RESEARCH ARTICLE

Abnormal Metabotropic Glutamate Receptor Expression and Signaling in the Cerebral Cortex in Diffuse Lewy Body Disease is Associated with Irregular α-Synuclein/Phospholipase C ($PLC\beta_1$) Interactions

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Diffuse Lewy body disease (DLBD) is a degenerative disease of the nervous system, involving the brain stem, diencephalic nuclei and cerebral cortex, associated with abnormal α-synuclein aggregation and widespread formation of Lewy bodies and Lewy neurites. DLBD presents as pure forms (DLBDp) or in association with Alzheimer disease (AD) in the common forms (DLBDc). Several neurotransmitter abnormalities have been reported including those of the nigrostriatal and mesocorticolimbic dopaminergic system, and central noradrenergic, serotoninergic and cholinergic pathways. The present work examines metabotropic glutamate receptor (mGluR) expression and signaling in the frontal cortex of DLBDp and DLBDc cases in comparison with age-matched controls. Abnormal L-[^{3}H]glutamate specific binding to group I and II mGluRs, and abnormal mGluR1 levels have been found in DLBD. This is associated with reduced expression levels of phospholipase C β1 (PLCβ1), the effector of group I mGluRs following protein G activation upon glutamate binding. Additional modification in the solubility of PLCβ1 and reduced PLCβ1 activity in pure and common DLBD further demonstrates for the first time abnormal mGluR signaling in the cerebral cortex in DLBD. In order to look for a possible link between abnormal mGluR signaling and α-synuclein accumulation in DLBD, immunoprecipitation studies have shown α-synuclein/PLCβ1 binding in controls and decreased α-synuclein/PLCβ1 binding in DLBD. This is accompanied by a shift in the distribution of α-synuclein, but not of PLCβ1, in DLBD when compared with controls. Together, these results support the concept that abnormal α-synuclein in DLBD produces functional effects on cortical glutamatergic synapses, which are associated with reduced α-synuclein/PLCβ1 interactions, and, therefore, that mGluRs are putative pharmacological targets in DLBD. Finally, these results emphasize the emergence of a functional neuropathology that has to be explored for a better understanding of the effects of abnormal protein interactions in degenerative diseases of the nervous system.

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INTRODUCTION

Diffuse Lewy body disease (DLBD), also called dementia with Lewy bodies, is a frequent degenerative disease of the nervous system in the elderly, characterized by the presence of abnormal α-synuclein production and deposition in the form of biochemical and structural aggregates, currently manifested as Lewy bodies in the cytoplasm of neurons and as enlarged neurites in degenerated neuronal processes that have been called Lewy neurites. Lesions are mainly encountered in selected nuclei of the medulla, serotoninergic raphe nuclei, noradrenergic locus ceruleus, substantia nigra *pars compacta*, dopaminergic mesocorticolimbic system, cholinergic nucleus basalis of Meynert and pedunculopontine nucleus, as in Parkinson disease (PD), and in the hippocampus and entorhinal cortex, diencephalic nuclei, amygdala, gyrus cinguli and isocortex (3, 19, 28, 31, 32, 35, 38, 71). PD and DLBD are included within the spectrum of Lewy body diseases (LBDs) that have been categorized, for operational purposes, as midbrain, limbic and neocortical LBDs (21, 53, 54). The limits of these categories are not precise, and the use of new antibodies raised against oxidized α-synuclein has shown widespread α-synuclein pathology in the striatum in LBDs, higher in DLBD and lower in PD (18). Alzheimer disease (AD) is a frequent association in DLBD. The common form of DLBD (DLBDc) is characterized by abundant senile plaques and neurofibrillary tangles, particularly in the temporal cortex, whereas the pure form of DLBD (DLBDp) comprises a subgroup of patients in whom the AD pathology is minimal (27, 43, 44). Although this association has produced much controversy, operational criteria for AD changes in DLBD are the same as those applied in conventional AD regarding βA4-amyloid burden (stages A-C) and neurofibrillary degeneration (I, II: entorhinal, III-IV: limbic and V, VI: isocortical stages) (5).

The main clinical manifestations of DLBD are parkinsonism and dementia, characterized by cognitive decline, recurrent visual hallucinations, and fluctuating cognitive state and level of consciousness. Cognitive decline includes impairment of memory, language, visuospatial ability, attention, learning visuoconstructive ability and psychomotor speed. Repeated falls, depression, rapid eye movement sleep behavior disorder, and neuroleptic sensitivity are additional clinical features in DLBD (31, 53, 54).

Several studies have shown a correlation between Lewy bodies in the cerebral cortex and Lewy neurites in amygdala with cognitive impairment (10, 30, 46, 52, 67).

Cortical synaptic loss, as suggested by decreased expression of synaptophysin in the frontal cortex, probably contributes to cognitive impairment in DLBD as well (26). Yet clinical manifestations, and particularly cognitive impairment in parkinsonism, are dependent on much more sophisticated biochemical shifts (34). For example, patients with hallucinations tend to have marked cortical cholinergic deficits and relatively preserved serotoninergic systems (58, 59, 60). Moreover, AD changes in DLBDc further complicate the scenario of cognitive decline.

G-protein-coupled receptors (GPCRs) comprise the largest superfamily of proteins in the body with thousands of putative ligands including biogenic amines, peptides, glycoproteins, lipids, nucleotides and ions (24, 42). GPCRs are composed of seven sequences that span the plasma membrane in a counter-clockwise manner forming a recognition and connection unit that has the ability to interact with a G-protein. The activated receptor induces a conformational change in the associated G protein α-subunit leading to release of GDP followed by binding of GTP, which dissociates from the receptor and modulates several signaling pathways (48). These include activation or inhibition of adenylyl cyclases and activation of phospholipases, and regulation of potassium and calcium channels (24). One of the major subfamilies of GPCRs is related to the metabotropic neurotransmitter receptors. This family includes the metabotropic glutamate and GABA receptors, calcium receptors, vomeronasal pheromone receptors and taste receptors.

