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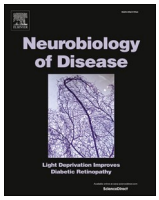

Sphingosine 1-phosphate receptor subtype 1 ($S1P_1$) activity in the course of Alzheimer's disease

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ABSTRACT

Some specific lipid molecules in the brain act as signaling molecules, neurotransmitters, or neuromodulators, by binding to specific G protein-coupled receptors (GPCR) for neurolipids. One such receptor, sphingosine 1-phosphate receptor subtype 1 ($S1P_1$), is coupled to $G_{i/o}$ proteins and is involved in cell proliferation, growth, and neuroprotection. $S1P_1$ constitutes an interesting target for neurodegenerative diseases like multiple sclerosis and Alzheimer's disease (AD), in which changes in the sphingolipid metabolism have been observed. This study analyzes $S1P_1$ receptor-mediated activity in healthy brains and during AD progression using *postmortem* samples from controls and patients at different Braak's stages. Additionally, the distribution of $S1P_1$ receptor activity in human brains is compared to that in commonly used rodent models, rats and mice, through functional autoradiography, measuring [^{35}S]GTPγS binding stimulated by the $S1P_1$ receptor selective agonist CYM-5442 to obtain the distribution of functional activity of $S1P_1$ receptors.

$S1P_1$ receptor-mediated activity, along with that of the cannabinoid CB_1 receptor, is one of the highest recorded for any GPCR in many gray matter areas of the brain, reaching maximum values in the cerebellar cortex, specific areas of the hippocampus and the basal forebrain. $S1P_1$ signaling is crucial in areas that regulate learning, memory, motor control, and nociception, such as the basal forebrain and basal ganglia. In AD, $S1P_1$ receptor activity is increased in the inner layers of the frontal cortex and underlying cortical white matter at early stages, but decreases in the hippocampus in advanced stages, indicating ongoing brain impairment. Importantly, we identified significant correlations between $S1P_1$ receptor activity and Braak stages, suggesting that $S1P_1$ receptor dysfunction is associated to disease progression, particularly in memory-related regions. The S1P signaling *via* $S1P_1$ receptor is a promising neurological target due to its role in key neurophysiological functions and its potential to modify the progression of neurodegenerative diseases. Finally, rats are suggested as a preferred experimental model for studying $S1P_1$ receptor-mediated responses in the human brain.

1. Introduction

Some of the endogenous lipid-based signaling molecules are considered as neurotransmitters with agonist-like or neuromodulatory properties, which can be denominated neurolipids (similar to the term "neuropeptides"). Neurolipids are a particular class of neurotransmitters since they are originated from membrane lipid precursors by the action of different enzymes such as phospholipases or sphingomyelinases, among others (Manuel et al., 2020). Neurolipids are synthesized on demand from membrane lipid precursors in a calcium-dependent manner, rather than being stored in presynaptic vesicles, and can be metabolized both enzymatically and non-enzymatically by oxidative

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degradation (Shimizu, 2009). The anatomical distribution or the activity of neurolipid receptors for the endocannabinoid system has been described (Herkenham et al., 1991), as well as for the lysophosphatidic acid (LPA) (González De San Román et al., 2015) or the sphingosine 1-phosphate (S1P) (Jiang et al., 2021). S1P was first described as a lysophospholipid acting through the endothelial differentiation gene (EDG) GPCRs family. Later this family of receptors was differentiated in S1P and LPA receptors, thus, EDG-1 was renamed as $S1P_1$ (Chun et al., 2002; Hla et al., 2001; Lee et al., 1998). Like other neurolipids, the $S1P_1$ receptor transduces intracellular signaling through the activation of $G_{i/o}$ proteins, leading to a wide variety of biological responses, such as effects on the immune response, the regulation of the cellular barrier integrity, angiogenesis, cell proliferation, and migration (Ben Shoham et al., 2012; Camp et al., 2020; Garcia et al., 2001; Liu et al., 2019; Matloubian et al., 2004; Pyne and Pyne, 2017; Spiegel and Weinstein, 2004). The pharmacological study of S1P receptors in the CNS made a leap with the development of fingolimod, a high-affinity agonist for $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ receptors after its *in vivo* phosphorylation, which is able to downregulate $S1P_1$, so this compound is also considered a "functional $S1P_1$ antagonist" (Brinkmann et al., 2002; Gräler and Goetzl, 2004). Regarding the pharmacological study of the $S1P_1$ receptor, the activity of the $S1P_1$ receptor has been studied by the [^{35}S]GTPγS assay in rat tissue using S1P and in mouse measuring the stimulation mediated by the partial agonist for $S1P_1$, SEW2871 (Sim-Selley et al., 2018, 2009; Waeber and Chiu, 1999), and the density of the $S1P_1$ receptor has recently been described using a new radioligand assay with [^{3}H]CS1P1 (Jiang et al., 2021).

The role of sphingolipids (SL) in neurodegenerative diseases, especially AD, is becoming increasingly important (Alaamery et al., 2020; Alessenko and Albi, 2020; Czubowicz et al., 2019). Some studies have related the membrane microdomains or "lipid rafts" rich in cholesterol and SL to the amyloidogenic processing of APP (Holmes et al., 2012; Kalvodova et al., 2005; Osenkowski et al., 2008). In particular, the accumulation of the Aβ in the lipid rafts activates the sphingomyelin (SM) hydrolysis, which could be mediated by the increase in the expression of neutral (nSMase) and acidic (aSMase) SMases in AD (Filippov et al., 2012; Haughey et al., 2010). Therefore, these SMase activations increase ceramide levels, as has been detected during moderate stages of dementia and would decrease during the progression of the disease to severe stages (Katsel et al., 2007). Similarly, aSM and nSM levels are found to be altered, increased or decreased depending on the brain area, in animals carrying the A53T mutation at early stages, which increases the formation of toxic alpha-synuclein species (Kalinichenko et al., 2024). In this regard, the accumulation of alpha-synuclein aggregates in AD patients appears to be associated with an increase in amyloid pathology (Pichet Binette et al., 2024). In addition, a loss of ceramide synthase 2 (CERS2), enzyme responsible for synthesis of very long chain ceramides, that are precursors of myelin lipids, has been reported in multiple brain regions of subjects with early and moderate stages of AD pathology. Interestingly, this CERS2 loss precedes the NFT pathology in temporal and frontal gray matter (Couttas et al., 2016). Moreover, the expression of sphingosine kinase 1 (SphK1) and the activity of sphingosine kinase 2 (SphK2) are decreased in AD, while the S1P lyase expression is enhanced leading to a decreased level of the own S1P neurolipid (Ceccom et al., 2014; Couttas et al., 2018, 2014). Consequently, the levels of S1P are decreased in the cytosolic fraction of the gray matter at the frontotemporal brain cortex of AD patients (He et al., 2010). In addition, the $S1P_1$ receptor is also decreased in frontal and temporal cortex when detected by immunoblotting (Ceccom et al., 2014). In a recently published study focused in frontal cortex, we have reported a marked reduction in $S1P_1$ receptor activity, particularly in the white matter (WM) associated to the frontal cortex, in some AD cases compared with controls and mild cognitive impairment (MCI) belonging to the Rush Religious Orders Study (RROS) cohort. This WM activity negatively correlates with CB_1 receptor activity in gray matter in layers V-VI (Moreno-Rodriguez et al., 2024).

