *post mortem* human brain

		Agonist-stimulated [^{35}S]GTPγS binding (nCi/g t.e.)					
Brain area	Basal level	WIN55212-2	DAMGO	8-OH-DPAT	Sumatriptan	Carbachol	UK14304
Hippocampus							
DG ml	21 ± 6	305 ± 165	36 ± 8	23 ± 8	40 ± 15	20 ± 4	96 ± 44
DG Hil.	13 ± 3	142 ± 90	21 ± 5	13 ± 4	19 + 8	23 ± 6	48 ± 17
CA3	18 ± 4	328 ± 208	29 ± 10	19 ± 6	23 ± 7	23 ± 6	63 ± 33
CA1	18 ± 5	284 ± 134	24 ± 4	51 ± 12	55 ± 11	22 ± 9	86 ± 42
Subiculum	33 ± 16	423 ± 292	34 ± 12	60 ± 38	95 ± 68	32 ± 16	163 ± 118
Entorhinal cortex	31 ± 17	353 ± 251	44 ± 24	34 ± 17	51 ± 33	28 ± 15	89 ± 34

Data are mean ± S.E.M. of $n = 3$ cases. DG ml, molecular layer of the hippocampal dentate gyrus; DG Hil., hilus of the hippocampal dentate gyrus; CA3, pyramidal layer of the hippocampal CA3 region; CA1, pyramidal layer of the hippocampal CA1 region.

Table 3. Specific guanosine 5′-(γ-[^{35}S]thio)triphosphate binding sites in the brainstem from *post mortem* human brain

		Agonist-stimulated [^{35}S]GTPγS binding (nCi/g t.e.)					
Brain area	Basal level	WIN55212-2	DAMGO	8-OH-DPAT	Sumatriptan	Carbachol	UK14304
Brainstem							
SNR	9 ± 1	51 ± 14	9 ± 2	12 ± 1	11 ± 3	11 ± 3	29 ± 1
SNC	7 ± 1	34 ± 8	7 ± 2	11 ± 0.5	7 ± 3	10 ± 3	25 ± 3
VTA	5 ± 2	17 ± 4	6 ± 3	7 ± 2	2 ± 1	8 ± 4	28 ± 7
PAG	8 ± 2	20 ± 6	6 ± 2	9 ± 1	8 ± 4	11 ± 3	17 ± 1
IPN	9 ± 2	48 ± 21	19 ± 8	8 ± 1	8 ± 2	15 ± 5	17 ± 2
Dorsal raphe	7 ± 2	23 ± 7	9 ± 2	13 ± 3	8 ± 1	9 ± 1	21 ± 3
Red nucleus	5 ± 2	7 ± 4	2 ± 1	3 ± 2	2 ± 1	3 ± 2	7 ± 3
SC	8 ± 1	16 ± 5	6 ± 2	6 ± 2	6 ± 2	10 ± 3	13 ± 1

Data are mean ± S.E.M. of $n = 3$ cases. SNR, substantia nigra pars reticulata; SNC, substantia nigra pars compacta; VTA, ventral tegmental area; PAG, periaqueductal gray matter; IPN, interpeduncular nucleus; SC, superior colliculus.

Table 4. Specific guanosine 5′-(γ-[^{35}S]thio)triphosphate binding sites in the striatal and cerebellar brain areas from *post mortem* human brain

		Agonist-stimulated [^{35}S]GTPγS binding (nCi/g t.e.)					
Brain area	Basal level	WIN55212-2	DAMGO	8-OH-DPAT	Sumatriptan	Carbachol	UK14304
Striatum							
Caudate	21 ± 2	272 ± 92	87 ± 25	23 ± 1	18 ± 4	32 ± 6	66 ± 9
Putamen	26 ± 3	337 ± 98	98 ± 16	28 ± 2	28 ± 4	41 ± 8	72 ± 10
Accumbens	29 ± 3	325 ± 74	156 ± 16	28 ± 2	31 ± 2	41 ± 2	58 ± 4
Cerebellum							
Cortex	11 ± 1	121 ± 82	17 ± 6	7 ± 1	5 ± 2	13 ± 5	23 ± 4

Data are mean ± S.E.M. of $n = 4$ cases.

autoradiography,[44,61] and with the μ-opioid receptor mRNA localization.[22,45]