Metabotropic glutamate receptors (mGluRs) are classified as group I, II and III (12, 55, 57). Receptors of group I, which includes $mGluR_1$ and $mGluR_5$, are coupled with a $G_{q/11}$ protein and stimulate phospholipase $C\beta_1$ ($PLC\beta_1$) following protein G activation upon glutamate binding. Receptors of groups II and III inhibit adenylyl ciclase and activate G-protein coupled potassium channels (GIRKs). $PLC\beta_1$ activation stimulates phosphatidylinositol (PI) turnover, leading to phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis and resulting in the generation of 2 intracellular messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which in turn promote the release of intracellular Ca^{2+} stores and the activation of protein kinase C (12, 25, 62, 65, 66).

Case number	Gender	Age	P-m delay	Diagnosis
1	M	60	8	DLBDp
2	M	68	12	DLBDp
3	M	85	7	DLBDp
4	M	81	16	DLBDp
5	F	72	3	DLBDp
6	F	70	8	DLBDp
7	F	77	5	DLBDp
8	F	70	8	DLBDp
9	M	78	6	DLBDc+AD I
10	F	81	6	DLBDc+AD I
11	F	71	5	DLBDc+AD II
12	M	76	9	DLBDc+AD II
13	F	78	7	DLBDc+AD III
14	M	84	5	DLBDc+AD III
15	F	76	8	DLBDc+AD V
16	F	84	4	DLBDc+AD V
17	F	78	13	DLBDc+AD V
18	F	84	1	DLBDc+AD V
19	F	91	5	DLBDc+AD VI
20	F	80	4	DLBDc+AD VI
21	M	82	11	C
22	H	63	7	C
23	M	46	10	C
24	M	80	11	C
25	H	79	7	C

Table1. Summary of the main clinical and neuropathological data in the present series. M: male; F: female; DLBDp, DLBDc: Diffuse Lewy body disease pure and common forms, respectively; AD: Alzheimer's disease stage I-VI of Braak and Braak; P-m delay: post-mortem delay between death and tissue processing, in hours.

Interestingly, group I and group III mGluRs modulate synaptic transmission in the striatopallidal complex (69, 73), and activation of group II mGluRs inhibits synaptic excitation of the substantia nigra pars reticulata (6). Blockade of group I mGluRs inhibits akinetic deficits in a rat model of parkinsonism (7, 56). Moreover, several lines of evidence implicate mGluRs as putative pharmacological targets in controlling motor deficits in PD (20, 63). However, practically nothing is known about mGluRs in the cerebral cortex in DLBD.

Synucleins have emerged as a novel class of substrates for G protein-coupled receptor kinases (64). Synucleins interact with phospholipase D2 (11, 36), and phosphorylation of α-synuclein at Ser^{129} inhibits the interaction of α-synuclein with phospholipids or phospholipase D2 (64). Synuclein interaction with phospholipase D isozymes inhibits phospholipase activation in certain paradigms (1). These data offer exciting insights based on the hypothesis that abnormal α-synuclein in LBDs may result in abnormal interactions with crucial proteins of the metabotropic receptors signaling pathways, thus leading to impaired neurotransmitter function.

In line with this rationale, the present work is focused on the study of mGluRs in the cerebral cortex in DLBD pure and common forms, with special attention to L-[^{3}H] glutamate binding and modifications in the glutamate binding to group I, group II and group III receptors, total protein expression levels of $mGluR_1$ and $PLC\beta_1$, $PLC\beta_1$ activity, and $PLC\beta_1$ solubility and interactions with α-synuclein in control and DLBD brains. Results of the present study show abnormal α-synuclein/$PLC\beta_1$ interactions associated with impaired mGluR function in the cerebral cortex in DLBD.

MATERIAL AND METHODS

Tissue samples. Brain samples were obtained from the Institute of Neuropathology Brain Bank following the guidelines of the local ethics committee. The brains of 8 patients with DLBD pure form, 12 patients with DLBD common form and 5 age-matched controls were obtained from 3 to 13 hours after death and were immediately prepared for morphological and biochemical studies. A summary of the main clinical characteristics is presented in Table 1. At autopsy, half of each brain was fixed in formalin, while the other half was cut in coronal sections one-cm thick, frozen on dry ice and stored at -80°C until use. In addition, samples 2-mm thick of the cerebral isocortex, cingulum, hippocampus and entorhinal cortex, and brain stem were

fixed with 4% paraformaldehyde for 24 hours, cryoprotected with 30% saccharose, frozen on dry ice and stored at -80°C until use. For diagnostic morphological studies, the brains were fixed by immersion in 10% buffered formalin for 2 or 3 weeks. The neuropathological study was carried out on de-waxed 4-µm thick paraffin sections of the frontal (area 8), primary motor, primary sensory, parietal, temporal superior, temporal inferior, anterior cingulated, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen and pallidum; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (2 levels), pons and medulla oblongata; and cerebellar cortex and dentate nucleus. The sections were stained with haematoxylin and eosin, luxol fast blue-Klüver Barrera, and, for immunohistochemistry to glial fibrillary acidic protein, CD68 and *Licopericum esculentum* lectin for microglia, βA4 amyloid, tau, αB-crystallin, α-synuclein and ubiquitin. Validated neuropathological criteria for the diagnosis of DLBDp and DLBDc are detailed elsewhere (21, 53, 54).

All the samples were processed for all biochemical studies. Immunohistochemistry and confocal microscopy was limited to cases with DLBDp.

Plasma membranes isolation. Plasma membranes from brain samples were isolated as described by Kessler et al (39) with some modifications. Samples were homogenized in 20 volumes of isolation buffer (50 mM Tris-HCl, pH 7.4 containing 10 mM $MgCl_2$ and protease inhibitors) in Dounce homogenizer (10xA, 10xB). After homogenization, brain preparations were centrifuged for 5 minutes at 1000g in a Beckman JA 21 centrifuge. Supernatant was centrifuged for 20 minutes at 27 000g and the pellet was finally resuspended in isolation buffer. Protein concentration was measured by the method of Lowry, using bovine serum albumin (BSA) as a standard.