Thus, there is multiple evidence establishing a relationship between the neurolipid systems and the pathology of AD. Specifically, the components that constitute the S1P system seem to be altered in their activity or expression; however, there are still no data on the activity of the $S1P_1$ receptor in patients with AD. Therefore, the present study aims to analyze the anatomical localization of the functional coupling of $G_{i/o}$ protein to $S1P_1$ receptors in *postmortem* human brain samples from 22 males and 24 females selecting representative brain areas enriched in $S1P_1$ receptors: frontal cortex, temporal cortex, caudate-putamen, hippocampus, amygdala, nucleus basalis of Meynert and cerebellum of *postmortem* human brain. The activity of the $S1P_1$ receptor has been studied during the progression of AD patients and classified according to Braak's classification to observe the possible evolution in the activity of this receptor with the course of the disease. Finally, the comparison of the human brain $S1P_1$ activity with that in rat and mice models has also been studied to validate these species as experimental models for the study of neurological diseases such as multiple sclerosis and dementia of the Alzheimer's type (DAT).

2. Materials and methods

2.1. Subjects and animals

2.1.1. Postmortem human brain samples

Postmortem human brain samples were obtained from different cerebral tissue banks: The samples from Biobank of the Basque Country were obtained at autopsy after getting informed consent in accordance to the ethics committees of the University of Basque Country (UPV/EHU) (M30/2024/066), following the Code of Ethics of the World Medical Association (Declaration of Helsinki), and warranting the privacy rights of the human subjects. The other samples and data from donors included in this study were provided by the Principado de Asturias BioBank (PT13/0010/0046), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees. These samples were initially differentiated in two main groups, control and AD patients. Control samples ($n = 8$) had not shown any evidence of metabolic or neurological disease, and after the neuropathological study, no abnormality was observed in the brain. However, AD samples ($n = 38$) were separated after the neuropathological study into three different groups according to the Braak's stages (AD I – II, AD III – IV and AD V – VI), which describe the progression of AD according to the spread of tau pathology. In the early stages (I–II), the pathology is primarily confined to the transentorhinal cortex; in the intermediate stages (III–IV), it extends to the limbic regions; and in the advanced stages (V–VI), the pathology reaches the neocortex, correlating with more severe cognitive impairment (Braak et al., 2006). The brain samples were frozen at −80 °C after autopsy (*postmortem* time 9–18 h). These samples were used for autoradiography assays. The mean demographic data are shown in the following table (Table 1).

2.2. Labeling of activated $G_{i/o}$ proteins by [^{35}S]GTPγS binding assay

For the performance of functional autoradiography of $S1P_1$ receptors, fresh 20 μm sections obtained from *postmortem* human and

Table 1
Demographic data from control and AD human samples. Data are mean ± SEM.

Samples	n			Age (years)			*Postmortem* (hours)		
	Total	Male	Female						
Control	8	5	3	74	±	4.7	9	±	2.3
AD									
I - II	14	8	6	81	±	3.4	12	±	2.2
III - IV	14	5	9	84	±	3.3	14	±	3.0
V - VI	10	4	6	84	±	4.1	18	±	2.9

rodent brain samples were air dried, followed by two consecutive incubations in HEPES-based buffer (HEPES 50 mM, NaCl 100 mM, $MgCl_2$ 3 mM, EGTA 0.2 mM and BSA 0.5 %; pH 7.4) for 30 min at 30 °C to remove the endogenous ligands. Slices were incubated for 2 h at 30 °C in the same buffer but supplemented with GDP 2 mM, DTT 1 mM and 0.04 nM [^{35}S]GTPγS. This assay allows for the detection of receptor activity by quantifying the activation of $G_{i/o}$ proteins following stimulation by the $S1P_1$-specific agonist CYM-5442, thereby measuring the functional state of the $S1P_1$ receptor. Basal binding was determined in two consecutive slices in the absence of the agonist. For the agonist-stimulated binding measurement, another consecutive slice was incubated with the same reaction buffer in the presence of CYM-5442. The specific $S1P_1$ receptor antagonist W146 (10 μM) together with CYM-5442 (10 μM) was used in other consecutive slices to ascertain that the activation was mediated by that specific receptor subtype. Non-specific binding was defined by competition with GTPγS (10 μM) in another consecutive section. Then, slices were washed twice in cold (4 °C) 50 mM HEPES buffer (pH 7.4), dried and exposed to β-radiation sensitive film with a set of [^{14}C]-standards calibrated for ^{35}S. After 48 h the films were developed, scanned and quantified by transforming optical densities into nCi/g tissue equivalent (nCi/g t.e.) units using a calibration curve defined by the known values of the [^{14}C]-standards (FIJI Software, Bethesda, MD, USA). Background and non-specific binding were subtracted from all experimental conditions. Then, the net stimulations were calculated by subtracting the basal binding.

2.3. Statistical analyses

Non-parametric Kruskal-Wallis tests followed by Dunn's *post hoc* tests were performed for multiple comparisons between the *postmortem* human brain samples when controls and patients were compared. In addition, to evaluate the influence of gender and Braak's stages, a two-way ANOVA was conducted, followed by Tukey's *post hoc* test for pairwise comparisons. This allowed for the assessment of interactions between these factors on the measured outcomes. Correlation analyses were performed to explore the relationships between $S1P_1$ receptor activity and key pathogenic factors, such as Braak's stages and patient age. Pearson correlation coefficients (r) were calculated for each brain region analyzed, and *p*-values were determined to assess statistical significance. All statistical analyses were conducted using GraphPad Prism (San Diego, CA, USA) and Microsoft Office Excel. Data are presented as mean ± standard error of the mean (SEM), and statistical significance was considered at $p < 0.05$.

3. Results

3.1. $S1P_1$ receptor activity in human postmortem brain

The activity of the $S1P_1$ receptor was analyzed in *postmortem* human brain tissue, exploring the distribution of this receptor in the CNS and comparing it with the most common animal models used in neuropharmacological research, *i.e.*, rat and mouse.