Although [^{35}S]GTPγS binding stimulation by WIN55212-2[48,52,53] and DAMGO[52,55] have previously been measured in rodent tissue showing an anatomical distribution comparable to that found in the present study in *post mortem* human tissue, the reported percentages of stimulation were somewhat different to those obtained in human brain. Thus, the incubation in the presence of the cannabinoid receptor agonist at 100 μM produced stimulations 20 times higher in human *post mortem* tissue than in rat brain at 10 μM for several areas such as the striatum, while the percentages of response were similar in other brain regions, such as the substantia nigra.[48,53] In the case of [^{35}S]GTPγS binding stimulation by DAMGO, the maximal stimulation was observed in the human nucleus accumbens, with similar levels to those observed in rat brainstem nuclei.[55] Nevertheless, the brainstem was weakly stimulated in the human brain. The differences in receptor densities as well as in the coupling process to G-proteins would account for the different distributions observed between rodent and human brains, as will be discussed further below.

The agonist 8-OH-DPAT was used to analyse the stimulation of [^{35}S]GTPγS binding coupled to the 5-HT$_{1A}$ receptor subtype. The autoradiograms showed an anatomical pattern coincident with that obtained by classical receptor autoradiography with [^{3}H]8-OH-DPAT[21,42] or [^{3}H]WAY100635.[18] However, scarce expression of the 5-HT$_{1A}$ receptor in the human striatum has been reported,[30] whereas [^{35}S]GTPγS binding stimulated by 8-OH-DPAT, shown in the present

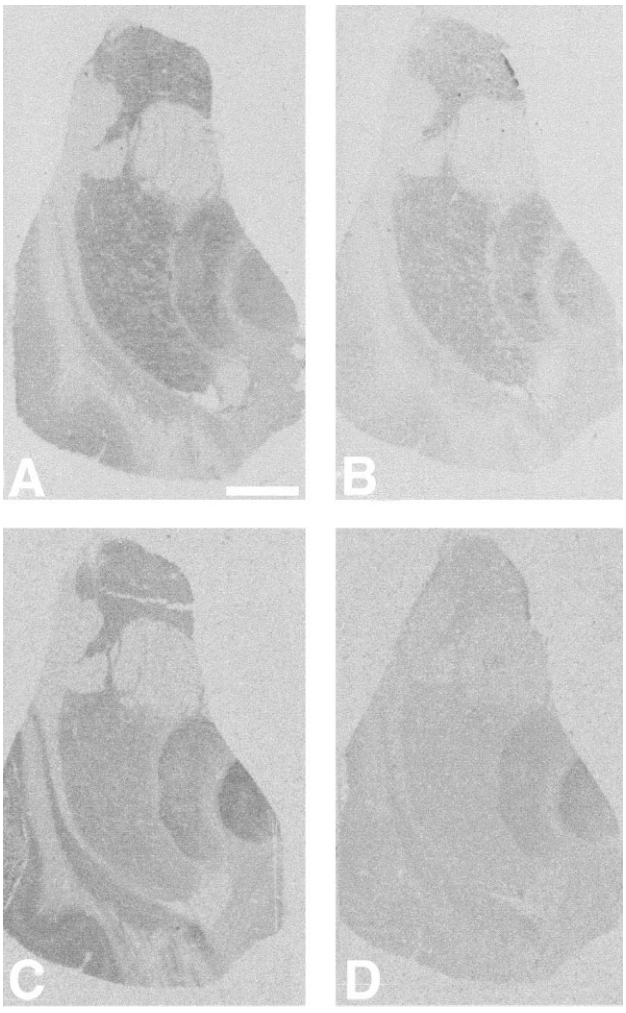

Fig. 6. Autoradiographic images of consecutive tissue sections from human striatal area, showing the cannabinoid CB_1 receptor-stimulated [^{35}S]GTPγS binding (A, B) and [^{3}H]CP55940 binding (C, D) distributions. A corresponds to [^{35}S]GTPγS (0.05 nM) binding in the presence of the CB_1 receptor agonist WIN55212-2 (100 μM) and B to the [^{35}S]GTPγS binding in the absence of agonist (basal level). C shows the [^{3}H]CP55940 binding in the absence of WIN55212-2 and D shows that in the presence of WIN55212-2 (non-specific binding). Note the similar distribution pattern between A and C. Scale bar = 5 mm.