Metabotropic glutamate receptor binding to plasma membranes. L-[^{3}H]Glutamate (48.1 Ci/mmol) was purchased from PerkinElmer (Madrid). L-glutamic and α-amino-3-hydroxy-5-methyl-isoxazole-4 propionic (AMPA) acids were obtained from Tocris (London). N-methyl-D-aspartic acid (NMDA), Kainate and DL-threo-β-hydroxyaspartic acid (TBHA) were from Sigma (Madrid). L-[^{3}H]Glutamate binding assays to brain plasma membranes were performed as described previously (2, 50). Briefly, to determine mGluR binding, 50 µg of protein was incubated for 60 minutes at 25°C in the presence of 100 µM AMPA, 100 µM kainate and 100 µM NMDA, in order to block ionotropic glutamate receptor binding, and different L-[^{3}H]Glutamate concentrations (50 nM-1960 nM) with 10 mM potassium phosphate pH 7.4. Non-specific binding was obtained in the presence of unlabeled L-glutamate. All assays were performed in the presence of 1 mM DL-threo-β-hydroxyaspartic acid (THBA), a L-glutamate uptake inhibitor (41).

Immunodetection of mGluR1 and the phospholipase C β_1 isoform. One hundred micrograms of protein were subjected to 7.5% polyacrilamide gel electrophoresis in the presence of SDS. Western blotting was performed as described earlier (49). Immunodetection was carried out by incubating the nitrocellulose membranes with specific rabbit polyclonal antibody to $mGlu_1$ at a dilution of 1:1000 and isoenzyme-specific monoclonal antibody anti-PLCβ_1 at a dilution of 1:400 (Upstate, Reactiva, Madrid). After washing, blots were incubated with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse IgG, respectively, diluted 1:3000. Antigen was visualized using the ECL chemiluminescence detection kit from Amersham and specific bands were quantified by densitometry in a GS-690 densitometer (BioRad, Madrid).

Phospholipase C assay. Phospholipase C activity in plasma membranes was assayed in the presence of exogenous phosphatidylinositol-4,5-bisphosphate, ([^{3}H]PIP_2, 8 Ci/mmol, PerkinElmer, Madrid) as described by Tiger et al (72). [^{3}H]PIP_2 was dried under a N_2 stream, dissolved in 2 mM sodium deoxycholate, 50 mM Tris-HCl pH 6.5 and sonicated using an Ultrasonic Processor UP 200 S. Phospholipase C assay was carried out for 10 minutes at 37°C, incubating [^{3}H]PIP_2 (17 000 dpm) with 20 µg of plasma membrane protein in 100 µl of buffer (100 mM NaCl, 1 mM sodium deoxycholate, 1 mM EGTA, 250 nM Cl_2Ca, 40 mM ClLi and 50 mM Tris-HCl pH 6.8) The incubation was terminated by adding 360 µl of chloroform/methanol/HCl (1:2:0.2 v/v) and putting the tubes on ice. After the addition of 120 µl 2M KCl and 160 µl of chloroform, the tubes were centrifuged for 5 minutes at 3500g. Upper aqueous phase (250 µl) was mixed with 3.5 ml of Optiphase-Hi-Safe® for scintillation counting.

α-Synuclein and PLC solubility and aggregation. Brain samples (0.2 g) of the frontal cortex were homogenized in a glass homogenizer in 1.5 ml of ice-cold PBS$^+$ (sodium phosphate buffer, pH 7.0, plus protease inhibitors), sonicated and centrifuged at 2650g at 4°C for 10 minutes. The pellet was discarded and the resulting supernatant was ultracentrifuged at 100 000g at 4°C for one hour. The supernatant (S2) was kept as the PBS-soluble fraction. The resulting pellet was re-suspended in a solution of PBS, pH 7.0, containing 0.5% sodium deoxycholate, 1% Triton and 0.1% SDS, and it was ultracentrifuged at 100000g at 4°C for one hour. The resulting supernatant (S3) was kept as the deoxycholate-soluble fraction. The corresponding pellet was re-suspended in a solution of SDS 2% in PBS and maintained at room temperature for 2 hours. Immediately afterwards, the samples were centrifuged at 100 000g at 25°C for one hour, and the resulting supernatant (S4) was the SDS-soluble fraction. Equal amounts of each fraction were mixed with reducing sample buffer and processed for 10% SDS-PAGE electrophoresis and Western blot analysis. The membranes were incubated with anti-α-synuclein (Chemicon, Barcelona) at a dilution of 1:2000 or with anti-PLCβ_1 (Santa Cruz, Quimigranel, Barcelona) at a dilution of 1:100. For comparative purposes, membranes were also incubated with rabbit anti-PLCγ antibodies (Neomarkers) at a dilution of 1:500. The protein bands were visualized by the ECL method (Amersham, Barcelona).

PLCβ_1 immunoprecipitation and immunoblot. Samples (0.2 g) of the frontal cortex (area 8) were homogenized in a glass homogenizer in 1.5 ml of ice-cold lysis buffer (PBS, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 2650g for 10 minutes at 4 °C. The supernatant S1 was further centrifuged at 100 000g for one hour at

4°C to generate the supernatant S2. Protein concentrations were determined using the BCA method with BSA as a standard. S2 fractions were diluted to roughly one µg/µl total protein with PBS before beginning the immunoprecipitation. Mouse monoclonal anti-PLCβ1 antibody (5 µg) (Upstate, Reactiva, Barcelona) was added to one mg of total protein from S2 fraction, and the mixture was gently rocked at 4°C overnight. The immunocomplexes were captured by adding 100 µl (50 µl packed beads) of washed Protein G agarose bead slurry (Amersham Pharmacia, Barcelona). The reaction mixture was gently rocked at 4°C for 2 hours and the agarose beads were collected by pulsing (5 seconds in the microcentrifuge at 14 000g), and draining off the supernatant. The beads were washed 3 times with ice-cold PBS and, finally, the agarose beads were re-suspended in 32.5 µl 4x Laemmli sample buffer and boiled for 5 minutes. The beads were collected using a microcentrifuge pulse, and 10% SDS-polyacrilamide gels were electrophoresed by using Mini-Protean II (BioRad, Madrid) and proteins were then transferred to nitrocellulose membranes (BioRad).