Note the high activity of $S1P_1$ receptors in all of the human brain

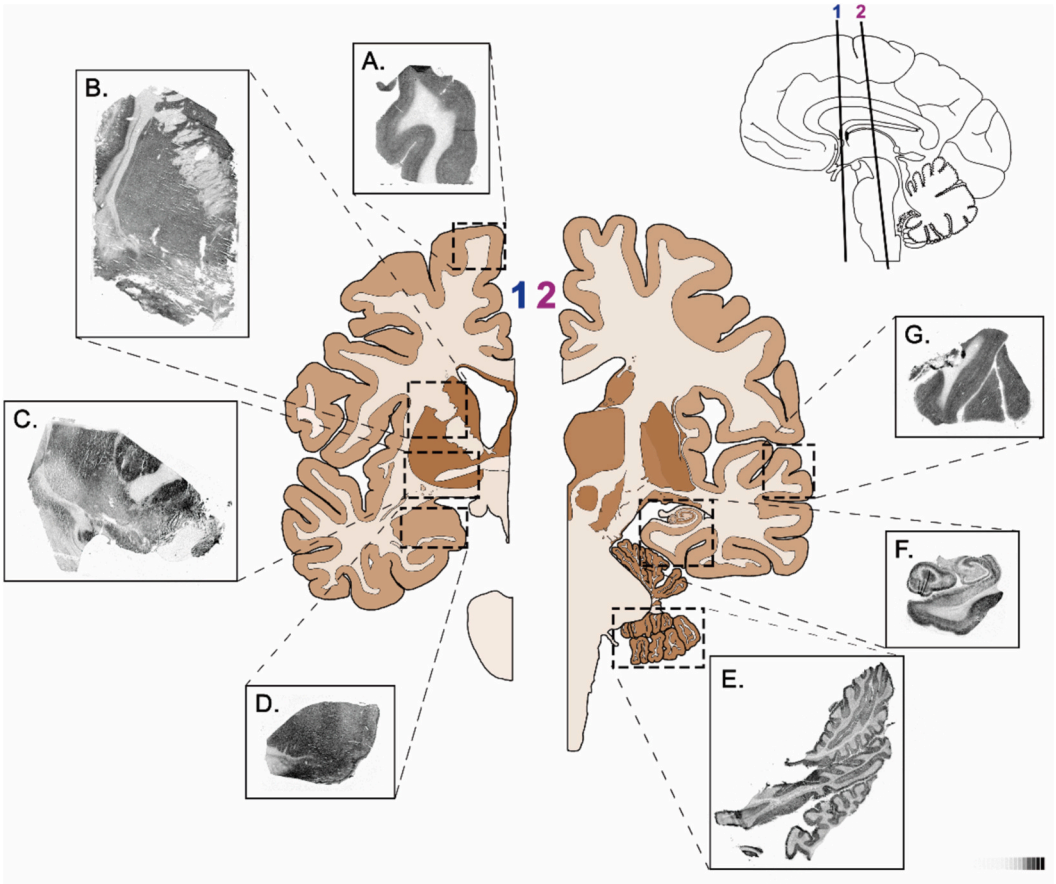

Fig. 1. Representative autoradiograms of [^{35}S] GTPγS stimulated by specific agonist of $S1P_1$receptor, CYM-5442 (10 μM), in human **A.** frontal cortex, ***B. striatum*, C.** basal forebrain, **D.** amygdala, **E.** cerebellum, **F.** hippocampus and **G.** temporal cortex. **1** corresponds to the coronal section of the brain at the level of the anterior commissure viewed from its posterior surface. **2** corresponds to the coronal section of the brain through the red nucleus viewed from its posterior surface. Right bottom [^{14}C]-Standard (0–35,000 nCi/g t.e.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

areas that were analyzed. In the control group frontal cortex, we observed that the layers V-VI were more stimulated than layers I – II and III-IV (Layer I – II: 1056 ± 120 nCi/g t.e.; Layer III – IV: 1142 ± 213 nCi/g t.e.; Layer V – VI: 1347 ± 168 nCi/g t.e.; Fig. 1A); in temporal cortex the stimulation measured in III-IV was higher than that measured in other layers (Layer I – II: 726 ± 108 nCi/g t.e.; Layer III – IV: 930 ± 198 nCi/g t.e.; Layer V – VI: 781 ± 157 nCi/g t.e.; Fig. 1G). This pattern changes in entorhinal cortex, where the layers V – VI were highly stimulated compared to layers I – II and III – IV (Layer I – II: 969 ± 145 nCi/g t.e.; Layer III – IV: 968 ± 166 nCi/g t.e.; Layer V – VI: 1570 ± 298 nCi/g t.e.; Fig. 1F), and in periamygdalar cortex, where all the layers were stimulated more homogeneously (Layer I – II: 917 ± 191 nCi/g t.e.; Layer III – IV: 769 ± 132 nCi/g t.e.; Layer V – VI: 780 ± 141 nCi/g t.e.; Fig. 1D). The white matter areas underneath to the different cortices were weakly stimulated (Frontal cortex: 167 ± 56 nCi/g t.e.; temporal cortex: 36 ± 21 nCi/g t.e.; entorhinal cortex: 296 ± 72 nCi/g t.e.; periamygdalar cortex: 139 ± 31 nCi/g t.e.; Fig. 1E, G and Table 2).

Regarding the basal ganglia, the globus pallidus showed high $S1P_1$ activity (1318 ± 198 nCi/g t.e.; Fig. 1C), but the striatum, including the caudate and putamen nuclei, showed moderated stimulations (921 ± 200 nCi/g t.e.; 751 ± 174 nCi/g t.e.; respectively; Fig. 1B). The anterior commissure, constituted by white matter fibers, was sparsely stimulated by the $S1P_1$ specific agonist (350 ± 98 nCi/g t.e.; Fig. 1C). In addition, nbM was measured in the basal forebrain and showed high stimulation (1592 ± 153 nCi/g t.e.; Fig. 1C and Table 2).

In the amygdala, we found that the activity of $S1P_1$ receptor was homogenous. Despite this homogeneity, different areas could be distinguished, as central (1014 ± 184 nCi/g t.e.; Fig. 1D) and lateral nucleus (1016 ± 235 nCi/g t.e.; Fig. 1D) that were more stimulated than cortical nucleus (794 ± 212 nCi/g t.e.; Fig. 1D) or basal nucleus, including the magnocellular and parvocellular parts (832 ± 226 nCi/g t.e.; 888 ± 199 nCi/g t.e. respectively; Fig. 1D and Table 2).

In the hippocampus, the pyramidal layer was sparsely stimulated, while the oriens and radiatum layers were strongly stimulated. Furthermore, in the dentate gyrus, the molecular layer showed a high $S1P_1$ activity, but the granular layer was sparsely stimulated. About the lacunosum moleculare, it was strongly stimulated (1877 ± 259 nCi/g t.e.; Fig. 1F and Table 2).

Finally in the cerebellum, the molecular layer showed a high activity (1715 ± 70 nCi/g t.e.; Fig. 1E), yet the granular layer was sparsely stimulated (160 ± 79 nCi/g t.e.; Fig. 1E and Table 2).

Overall, no differences were found between $S1P_1$ receptor activity when comparing male and female samples in all the areas that were analyzed (Supplemental material S1; Table S1).

3.2. $S1P_1$ receptor activity during Alzheimer's disease progression

The abundance of $S1P_1$ signaling in areas related to memory and learning, together with the reported modifications on other neurolipid neurotransmitter systems in AD, led us to consider also the involvement of S1P signaling *via* $S1P_1$ receptor in AD. Therefore, we studied the activity of the $S1P_1$ receptor in AD patients by measuring the $S1P_1$-activated $G_{i/o}$ proteins using the [^{35}S]GTPγS binding assay in human *postmortem* brain samples corresponding to control age-matched cases compared to AD patients in the different Braak's stages of neurocognitive impairment, and classified in 3 groups: AD I – II (mild stage), AD III – IV (moderate stage) and AD V – VI (severe stage). Despite performing an analysis to assess the potential influence of sex/gender on $S1P_1$ receptor activity, no significant differences were observed. The only factor that yielded statistically significant changes in receptor activity was the Braak's stage, indicating that disease progression plays a critical role in altering $S1P_1$ receptor function (Table S4).

Thus, we found that the activity of the $S1P_1$ receptor was decreased in layer I – II of the frontal cortex in all the stages of the AD patients (Control: 1056 ± 120 nCi/g t.e. *vs* AD I – II: 510 ± 83 nCi/g t.e. $*^a$ $p < 0.05$; *vs* AD III – IV: 545 ± 52 nCi/g t.e. $*^b$ $p < 0.05$; *vs* AD V – VI: 406 ± 70 nCi/g t.e. $***^c$ $p < 0.001$). However, the activity of $S1P_1$ receptor in the layer V – VI of frontal cortex was upregulated in the first stages of the disease (Control: 1347 ± 168 nCi/g t.e. *vs* AD I – II: 2117 ± 130 nCi/g t.e. $*^a$ $p < 0.05$) and subsequently, were decreased compared to these initial stages (AD I – II: 2117 ± 130 nCi/g *vs* AD V – VI: 1301 ± 209 nCi/g t.e. $*^d$ $p < 0.05$). Regarding the white matter, the activity of $S1P_1$ receptors was upregulated in the stages AD I – II and AD III – IV in contrast to controls (Control: 167 ± 56 nCi/g t.e. *vs* AD I – II: 537 ± 59 nCi/g t.e. $*^a$ $p < 0.05$; *vs* AD III – IV: 541 ± 58 nCi/g t.e. $**^b$ $p < 0.01$) (Figs. 2 and 3, Table 2).