study, or the previously described [^{3}H]8-OH-DPAT binding sites showed moderate levels.[21,42] The distribution was coincident in the hippocampus and prefrontal cortex.[30] These discrepancies could suggest a different origin of 8-OH-DPAT binding sites in different brain areas. The 8-OH-DPAT-stimulated [^{35}S]GTPγS binding densities were high in the hippocampal area, especially the CA1 pyramidal layer and subiculum. Nevertheless, the dorsal raphe, a nucleus with a high density of 5-HT_{1A} receptors, did not show a high [^{35}S]GTPγS binding in the presence of 8-OH-DPAT. So, even if the dorsal raphe is a region rich in 5-HT_{1A} receptors, their coupling to G-proteins appears to be different in this region. This would not be surprising, as the transductional mechanisms linked to the activation of 5-HT_{1A} receptors appear to be different depending on their pre- or postsynaptic localization.[9,57] In general terms, the anatomical pattern of distribution of 8-OH-DPAT-stimulated [^{35}S]GTPγS binding in human brain sections shows a good correlation with that reported previously in mammalian brain.[10,33,62]

The [^{35}S]GTPγS binding stimulation with the 5-HT agonist sumatriptan showed the highest stimulation in the hippocampal region and cortical areas. However, *in vitro* studies with [^{3}H]sumatriptan revealed high autoradiographic densities in the substantia nigra, cortex and putamen in human brain.[4,40] Moreover, the autoradiographic localization of [^{3}H]sumatriptan binding sites closely resembles the mRNA expression distribution in guinea-pig brain.[33] In the human brain, sumatriptan binds to the 5-$HT_{1B/1D}$ and 5-HT_{1F} receptor subtypes, at least.[40] Taking into account that the cortical regions and the hippocampus show a relatively high expression of the 5-HT_{1F} subtype, it is possible that the [^{35}S]GTPγS binding stimulation by sumatriptan is due mainly to 5-HT_{1F} receptor activation. The marked lack of correlation between the anatomical distribution of [^{3}H]sumatriptan binding sites and that of sumatriptan-stimulated [^{35}S]GTPγS binding has no definitive explanation at present. In addition, the possibility exists that a relevant part of the [^{35}S]GTPγS binding sites stimulated by sumatriptan over the hippocampus belong to the 5-HT_{1A} receptor subtype, in a similar way to what has been proposed by Waeber and Moskowitz[62] for [^{35}S]GTPγS binding stimulation by the putative 5-$HT_{1B/1D}$ agonist 5-*O*-carboxymethyl-glycyl-tyrosinamide. Clearly, caution is needed in interpreting this part of the study and further pharmacological work will be required to clarify this issue.

In consecutive sections, the cholinergic muscarinic agonist carbachol was used to stimulate [^{35}S]GTPγS binding. Carbachol has been demonstrated to be selective for the M_2 muscarinic receptor subtype, but also to show a good affinity for the M_4 subtype.[59] The M_2 subtype distribution has been detected in rat brain[60] by immunohistochemistry and *in situ* hybridization techniques, showing the highest densities in the basal forebrain, striatum, hippocampus and hypothalamus. Moderate to low M_2 subtype autoradiographic densities have been described in human[47] and rat brain[60] in the striatal nucleus, where we found the highest stimulation of [^{35}S]GTPγS binding by carbachol. This stimulation in the striatal nucleus could be explained by an M_4 muscarinic receptor subtype-mediated effect. Autoradiographic studies for localization of the M_2 subtype in human brain have used [^{3}H]oxotremorine as a ligand instead of the radiolabelled carbachol molecule.[47] The higher selectivity of oxotremorine for M_2 vs M_4, in contrast to carbachol, could also account for the discrepancies between the present findings and radioligand binding studies.