The blotted membranes were treated with PBS containing 3% skimmed milk for 20 minutes at room temperature with constant agitation, and then incubated with 5 µg/ml of monoclonal, rabbit polyclonal anti-PLCβ1 (Santa Cruz) at a dilution of 1:100, or with the rabbit policlonal anti-α-synuclein antibody (Chemicon) used at a dilution of 1:1000 in PBS containing 3% bovine albumin with agitation at 4°C overnight. The protein bands were visualized using the chemiluminescence ECL method (Amersham), and the membranes were exposed to autoradiographic films (Hyperfilm, Amersham). Control of protein loading was carried out by incubating the membranes with anti-β-actin used at a dilution of 1:5000.

Cell fractionation by sucrose density gradients. Samples (0.2 g) from DLBD, pure and common forms, and control cases were homogenized in a glass homogenizer in 2 ml of ice-cold extraction buffer (5 mM Tris, 250 mM sucrose, 1 mM EGTA pH 7.4), plus protease and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), sonicated and centrifuged at 2650g for 5 minutes at 4°C. The supernatant was reserved and the pellet was resuspended in one ml of the same extraction buffer and centrifuged at 2650g for 5 minutes at 4°C. The pellet was discarded and the supernatant was pooled with the first supernatant. This postnuclear supernatant was layered onto a 10-ml, linear 0.4-2 M sucrose gradient buffered with 5 mM Tris, 1 mM EGTA pH 7.4 in 14 × 95-mm polyallomer tubes (Beckman Instruments, Madrid). Gradients were centrifuged at 100 000g for 3 hours at 4°C (Beckman Instruments). Fractions of 500 µl were collected from the bottom of the tube and stored at -80°C.

Samples from the gradient fractions were subjected to 10% electrophoresis and western blotting with the following antibodies: rabbit polyclonal anti-α-synuclein (Chemicon) used at a dilution of 1:2000, rabbit polyclonal anti-PLCβ1 (Santa Cruz) used at at a dilution of 1:500, mouse monoclonal anti-synaptosomal associated protein of 25 kDa (SNAP-25) (Chemicon) 1:1000, mouse monoclonal anti-synaptotagmin (a gift of Dr J. Blasi, Barcelona, Spain) used at a dilution of 1:1000, and mouse monoclonal anti-α-tubulin (Sigma) used at a dilution of 1:5000. The protein bands were visualized by the ECL method (Amersham).

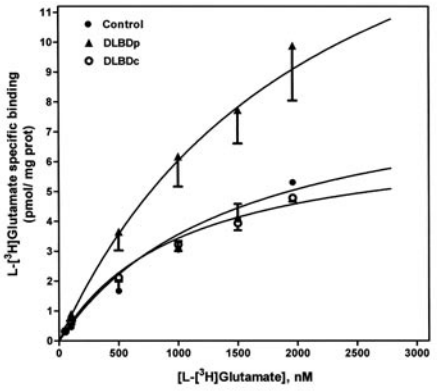
Immunohistochemistry. Confocal microscopy. Paraformaldehyde-fixed, cryostat sections, 15-µm thick, were processed for α-synuclein and $PLC\beta_1$ immunohistochemistry following the streptavidin LSAB method (Dako). After incubation with methanol and normal serum, the sections were incubated free-floating with one of the primary antibodies at 4°C overnight. Antibodies to α-synuclein (Chemicon) were used at a dilution of 1:1500; antibodies to $PLC\beta_1$ (Santa Cruz) were used at a dilution of 1:100. Following incubation with the primary antibody, the sections were incubated with LASB for one hour at room temperature. The peroxidase reaction was visualized with diaminobenzidine, NH_4NiSO_4 and H_2O_2. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody.

De-waxed sections of the entorhinal cortex in AD cases were stained with a saturated solution of Sudan black B (Merck) for 30 minutes to block the autofluorescence of lipofuscin granules present in nerve cell bodies, rinsed in 70% ethanol and washed in distilled water. The sections were incubated, at 4°C overnight, with the mouse α-anti-synuclein antibody (Neomarkers) used at a dilution of 1:100 and mouse anti-$PLC\beta_1$ antibody (santa Cruz Biotechnology) used at a dilution of 1:200 in a vehicle solution composed of Tris buffer, pH 7.2, containing 15 mmol/L NaN_3, and protein (Dako). After washing in PBS, the sections were incubated in the dark with the cocktail of secondary antibodies, diluted in the same vehicle solution as the primary antibodies, during 45 minutes at room temperature. Secondary antibodies were Alexa488 anti-mouse (red) and Alexa546 anti-rabbit (green) (both from Molecular Probes), and they were used at a dilution of 1:400. After washing in PBS, the sections were mounted in immuno-Fluore Mounting medium (ICN Biomedicals), sealed and dried overnight. Sections were examined in a Leica TCS-SL confocal microscope.

Statistical and data analysis. Data statistical analysis was performed using the Student *t*-test. Differences between mean values were considered statistically significant at $p<0.05$. The binding data were analyzed with the GraphPad Prism 3.03 program (GraphPad Software, San Diego, Calif).

RESULTS

Metabotropic glutamate receptor. L-[^{3}H]Glutamate binding assay performed in the presence of ionotropic agonists kainate, AMPA and NMDA revealed a single and saturable metabotropic binding site in plasma membranes from human brain. The total number of binding sites (B_{max}) was obtained by scatchard and non-linear regression analysis from saturation curves data. As shown in Figure 1, B_{max} value was slightly but significantly decreased (78% of control, $p<0.01$) in DLBDc. However, an increase in B_{max} value (217% of control, $p<0.001$) was found in DLBDp. In addition, K_D values were significantly decreased (68.9% of control value, $p<0.01$) and increased (147% of control value, $p<0.01$), respectively, in DLBDc and DLBDp, suggesting that metabotropic glutamate receptor affinity was significantly higher



	B_{max} (pmol/mg prot)		K_D (nM)	
Control	8.89 ± 1.16	(5)	1497.5 ± 303.5	(5)
DLBDp	19.31 ± 1.78	(8) ***	2202.9 ± 430	(6) **
DLBDc	6.98 ± 0.27	(10) **	1032.1 ± 108.9	(10) **

Figure 1. *Saturation binding curve for metabotropic glutamate receptors.* Plasma membranes (50 µg protein) from control, DLBD pure form (DLBDp) and common form (DLBDc) cases were incubated with L-[^{3}H]Glutamate in a concentration range from 50 nM to 1960 nM as described in Methods. Data points are means ± SEM of 5 to 10 experiments (5 controls, 8 DLBDp and 10 DLBDc cases) performed in duplicate, each using different membrane preparations. K_D and B_{max} values from Scatchard analysis of saturation curves are shown in the inset. **p<0.01, ***p<0.001 significantly different from control.