In the same way, the activity of $S1P_1$ receptor was upregulated in white matter of the stages AD I – II and AD III – IV of all the other cortical areas that were analyzed including the temporal cortex (Control: 36 ± 21 nCi/g t.e. *vs* AD I – II: 153 ± 16 nCi/g t.e. $***^a$ $p < 0.001$; *vs* AD III – IV: 123 ± 7 nCi/g t.e. $**^b$ $p < 0.01$), the entorhinal cortex (Control: 296 ± 72 nCi/g t.e. *vs* AD III – IV: 956 ± 221 nCi/g t.e. $*^b$ $p < 0.05$) and the periamygdalar cortex (Control: 139 ± 31 nCi/g t.e. *vs* AD I – II: 401 ± 45 nCi/g t.e. $**^a$ $p < 0.01$; *vs* AD III – IV: 337 ± 33 nCi/g t.e. $*^b$ $p < 0.05$) (Figs. 2 and 3, Table 2). In addition, we found a decrease in the activity of $S1P_1$ receptor in the layer I – II of the entorhinal cortex during the stages of AD V – VI (Control: 969 ± 145 nCi/g *vs* AD V – VI: 380 ± 67 nCi/g t.e. $*^d$ $p < 0.05$) (Figs. 2 and 3, Table 2).

Finally, the activity of $S1P_1$ receptor decreased in some hippocampal areas during the severe stages of the disease (AD V – VI), *e.g.* in radiatum layer of the CA1, in oriens and radiatum layers of the CA2, in oriens and radiatum layers of the CA3, in oriens, pyramidal and radiatum layers of the CA4, in oriens, pyramidal and radiatum layers of the subiculum, in the molecular layer of the dentate gyrus and in the lacunosum moleculare (Figs. 2 and 3).

3.3. $S1P_1$ receptor activity correlates with Braak's stages

We analyzed the correlation between $S1P_1$ receptor activity and two key factors influencing Alzheimer's disease progression: Braak's stages and age, across multiple cortical and hippocampal regions (Table S5). Significant negative correlations between Braak's stages and $S1P_1$ receptor activity were observed in the frontal cortex (Layer I-II, $r = -0.5903$, $***p = 0.0002$; Fig. 4A), entorhinal cortex (Layer I-II, $r = -0.5115$, $*p = 0.0178$ Fig. 4B), and hippocampus, including CA1 *radiatum* ($r = -0.5293$, $**p = 0.0054$; Fig. 4C), CA2 (*oriens*: $r = -0.5588$, $**p = 0.0069$; Fig. 4D; and *radiatum*: $r = -0.5595$, $**p = 0.0084$ Fig. 4E), CA3 *radiatum* ($r = -0.4386$, $*p = 0.0467$; Fig. 4F) and CA4 *radiatum* ($r = -0.457$, $*p = 0.0428$; Fig. 4G). Additionally, in the dentate gyrus molecular layer, a significant correlation was observed ($r = -0.565$, $**p = 0.0061$; Fig. 4H), as well as in the *radiatum* layer of the subiculum ($r = -0.4236$, $*p = 0.0495$; Fig. 4I) and in the *lacunosum moleculare* ($r = -0.6304$, $**p = 0.0029$; Fig. 4J), indicating that as Braak's stages increases, $S1P_1$ receptor activity decreases, particularly in memory-related brain regions. No significant correlations were observed between $S1P_1$ receptor activity and patient age in any of the analyzed regions, highlighting that receptor dysfunction is more closely linked to disease progression rather than age itself. (Table S5)

3.4. Anatomical comparison of $G_{i/o}$-proteins activated by the $S1P_1$ receptor agonist CYM-5442 in rodents and human brain

$S1P_1$ receptor activity was also detected in the experimental animals most commonly used for the study of neurodegenerative diseases, rats and mice, and compared with that observed in human tissues (Supplemental material S2).The activity of the $S1P_1$ receptor was conserved in human compared to the rat brain (Fig. 5). In the cortex, the layers III - IV were strongly stimulated in both types of rodents (mouse and d rat) and in human, but the layers V – VI showed a higher activity in both rodent brains than in human brain (Fig. 5A;, Figs. S1 and S2; Tables S2 and S3). The activity of the $S1P_1$ receptor was similar in the striatal areas of humans and rodents. Moreover, the nucleus basalis magnocellularis in

Table 2
Net binding of [^{35}S]GTPγS in human control brain (nCi/g t.e.) and Alzheimer's disease stimulated by the specific agonist of $S1P_1$ receptor, CYM-5442 (10 μM) in cortical areas (frontal, temporal, entorhinal and periamygdalar), amygdala, hippocampus and basal forebrain.

AREA	CONTROL (nCi/g t.e.)			AD I - II (nCi/g t.e.)			AD III - IV (nCi/g t.e.)			AD V - VI (nCi/g t.e.)		
Frontal Cortex		(n = 8)			(*n* = 10)			(*n* = 11)			(*n* = 8)	
Layer I - II	1056	±	120	510	±	83*[a]	545	±	52*[b]	406	±	70***[c]
Layer I	1102	±	119	450	±	88**[a]	554	±	53**[b]	496	±	77**[c]
Layer II	1054	±	155	524	±	61*[a]	547	±	44*[b]	370	±	77***[c]
Layer III - IV	1142	±	213	950	±	143	1067	±	114	738	±	160
Layer V - VI	1347	±	168	2117	±	130*[a]	1596	±	136	1301	±	209*[d]
White Matter	167	±	56	537	±	59*[a]	541	±	58**[b]	434	±	86
Temporal Cortex		(n = 8)			(*n* = 13)			(*n* = 14)			(n = 10)	
Layer I - II	726	±	108	676	±	58	642	±	90	667	±	113
Layer III - IV	930	±	198	810	±	114	757	±	113	675	±	121
Layer V - VI	781	±	157	974	±	131	925	±	145	830	±	143
White Matter	36	±	21	153	±	16***[a]	123	±	7**[b]	92	±	14
Entorhinal Cortex		(*n* = 7)			(*n* = 4)			(*n* = 6)			(*n* = 7)	
Layer I - II	969	±	145	527	±	206	760	±	178	380	±	67*[c]
Layer III - IV	968	±	166	671	±	226	916	±	225	408	±	58
Layer V - VI	1570	±	298	1170	±	144	1473	±	323	772	±	140
White Matter	296	±	72	640	±	204	956	±	221*[b]	353	±	45
Periamygdalar Cortex		(n = 8)			(n = 11)			(n = 10)			(n = 7)	
Layer I - II	917	±	191	909	±	131	963	±	171	842	±	175
Layer III - IV	769	±	132	884	±	131	1022	±	172	898	±	246
Layer V - VI	780	±	141	999	±	161	1072	±	190	974	±	254
White Matter	139	±	31	401	±	45**[a]	337	±	33*[b]	270	±	68
Amygdala		(n = 8)			(n = 11)			(n = 10)			(n = 7)	
Basal nucleus (magnocellular)	832	±	226	848	±	151	1011	±	220	866	±	207
Basal nucleus (parvocellular)	888	±	199	904	±	167	1087	±	217	790	±	175
Central nucleus	1014	±	184	994	±	160	1173	±	270	813	±	107
Cortical nucleus	794	±	212	989	±	138	978	±	181	710	±	144
Lateral nucleus	1016	±	235	1106	±	131	1332	±	262	1009	±	231
Hippocampus		(n = 7)			(n = 6)			(n = 7–9)			(n = 7–8)	
CA1												
Oriens	1328	±	302	1633	±	256	1178	±	201	688	±	126
Pyramidal	525	±	75	482	±	60	430	±	87	285	±	73
Radiatum	1617	±	311	1546	±	285	911	±	153	557	±	95**[c,*d]
CA2												
Oriens	1392	±	124	1353	±	241	1241	±	189	676	±	93*[c]
Pyramidal	434	±	70	465	±	69	460	±	104	233	±	60
Radiatum	1518	±	261	1871	±	147	1067	±	144	641	±	103*[c,**d]
CA3												
Oriens	1526	±	242	1449	±	291	1437	±	200	636	±	95*[c,*e]
Pyramidal	474	±	66	477	±	122	584	±	140	247	±	63
Radiatum	1588	±	230	1491	±	297	1536	±	214	600	±	134*[c]
CA4												
Oriens	1492	±	196	1169	±	271	1442	±	251	516	±	117*[c,*e]
Pyramidal	434	±	84	342	±	57	658	±	174	190	±	53*[e]
Radiatum	1820	±	287	1335	±	216	1905	±	308	563	±	107*[c,**e]
Dentate gyrus												
Granular	292	±	117	161	±	29	286	±	77	152	±	53
Hilus	922	±	269	526	±	151	562	±	104	267	±	64
Molecular	1485	±	182	1021	±	185	1279	±	200	453	±	138**[c,*e]
Subiculum												
Oriens	1520	±	318	1350	±	284	1307	±	199	511	±	74*[c,*e]
Pyramidal	679	±	80	561	±	108	813	±	204	242	±	46*[c,*e]
Radiatum	1403	±	212	1457	±	321	1304	±	196	496	±	22*[c,*d]
Lacunosum moleculare	1877	±	259	2057	±	142	1398	±	206	783	±	158*[c,**d]
Basal forebrain		(n = 7)			(n = 3)			(*n* = 5)			(*n* = 2)	
Nucleus basalis (Meynert)	1592	±	153	1664	±	161	1245	±	452	1496	±	181
Striatum		(n = 8)										
Caudate	921	±	200	n/a			n/a			n/a		
Putamen	751	±	174	n/a			n/a			n/a		
		(n = 6)										
Globus pallidus	1318	±	198	n/a			n/a			n/a		
		(n = 5)										
Anterior commissure	350	±	98	n/a			n/a			n/a		
Cerebellum		(n = 5)										
Granular layer	160	±	79	n/a			n/a			n/a		
Molecular layer	1715	±	70	n/a			n/a			n/a		
White Matter	48	±	24	n/a			n/a			n/a		