The [^{35}S]GTPγS binding stimulated by the selective α_2-adrenoceptor agonist UK14304 showed a distribution parallel to that described for [^{3}H]UK14304 binding sites[31,39] or the α_2-adrenoceptor antagonist [^{3}H]RX821002.[17] The described expression of the α_2-adrenoceptor in mammalian brain tissue, in particular the α_2-C10 mRNA that would correspond to the α_{2A}-adrenoceptor subtype, is also comparable to the radioligand distribution.[25,43,66] The α_2-C10 mRNA expression has been localized in the brainstem, cerebral cortex, hippocampus and cerebellum.[24,42,64] Thus, high [^{35}S]GTPγS binding stimulated by UK14304 densities were found in the subiculum, molecular layer of the dentate gyrus and CA1 area of the hippocampal formation. The cortical areas also showed a high density of stimulated [^{35}S]GTPγS binding sites, particularly high in layers I and III of the frontal cortex, in agreement with the autoradiographic distribution of the tritiated ligand.

The [^{35}S]GTPγS binding stimulated by the different agonists analysed in this study is similar to the already

known receptor distribution in most of the brain areas studied. An illustration of this general correlation can be observed for the CB_1 cannabinoid receptor in Fig. 6 at the level of the basal ganglia. Nevertheless, there are several mismatches in the distribution of some GPCRs, as has been described above. In this sense, it is worth mentioning that the [^{35}S]GTPγS binding stimulated by agonist is labelling only the effective coupling of the receptor to the G-protein. Subsequently, it may be possible, through radiometric binding, to label a population of receptors which are not effectively coupled to the G-protein signal transduction mechanisms. Consequently, this population of receptors would not be labelled using the [^{35}S]GTPγS technique. Moreover, the G-protein population is constituted, among others, by G_o-, G_i- and G_s-proteins, whose relative abundance in the brain is $G_o > G_i > G_s$. Thus, the [^{35}S]GTPγS binding technique recognizes the receptor-mediated activation of a heterogeneous pool of G-proteins coupled to the agonist-stimulated receptor.[56]

In a previous study performed on membrane homogenates of the frontal cortex from *post mortem* human brain, we verified that the level of stimulation by GPCR agonists is dependent on the [^{35}S]GTPγS binding to G_i/G_o-proteins.[16] The exposure of the membrane homogenates to *N*-ethylmaleimide, which selectively uncouples the receptor from G_i/G_o-proteins,[37,58] produced a full inhibition of the [^{35}S]GTPγS binding stimulation by the different agonists used in the present study. This result also confirms the preservation, in *post mortem* human tissue, of the coupling mechanism of the receptor to G_i/G_o-proteins.

The interaction of the different agonists with their respective receptors was confirmed by the inclusion in the assay of selective antagonists, which resulted in an inhibition of the agonist-stimulated [^{35}S]GTPγS binding. Although the inhibition (at 10 μM) was not always complete, it showed a clear anatomical selectivity. An increase in the antagonists' concentration could probably demonstrate inhibitions down to the basal levels. Nevertheless, the different pharmacological selectivity of the agonists and antagonists used makes a uniform interpretation of the results difficult. In some cases, this fact could result in an apparent stimulation of [^{35}S]GTPγS binding by an agonist specific for a type of receptor in regions that do not contain relevant densities of that receptor, and could explain, for instance, the limitations in understanding the sumatriptan–GR127935 data.[41] A detailed study of each agonist at different concentrations, as well as the complete antagonist inhibition curves in each brain area, would help to fully understand the selectivity of the drugs for different GPCRs. However, such a study goes beyond the objective of the present work, which was to demonstrate the applicability of the [^{35}S]GTPγS binding technique to *post mortem* human tissue slices.

CONCLUSIONS

[^{35}S]GTPγS autoradiography in *post mortem* human brain constitutes a functional method to study receptors with anatomical detail. Therefore, this technique allows one to quantify receptor-activated G-proteins in discrete brain nuclei. It allows the analysis of agonist efficacy, combined with a high level of resolution. In addition, [^{35}S]GTPγS autoradiography is not limited by the availability of radiolabelled ligands, providing anatomical and functional information about new drugs. The present study demonstrates that it can be used successfully on *post mortem* samples, in spite of the limitations of these kinds of tissue. Furthermore, [^{35}S]GTPγS autoradiographic labelling applied to *post mortem* human tissue opens up a broad field of research on human neuropsychiatric and neurodegenerative diseases where GPCRs are implicated, and provides functional and anatomical information about possible modifications of the effective coupling of the receptor to the G-proteins in these disorders. This technique, in combination with other anatomical methods, such as *in situ* hybridization, immunocytochemistry and receptor autoradiography, will help in the study of the different elements involved in the receptor-mediated functionality of the human brain.

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