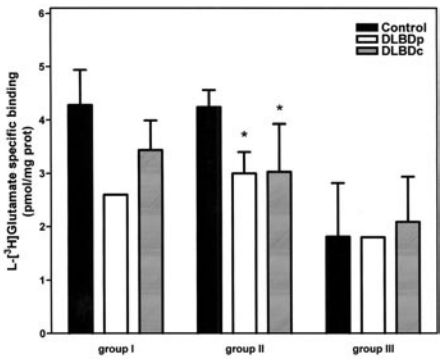


Figure 2. *Specific L-[^{3}H]Glutamate binding to different metabotropic glutamate receptor groups.* Plasma membranes (50 µg protein) from control, DLBD pure form (DLBDp) and common form (DLBDc) cases were incubated with 500 nM L-[^{3}H]Glutamate in the absence or the presence of 1 mM quisqualic acid (group I), 1 mM (2R,4R)-APDC (group II) or 1 mM L-AP4 (group III), as described in Methods. Data are means ± SEM of, at least, 3 experiments performed in duplicate, each using different membrane preparations. *p<0.05 significantly different from control value.

than in control in DLBDc and lower than in control in DLBDp.

To further analyze which mGluR groups were affected in DLBD, binding assays were performed to a single concentration of radioligand (500 nM) and in the presence of unlabelled 1 mM L-quisqualic acid (which is a very potent agonist of group I

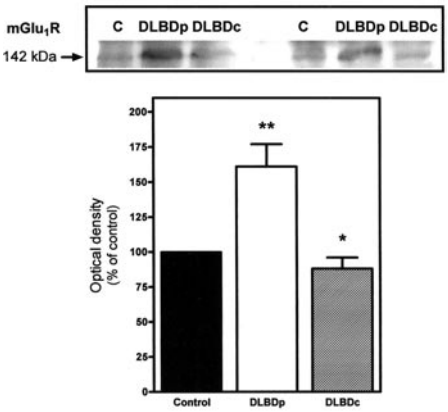


Figure 3. *Immunodetection of mGluR$_{1a}$ in plasma membranes from human cerebral cortex.* Identical quantities (100 µg) of plasma membranes from control, DLBD pure form (DLBDp) and common form (DLBDc) cases were subjected to 7.5% SDS-PAGE, electrophoretically transferred to nitrocellulose and tested with the specific antibody against mGluR$_{1a}$ protein, as described in Methods. Data are means ± SEM of the densitometric quantification of 4 independent immunoblots performed with different membrane preparations corresponding to all cases analyzed in this study. **p<0.01 significantly different from control. Inset shows bands of a representative experiment.

mGluRs), (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R,4R)-APDC, a selective agonist of group II mGluRs) or L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4, a selective agonist of group III mGluRs). As shown in Figure 2, specific L-[^{3}H]Glutamate binding to mGluRs of groups I and II was reduced in DLBD cases when compared with controls, whereas no apparent binding modifications were observed for group III mGluRs in DLBD cases when compared with controls.

As mGluR$_1$ is the main and most studied receptor subtype involved in physiological processes such as learning and memory processing, we analyzed its protein level by Western blotting using a specific antibody that recognizes mGluR$_1$. Figure 3 shows that the expression level of this receptor was significantly higher (161%) in DLBDp when compared with controls. Yet mGluR$_1$ protein level in DLBDc was significantly lower (88%) than in controls. Both results agree with the percentage of variation detected in B_{max} values from DLBDp (217% of control) and DLBDc (78% of control) (Figure 1), suggesting that B_{max} changes detected in DLBD cases by binding assays are mainly due to modifications in mGluR1.

Phospholipase C activity. One of the most widely described effector systems

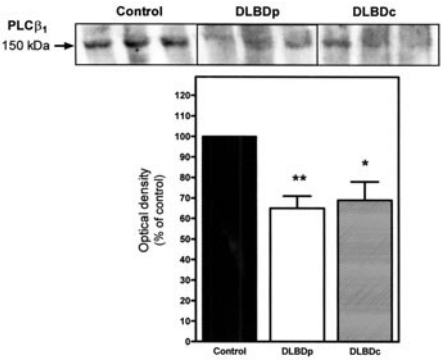


Figure 4. *Immunodetection of PLC β_1 isoform in plasma membranes from human cerebral cortex.* Identical quantities (100 µg) of plasma membranes from control, DLBD pure form (DLBDp) and common form (DLBDc) cases were subjected to 7.5% SDS-PAGE, electrophoretically transferred to nitrocellulose and tested with the isoform-specific monoclonal antibody against PLC β_1 isoform, as described in Methods. Figure shows densitometric quantification (means ± SEM) of 4 independent immunoblots performed with different membrane preparations corresponding to all cases analyzed in this study. *p<0.05, **p<0.01 significantly different from control. Inset shows bands of a representative experiment.

coupled to mGluRs is the β_1 isoform of phospholipase C (PLCβ_1). Therefore, we analyzed its presence in plasma membranes from DLBD and control cases by Western blotting. All cases were tested with anti-PLCβ_1 and distributed in 4 independent immunoblotting assays. As optical density values obtained from different blots cannot been compared, we averaged optical density for control, DLBDp and DLBDc cases. Then, we calculated the percentage of increase or decrease in the signal obtained in DLBD cases in relation to controls in each blot. Finally, we performed Student *t*-test with "100 ± 0 (1)" as control value and corresponding "mean ± SEM (4 gels)" DLBD value. As shown in Figure 4, steady state level of PLCβ_1 was significantly decreased in DLBD pure and common forms. This low quantity of β_1 isoform was, at least partially, responsible for the decreased PLC basal activity detected in DLBDp (416.2 ± 41.8 pmol/mg prot·min) and DLBDc (442.3 ± 48.9 pmol/mg prot.min) compared with controls (524.7 ± 54.2 pmol/mg prot.min).