Data are mean ± SEM values. (n): number of cases available. The *p* values were calculated by the Kruskal-Wallis non-parametric test followed by Dunn's *post hoc* test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). [a]Control *vs* AD I-II, [b]Control *vs* AD III-IV, [c]Control *vs* AD V-VI, [d]AD I-II *vs* ADV-VI, [e]AD III-IV *vs* AD V-VI. n/a: not available.

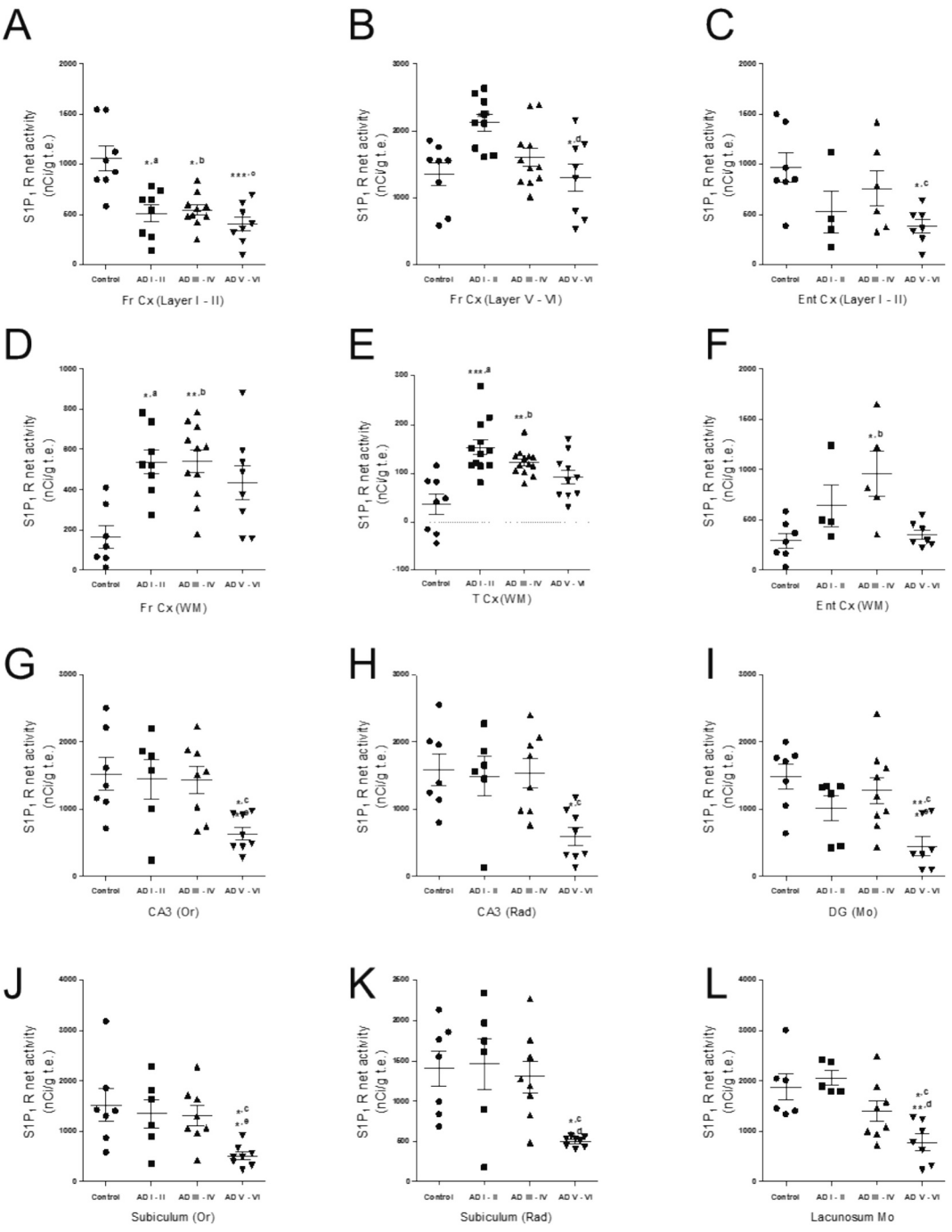

Fig. 2. Scatter plots showing the activity of S1P$_1$receptor during the progression of AD in frontal cortex (Fr Cx), entorhinal cortex (Ent Cx), temporal cortex (T Cx) and different areas of the hippocampus. A. The activity of S1P$_1$ receptor was decreased in layers I – II of the frontal cortex (Fr Cx ly I – II) during all the stages of the disease (AD I – II, $*^a$ $p < 0.05$; AD III – IV, $*^b$ $p < 0.05$; AD V – VI, $***^c$ $p < 0.001$). **B.** In contrast, the activities of S1P$_1$ receptor were upregulated in layer V – VI (Fr Cx ly V – VI) at the initial stages ($*^a$ $p < 0.05$), and subsequently were decreased ($*^d$ $p < 0.05$). **C.** The activities of S1P$_1$ receptor were decreased in layer I – II of entorhinal cortex (Ent Cx ly I – II) during the severe stages of the disease (AD V – VI, $*^c$ $p < 0.05$). **D.** The activity of the S1P$_1$ receptor was upregulated in the white matter (Fr Cx WM) in the initial and moderate stages (AD I – II, $*^a$ $p < 0.05$; AD III – IV, $**^b$ $p < 0.01$). **E.** The activity of the S1P$_1$ receptor was upregulated in the white matter of temporal cortex (T Cx WM) in the initial and moderate stages (AD I – II, $***^a$ $p < 0.001$; AD III – IV, $**^b$ $p < 0.01$). **F.** The activity of the S1P$_1$ receptor was upregulated in the white matter of entorhinal cortex (Ent Cx WM) during the moderate stages (AD III – IV, $*^b$ $p < 0.05$). **G.** The activities of S1P$_1$ receptor were decreased during the severe stages of the disease (AD V – VI) in oriens (Or) of CA3 (CA3, Or $*^c$ $*^e$ $p < 0.05$), **H.** in *radiatum* (Rad) of CA3 (CA3 Rad $*^c$ $p < 0.05$). **I.** in molecular layer of the dentate gyrus (DG Mo) (DG Mo $**^c$ $p < 0.01$ / $*^e$ $p < 0.05$), **J.** in Or of the subiculum (Subiculum, Or $*^c$ $*^e$ $p < 0.05$), **K.** in Rad of the subiculum (Subiculum, Rad $*^c$ $*^d$ $p < 0.05$) and L. in lacunosum moleculare (Lac Mo $*^c$ $p < 0.05$ / $**^d$ $p < 0.01$). The p values were calculated by the Kruskal-Wallis non-parametric test followed by Dunn's *post hoc* test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; [a]Control *vs* AD I-II, [b]Control *vs* AD III-IV, [c]Control *vs* AD V-VI, [d]AD I-II *vs* ADV-VI, [e]AD III-IV *vs* AD V-VI).