In order to study metabotropic glutamate receptor functionality, we determined the stimulatory effect exhibited by glutamate, GTP, dihydroxyphenylglycine (DHPG) or glutamate plus GTP on PLC activity. As shown in Figure 5, L-glutamate stimulated PLC activity (139% of basal activity) in

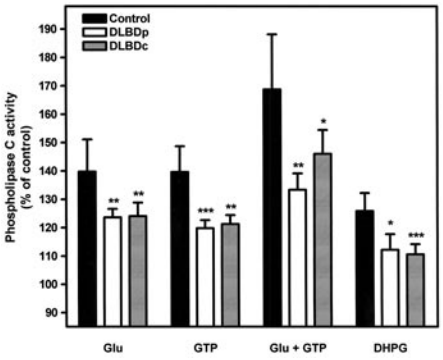


Figure 5. *Metabotropic glutamate receptors/ phospholipase C activity coupling.* Twenty micrograms of plasma membranes from control, DLBD pure form (DLBDp) and common form (DLBDc) cases were incubated with [^{3}H]PtdInsP$_2$ in the presence of 100 μM GTP (GTP), 1 mM L-Glutamate (Glu), 1 mM dihydroxyphenylglycine (DHPG) or glutamate plus GTP (Glu+GTP), and phospholipase C (PLC) activity determined, as described in Methods. Data are means ± SEM of 4 to 9 experiments performed in duplicate. *p<0.05, **p<0.01, ***p<0.001 significantly different from control. All data were significantly different (at least p<0.01) from the respective basal value in control and DLBD membranes. Basal PLC activities were 524.7 ± 54.2, 416.2 ± 41.8 and 442.3 ± 48.9 pmol/mg prot·min in control, DLBDp and DLBDc cases, respectively.

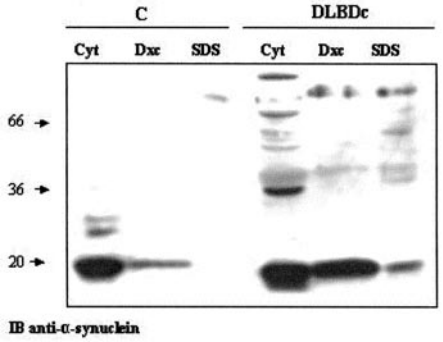


Figure 6. *α-Synuclein solubility.* Solubility and aggregation α-synuclein examined in brain homogenates of control (C) and DLBD cases blotted for α-synuclein. A specific band of 20 kDa is recovered in the PBS-soluble (Cyt) and deoxycholate-soluble (Dxc) fractions in C and DLBD cases, and in the SDS-soluble fraction in DLBD. In addition, bands of high molecular weight of 34 kDa, 66 kDa and higher are detected in the Cyt-, Dxc- and SDS-soluble fractions only in DLBD.

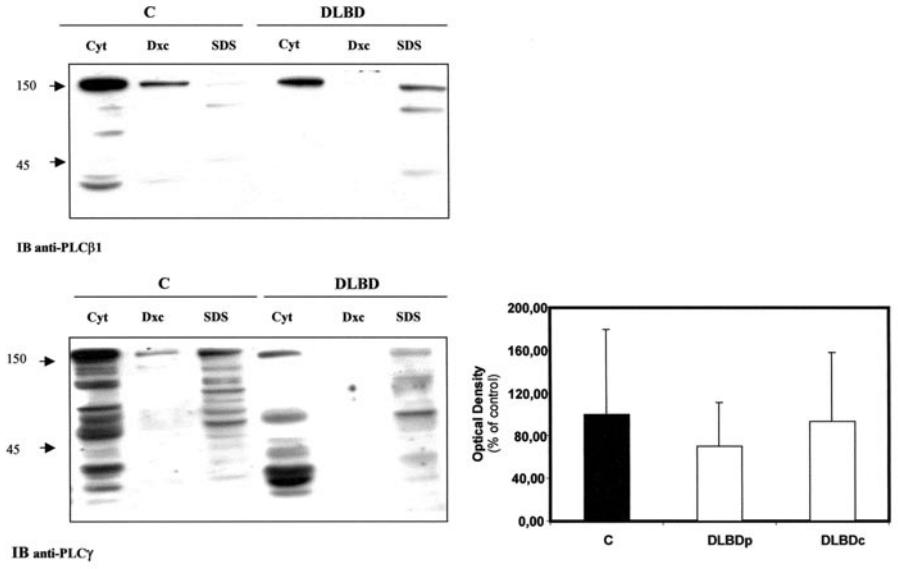


Figure 7. *PLCβ$_1$ solublity.* Solubility and aggregation of PLCβ$_1$ examined in brain homogenates of control (C) and DLBD cases, blotted for PLCβ$_1$ and processed in parallel. PLCβ$_1$ specific bands of about 150 kDa were recovered in the PBS-soluble (Cyt) and deoxycholate-soluble (Dxc) fractions in C and DLBD cases, although the intensity of the bands was higher in the control than the diseased brain. In addition, a strong PLCβ$_1$ band is found in SDS-soluble fraction in DLBD. Finally, several bands of variable molecular weight (45 kDa, 60 kDa and higher) are found in the Cyt-, Dxc- and SDS-soluble fractions in DLBD (upper panel).
In contrast to PLCβ$_1$, the solubility of PLCγ does not differ in DLBD when compared with control samples. Although the content of PLCγ appears to be reduced in some cases, no significant differences are observed between control and diseased brains when the data of individual cases are pooled in the different groups: control, DLBDp and DLBDc (lower panel).