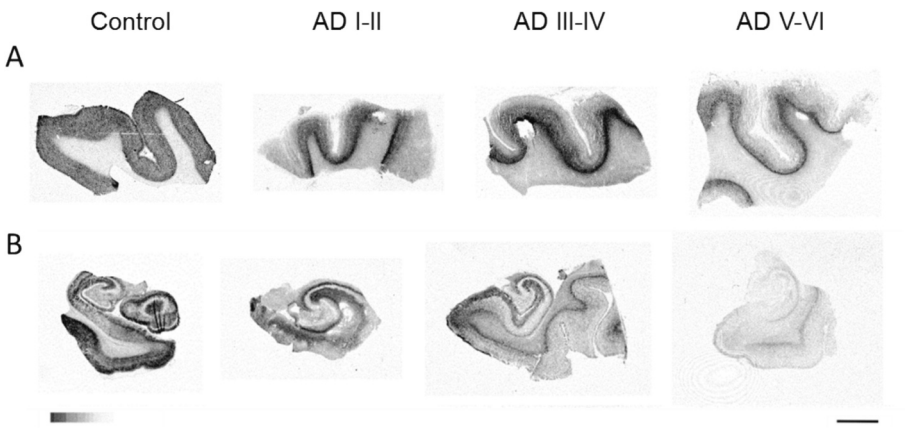

Fig. 3. Representative autoradiograms showing the [^{35}S] GTPγS binding stimulated by the specific agonist of S1P$_1$receptor, CYM-5442 (10 μM). A. Frontal cortex and **B.** hippocampus during the progression of AD (AD I – II, AD III – IV and AD V – VI). [^{14}C]-Standard (35000–0 nCi/g t.e.). Scale bar = 6 mm.

rodent brains and the nbM in human, which are assimilated to be comparable, showed one of the highest values of S1P$_1$ activity (Fig. 5B; Figs. S1 and S2; Tables S2 and S3). Regarding the hippocampus, in mouse brain the S1P$_1$ activity was lower than in rat brain. Nevertheless, the S1P$_1$ stimulation pattern was conserved in all the samples, being high in *oriens* and *radiatum* layers, more than in *pyramidal* layer (Fig. 5C; Figs. S1 and S2; Tables S2 and S3). Finally, in the cerebellum the molecular layer was the most stimulated layer in this area in both rodent and human brains, although the rat pattern is more similar to that observed in human (Fig. 5D; Figs. S1 and S2; Tables S2 and S3).

In general, the activity of the S1P$_1$ receptors mediated by CYM-5442 showed a similar distribution in human and rat brains, including the basalocortical pathway that regulates learning and memory processes but also in striatum, hippocampus or cerebellum. The present results indicate that rat models could be more similar to human beings for the study of S1P$_1$ receptors activity in neurodegenerative diseases, such as DAT.

4. Discussion

The present study quantifies the activity of S1P$_1$ receptors in brain areas involved in cognitive processes in the brains of both control subjects and AD patients at different stages of disease progression. On the other hand, the work shows the anatomical distribution of S1P$_1$ receptor activity in the most used rodent models of neurodegenerative diseases, that can be developed to describe the similarities and differences that may enlighten the understanding of the physiology of this type of receptor in the mammalian brain.

Sphingolipids, key components of cell membranes, have been recognized to play a critical role in learning and memory processes. The most abundant sphingolipids in the brain, ceramides and gangliosides, actively influence the structure of protein-containing cellular membranes. Modulation of these lipids affects protein signaling throughout membranes and neuronal plasticity overall. Once the sphingolipid balance is disrupted, normal cellular function is impaired, leading to a variety of psychiatric and neurological conditions characterized by cognitive deficits. S1P, a key metabolite of sphingolipids, plays a pivotal role in memory and learning by regulating neuronal plasticity and protein signaling across cellular membranes (reviewed in Rahmati-Dehkordi et al., 2024). Disruptions in S1P levels have been linked to cognitive impairments, contributing to the pathogenesis of AD (Couttas et al., 2014). S1P modulates the balance of ceramides and gangliosides, which are crucial for *de novo* learning and memory processes (reviewed in Chalfant and Spiegel, 2005; Kalinichenko et al., 2022).

The involvement of sphingolipids in the course of AD is acquiring a greater relevance during recent years. In fact, different enzymes involved in the metabolism of sphingolipids have been analyzed in AD. The observed modifications seem to be independent of the aging process, such as the upregulation of the gene expression of enzymes responsible for *de novo* synthesis of middle to long acyl chain Cer species, as well as the activation of SMase, resulting in increased ceramide levels during moderate stages of dementia (Katsel et al., 2007). Furthermore, the accumulation of the Aβ in membrane lipid rafts activates the SM hydrolysis, which could be mediated by this increase in the expression of neutral sphingomyelinases (nSMase) and acidic sphingomyelinases (aSMase) in AD (Filippov et al., 2012). However, the Cer regulation depends on the length of the chain. There is a loss of the enzyme responsible for the synthesis of very long chain ceramides, CERS2, which are precursors of myelin lipid, and this loss precedes the NFT pathology (Couttas et al., 2016). In addition, some components of the S1P system have also been directly evaluated in AD, showing that the expression of the enzymes that produce S1P, such as SphK1, or even their activity, such as SphK1 and SphK2, were decreased, while the expression of the enzyme that degrades the S1P, the S1P lyase, was enhanced leading to decreased S1P levels (Ceccom et al., 2014; Couttas et al., 2018, 2014; He et al., 2010). The status of the S1P$_1$ receptor signaling in AD was almost unknown; only a decrease of the S1P$_1$ receptor had been described in frontal and temporal cortex detected by immunoblotting (Ceccom et al., 2014), and as previously noted, we have recently reported that S1P$_1$ signaling is modified in WM of AD patients (Moreno-Rodriguez et al., 2024). Thus, we have evaluated the activity of the S1P$_1$ receptor with anatomical resolution in different brain areas during the progression of the AD, showing alterations in areas involved in memory processing, such as frontal and entorhinal cortices or hippocampus. Despite the well-documented sex/gender-specific changes in sphingolipid metabolism (Couttas et al., 2018), we did not observe statistically significant differences in S1P$_1$ receptor activity in the analyzed brain areas when stratified by sex/gender.