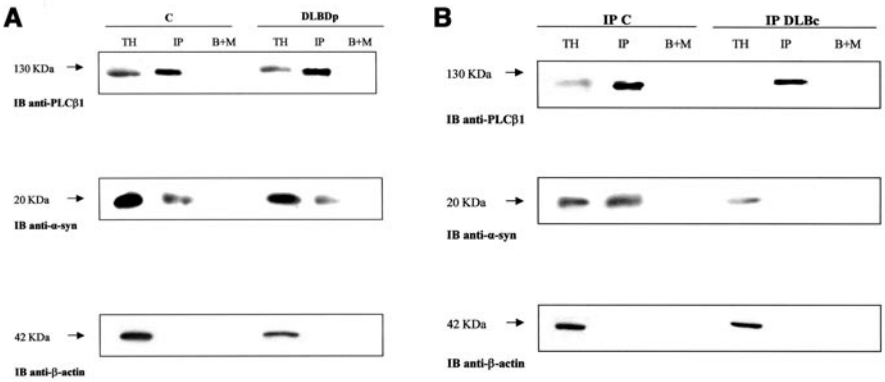


Figure 8. *Immunoprecipitation.* PLCβ$_1$ immunoprecipitation in brain homogenates of control (C) and DLBD cases blotted for PLCβ$_1$ and α-synuclein. PLCβ$_1$-immunoreactive bands are observed in total homogenates (TH) of C and DLBD cases, but only in the IP lane corresponding to the control and not in the lane corresponding to the diseased cases. No signal is found in the lane incubated only with protein G plus antibody (B+M). Similar results are seen in DLBD pure forms (DLBDp) (**A**) and common forms (DLBDc) (**B**). Anti-β-actin antibodies are used as a control of protein loading.

controls, and this effect was higher in the presence of GTP (168% of basal activity), thus confirming mGluR/PLC coupling through a G-protein. DHPG, selective group I mGluR agonist also stimulated PLC activity. Yet PLC activity elicited by glutamate, DHPG, GTP or glutamate plus GTP significantly decreased (70%-90% of control) in DLBD, indicating that mGluR/ PLC pathway responsiveness to mGlu receptor agonists is decreased in DLBD cases, in spite of the detected increase in B_{max} value in DLDBp.

α-Synuclein and PLCβ$_1$ solubility and aggregates. α-Synuclein (20 kDa) was recovered in the PBS-soluble (cyt) and in the deoxycholate-soluble fractions in control and DLBD cases, but α-synuclein-immunoreactive bands in the SDS-soluble fraction only in DLBD. In addition, several α-synuclein-immunoreactive bands of high molecular weight (36 kDa, 66 kDa and higher) in the PBS-soluble, deoxycholate-soluble and SDS-soluble fractions were recovered only in DLBD (Figure 6).

PLCβ$_1$-specific bands of 150 kDa were recovered in the PBS-soluble fraction (cyt) and deoxycholate-soluble fractions in control and DLBD cases, although the inten-

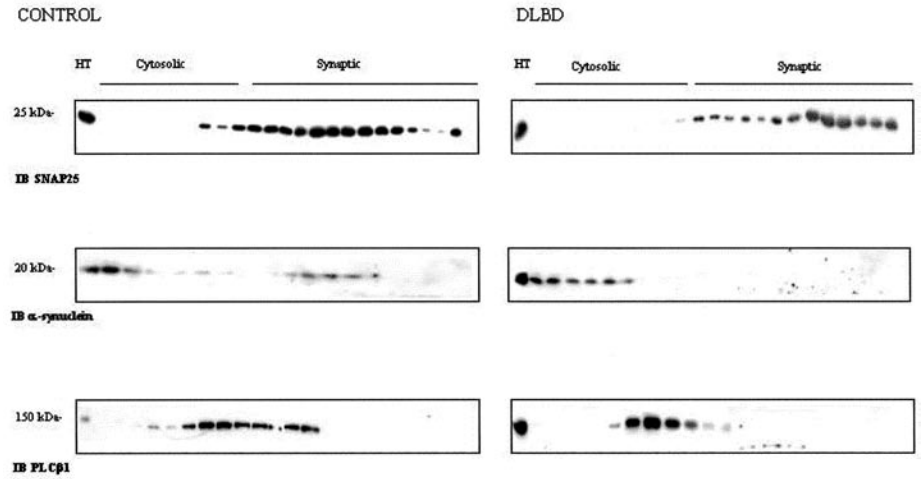


Figure 9. *Sucrose gradients.* Cell fractionation by sucrose density gradients of frontal homogenates in control and DLBD cases processed in parallel and blotted for SNAP-25, α-synuclein and PLCβ$_1$. Two main pools were recovered in the process. The synaptic pool was represented by SNAP-25 (25 kDa). While in control cases α-synuclein (20 kDa) was distributed in both pools, α-synuclein in the synaptic pool was dramatically decreased in DLBD. Yet PLCβ$_1$ (150 kDa) was detected in the cytosolic and synaptic pools equally in control and DLBD cases.

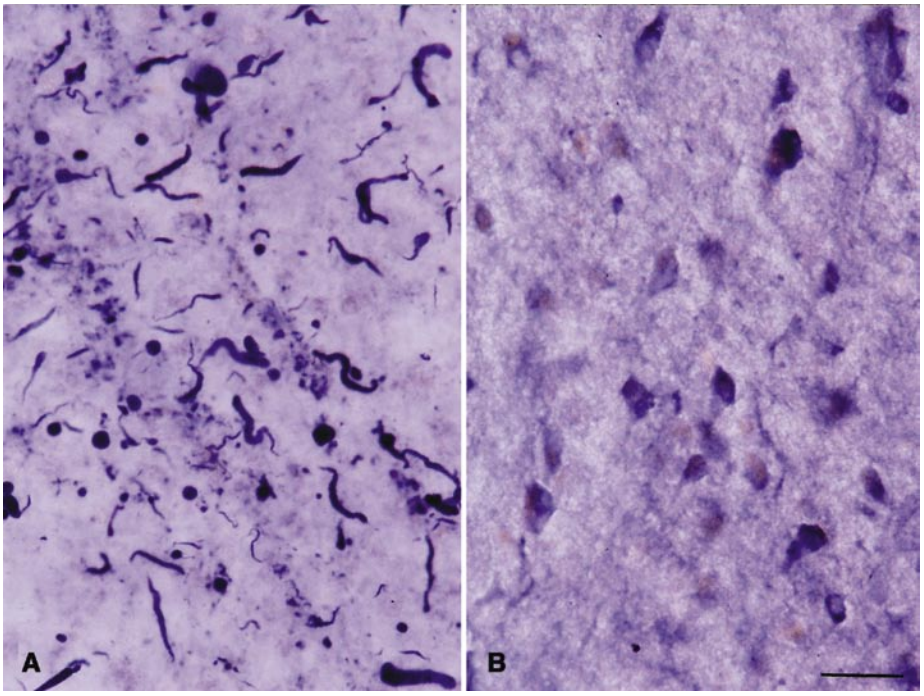


Figure 10. *α-synuclein and PLCβ$_1$ immunohistochemistry.* A similar area of consecutive sections processed for α-synuclein (**A**) and PLCβ$_1$ (**B**) in the cingulate cortex of a patient with DLBD pure form showing lack of PLCβ$_1$ immunostaining of Lewy bodies and Lewy neurites. Cryostat sections processed free-floating, without counterstaining, bar = 25 μm.