The layers I–II of frontal cortex tissue sections, from patients belonging to all the different stages of AD, showed a decrease in the activity of the S1P$_1$ receptor. However, the activities of S1P$_1$ receptor, were upregulated in layer V–VI of frontal cortex at the most initial stages of the disease. Since the expression of the S1P$_1$ receptor has been described as diminished in the frontal cortex gray matter of AD patients (Ceccom et al., 2014), it could be inferred that its activity would also be diminished. Nevertheless, the study from (Ceccom et al., 2014) only analyzed the severe AD patients and the layers where the decrease occurs by using the immunoblotting technique are not specified. In this sense, our results suggest that the decrease in the S1P$_1$ signaling in layers I–II would arise during the early AD stages and would be maintained throughout the disease. Moreover, we found an increase in the signaling of S1P$_1$ receptor in layer V–VI of frontal cortex at first stages of the disease that coincides with the described high CB$_1$ receptor activity. Those changes in the CB$_1$ receptor activity in layer V–VI were previously

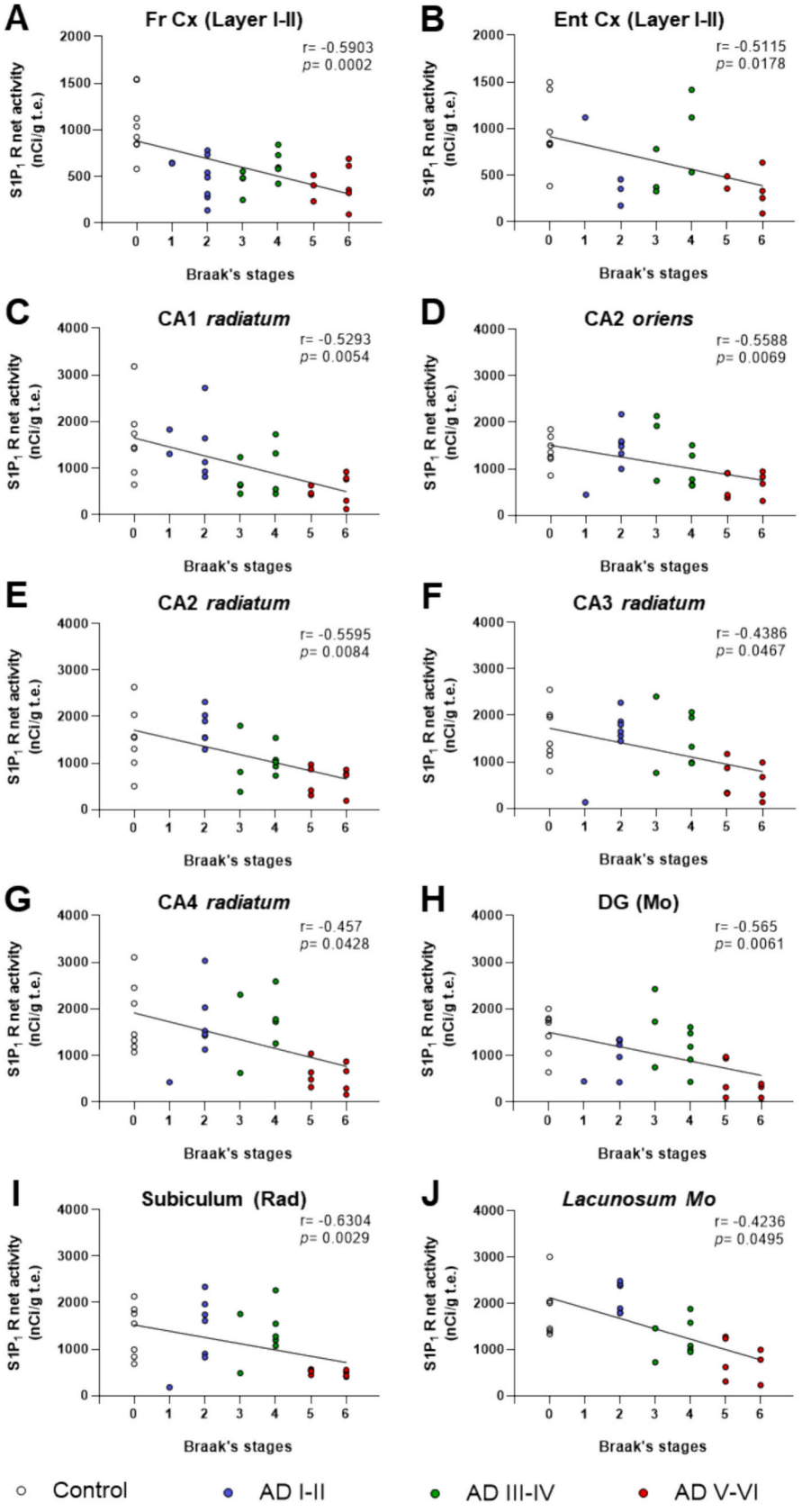

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Fig. 4. Correlation of Braak's stages with S1P activity in Alzheimer's Disease (AD) patients and controls. Scatter plots represent the relationship between Braak's stages (x-axis) and S1P activity measured in different regions (y-axis): **A.** frontal cortex layer I-II (Fr Cx), **B.** entorhinal cortex layer I-II (Ent Cx), **C.** CA1 *radiatum*, **D.** CA2 *oriens*, **E.** CA2 *radiatum*, **F.** molecular layer of the dentate gyrus (DG (Mo)), **G.** CA3 *radiatum*, **H.** *lacunosum moleculare* (*Lacunosum* Mo), **I.** CA4 *radiatum*, **J.** *radiatum* layer of the subiculum (Subiculum (Rad)); across control (white) and AD patients classified into different stages: AD I–II (blue), AD III–IV (green), and AD V–VI (red). Linear regression analyses were performed to assess the strength of correlation, with Pearson's correlation coefficient (r) and *p*-values provided for each comparison. Significant negative correlations between Braak's stages and $S1P_1$ receptor activity were observed, indicating a progressive decline in $S1P_1$ signaling with increasing disease severity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

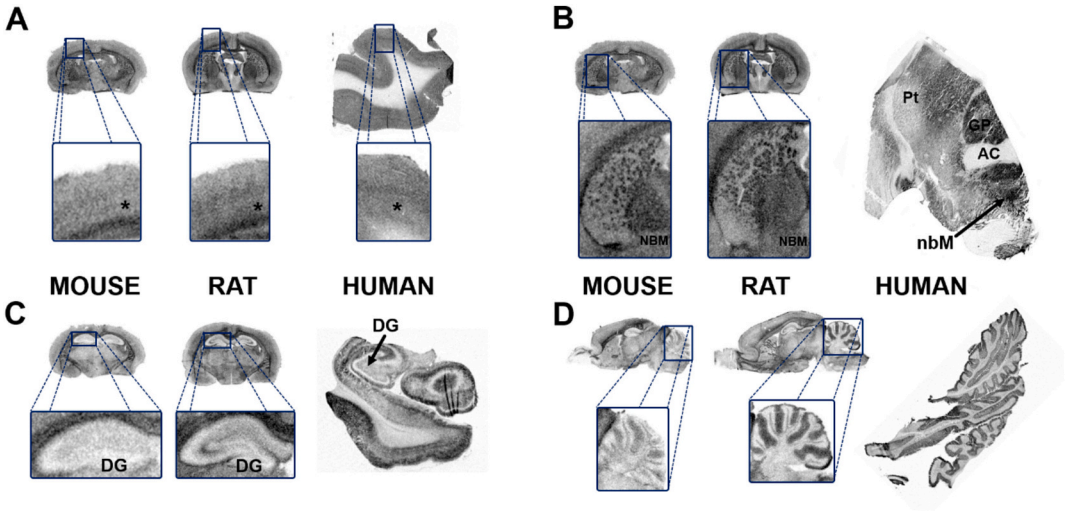

Fig. 5. Comparative autoradiograms showing the [^{35}S] GTPγS binding stimulated by the specific agonist of $S1P_1$receptor, CYM-5442, (10 μM) in mouse, rat and human. A. Note in cortical areas that the layers V – VI (labelled with an asterisk*) show a higher activity in rodent brains than in human brain. **B.** Mouse, rat and human striatum and basal forebrain (NBM: nucleus basalis magnocellularis; GP: *Globus Pallidus*; AC: Anterior Commissure; Pt: putamen; nbM: nucleus basalis of Meynert). **C.** Mouse, rat and human hippocampus, although the stimulation pattern is conserved in rodent brain, in mouse brain is much lower the activity of $S1P_1$ receptor. (DG: dentate gyrus).u **D.** Mouse, rat and human cerebellum, showing that the rat $S1P_1$ activity is more similar to the human.

reported by our group but in a different cohort of AD patients, suggesting that the increase during the initial stages of AD could indicate a neuroprotective action in response to initial neural damage (Manuel et al., 2014). Regarding the entorhinal cortex, changes in $S1P_1$ receptor signaling occur in the superficial layers (layer I-II) in the most severe phases of AD. (Ceccom et al., 2014) found a negative correlation between the Aβ deposits and the SphK1 expression as well as a positive correlation between the Aβ deposits and the S1P lyase expression in the entorhinal cortex, suggesting that the S1P levels could be related to the Aβ deposits in this area. This study, as already noted, only analyzed the severe stages of AD, in line with those we obtained showing decreased $S1P_1$ receptor signaling at the severe stages of AD.

Regarding the white matter, the activity of the $S1P_1$ receptor was increased in underlying white matter of all the cortical areas that were analyzed (frontal, temporal, entorhinal and periamygdalar cortex), at early and moderate stages of AD. We have recently reported results on $S1P_1$ receptor activity in samples from the Rush Religious Orders Study (RROS), classified according to CERAD criteria, showing alterations in receptor activity compatible with those obtained in the cohort used in the present study (Moreno-Rodriguez et al., 2024). The $S1P_1$ receptor regulates, in somehow, the survival and differentiation of oligodendrocytes, as well as myelination processes (Dukala and Soliven, 2016; Kim et al., 2018). Additionally, CerS2 enzyme, responsible for synthesizing very long chain ceramides (C24:1) in oligodendrocytes that can be regulated by S1P (Becker et al., 2008; Couttas et al., 2016; Laviad et al., 2008), serves as a precursor for myelin lipids like GalCer and sulfatides, which we have previously reported to be decreased in AD (Gonzalez de San Román et al., 2017). Moreover, the loss of CerS2 activity precedes NFT pathology in AD patients (Couttas et al., 2016). So, it seems that the activity of $S1P_1$ receptor in the white matter could be acting as a regulator of the oligodendrocytes in the early and moderate stages of the AD (Miron et al., 2008). In addition, the correlation analyses we performed between $S1P_1$ receptor activity and Braak's stages, particularly in the cortex and hippocampus, further highlight the significant relationship between receptor function and Alzheimer's disease progression. These correlations underscore the potential role of $S1P_1$ activity as a marker of disease severity and progression, particularly in regions critical for cognitive function.

In the hippocampus, the activity of $S1P_1$ receptor was decreased in almost all the analyzed hippocampal areas during the severe stages of the disease in line to that reported in the hippocampal region by immunoblotting technique (Ceccom et al., 2014). Decreased neurolipid receptor activity was also reported in the late stages of AD in a study of cannabinoid CB_1 receptor activity (Manuel et al., 2014), but in the present study assessing $S1P_1$ activity, there is no initial upregulation of activity as was observed for CB_1 receptors.

As mentioned before, the activity mediated by $S1P_1$ receptor in *postmortem* control human brain tissue was analyzed, and it was compared with the most widely used rodent experimental models for the study of the CNS; *i.e.*, rat and mouse. The anatomical distribution of the $S1P_1$ receptor in the human brain has been poorly studied and restricted to few immunohistochemical studies to determine the cellular distribution of this receptor (Nishimura et al., 2010), or the recently published study using the new [^{3}H]CS1P1 radioligand (Jiang et al., 2021; Nishimura et al., 2010). As indicated, we applied the same measurement approach used in *postmortem* human brain samples to measure the $S1P_1$ activity in rat and mice.

The $S1P_1$ activity patterns obtained in the human cortical areas were similar to those previously observed in rodent brains. However, there were few peculiarities in terms of stimulation in the different cortical areas. The layers III – VI of the frontal and temporal cortices showed higher stimulation than layers I – II and V – VI, while the layers V – VI of

the entorhinal cortex were highly stimulated compared to layers I – II and III – IV. This change of pattern compared to that found in rodents may be related to the different distribution of cells in human cortical areas (Ribeiro et al., 2013; Zilles et al., 2004). In general, the cortical $S1P_1$ activity was higher in the deeper layers of rodents compared to human brain. In addition, the white matter seems to be also more stimulated in rodents than in humans, possibly due to the different packing of the white matter described in rodents as opposed to the greater amplitude found in humans thanks to the gyrification of the cortex (Mota et al., 2019; Ventura-Antunes et al., 2013).

In relation to the activity of $S1P_1$ receptor in the basal ganglia, it was high at globus pallidus while the striatum, including the caudate and putamen nuclei, showed moderate levels. The latter, possibly due to the contribution of the highly myelinated striatopallidal fibers. Moreover, the nbM located at the basal forebrain, showed a high stimulation in a similar way to that found for the BFCN in rat. Interestingly, both structures, the nucleus basalis magnocellularis (B) in rodent brains and the nbM in human, showed the highest measurements of $S1P_1$ activity, probably indicating that $S1P_1$ receptors are regulating their physiological functions. Moreover, in view of these results it can be inferred that both rodents are a good model to study the role of this subtype of S1P receptor in this area, very probably modulating the cholinergic signaling that governs the memory and learning controlled by the basalocortical pathway. In the same way, the activity of $S1P_1$ receptor was similar in the striatal areas of rodents and humans, therefore the rodents could constitute also a good model to study the impairment of the nigrostriatal pathway in motor-related diseases including Parkinson's and Hungtinton's disease.

In the hippocampus, the $S1P_1$ activity pattern resembles that seen in the rat but not in the mouse brain. In this sense, the *oriens* and *radiatum* layers showed high levels, while in the pyramidal layer was weaker. In addition, the molecular layer of the dentate gyrus showed a high $S1P_1$ activity, but in the granular layer was low. It is assumed that the neurogenesis in humans, as well as in rodent brain, also occurs in this region. This process is regulated by glutamatergic inputs, which, as above mentioned, can be regulated by $S1P_1$ receptor activity in this area (Kempermann et al., 2015).

In summary, $S1P_1$ receptor is one of the most abundant and efficient GPCRs coupled to $G_{i/o}$ proteins in the human and rodent brain, with the highest activity in areas related with learning and memory processes. Moreover, it could be signaling a vast amount of different biological processes by regulating or modulating other systems, resulting in very relevant roles in the control of brain functioning. Furthermore, the activity mediated by $S1P_1$ receptor is regulated during AD progression, revealing an increase of the cortical receptor activity at initial stages of the disease. These results could open a new therapeutic approach based on the $S1P_1$ receptors as a target for the treatment of dementias, including Alzheimer's disease.

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CRediT authorship contribution statement

Jonatan Martínez-Gardeazabal: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Gorka Pereira-Castelo:** Methodology, Formal analysis. **Marta Moreno-Rodríguez:** Methodology, Formal analysis, Conceptualization. **Alberto Llorente-Ovejero:** Writing – review & editing, Methodology, Investigation. **Manuel Fernández:** Resources, Methodology. **Iván Fernández-Vega:** Resources, Methodology. **Iván Manuel:** Writing – original draft, Investigation, Conceptualization, Methodology, Supervision, Validation. **Rafael Rodríguez-Puertas:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2024.106713.

Data availability

Data will be made available on request.

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