sity of these bands was lower in diseased than in control cases. However, PLCβ$_1$ was increased in the SDS-soluble fraction in DLBD, and several bands of about 45 and 60 kDa appeared only in disease (Figure 7, upper panel).

For comparative purposes, another unrelated phospholipase PLCγ was tested. In contrast with PLCβ$_1$, no differences in solubility were detected. PLCγ in SDS fractions was equally observed in control and DLBD cases. Although PLCγ was apparently decreased in some cases, no significant differences were observed between control and diseased brains when the data of the different samples were pooled (Figure 7, lower panel).

PLCβ$_1$ immunoprecipitation. As shown in Figure 8, immunoprecipitation with the mouse monoclonal anti-PLCβ$_1$ antibody disclosed specific bands in control and DLBD cases following incubation with the rabbit polyclonal anti-PLCβ antibody. Incubation of the membranes with anti-α-synuclein showed specific bands (20 kDa) in control and DLBD total homogenates, and in control PLCβ$_1$-immunoprecipitate samples. Markedly reduced or no α-synuclein was recovered in PLCβ$_1$-immunoprecipitates from DLBD cases. These findings were not dependent on the relative amount of total protein between DLBD and control cases, as seen in IP lanes blotted for β-actin used as a control of protein loading. Similar results (Figure 8A, B) were obtained in all DLBDp and DLBDc cases

Cell fractionation by sucrose density gradients. Cell fractionation revealed that the distributions of tubulin, synaptotagmin (not shown) and SNAP-25 (Figure 9, upper panel) were similar in DLBD and control cases. However, the subfractionation distribution of α-synuclein (a band of about 20 kDa) in DLBD cases differed from controls, as the amount of α-synuclein was reduced in the so-called synaptic fractions (Figure 9, middle panel). The distribution of PLCβ1 (150 kDa) was similar in DLBD cases and controls (Figure 9, inner panel). Similar results were obtained in DLBDp and DLBDc cases.

PLCβ$_1$ immunohistochemistry. In order to rule out possible accumulation of PLCβ$_1$ in Lewy bodies and Lewy neurites, immunohistochemistry was used to show localization of PLCβ$_1$ immunoreactivity in the cell bodies of cortical neurons in control and DLBD cases. However, Lewy bodies and Lewy neurites were not stained with anti-PLCβ$_1$ antibodies, as revealed in consecutive sections stained with anti-α-synuclein (Figure 10). This was supported by double-labeling immunohistochemistry and confocal microscopy showing that α-synuclein aggregates did not accumulate PLCβ$_1$ (Figure 11).

DISCUSSION

Several studies have dealt with neurotransmitter deficits in PD and DLBD, most of these correlating with neuronal loss of the corresponding vulnerable nuclei to LBDs. Less is known about possible abnormalities of neurotransmitter receptors that may sustain impaired cognitive functions. Yet recent studies have shown re-

duced dopaminergic (70) and acetylcholine nicotinic (51, 61) receptors in the cerebral cortex in DLBD, thus probably accounting, in part, for the cognitive impairment in these patients.

The present work has shown abnormal L-[^{3}H]glutamate specific binding to group I and II mGluRs, and abnormal mGluR$_1$ levels in the cerebral cortex in DLBD. In addition to abnormal glutamate binding, reduced PLCβ$_1$ levels and reduced PLCβ$_1$ activity have been found in DLBD, pure and common forms. These findings demonstrate abnormal mGluR receptor signaling in the cerebral cortex in DLBD.

GluR δ1 and δ2 are members of the ionotropic glutamate receptor family sharing 20% to 30% sequence identity with NMDA and AMP/kainate receptor subunits. However, they are not activated by glutamate, radiolabeled glutamate or other typical GluR agonists; and, therefore, members of this family have been classified as orphan glutamate receptors. Furthermore, GluR δ2 is practically restricted to cerebellar parallel fiber-Purkinje cell synapses (75). Since we performed L-[^{3}H] glutamate binding assays in plasma mmebranes from cerebral cortex in the presence of ionotropic agonists NMDA, AMPA and kainate, we can definitevely conclude that the detected binding is due to metabotropic glutamate receptors (2).

Reduced [^{3}H]glutamate metabotropic binding in the subiculum and CA1 region of the hippocampus (16), and decreased PLCβ$_1$ and PKC levels and activity (13, 74), have been reported in AD. It can be suggested that associated AD changes may explain mGluR deficits in DLBDc. However, the present findings clearly indicate abnormal mGluR patterns in DLBDp, thus demonstrating primary mGluR impairment in DLBD. Interestingly, the expression levels of mGluR$_1$ are higher in DLBDp and lower in DLBDc when compared with control values. Whether this is a reflection of up-regulation or low turnover of mGluR$_1$ receptors in DLBDp is not known, but this aspect further emphasizes peculiar abnormalities in DLBD not associated with AD changes.

It is tempting to speculate that α-synuclein plays a role in the abnormal mGlu receptor signaling in DLBD. Previous studies have shown insoluble α-synuclein and formation of biochemical aggregates in DLBD cases (8, 33). Multiple α-synuclein bands of variable solubility have also been observed in the present study. Furthermore, fractionation studies have shown a discrete shift consistent with modifications in the motility of DLBD α-synuclein in sucrose gradients. Western blots of formic acid-treated Lewy bodies isolated from DLBD cortex show partially truncated forms of α-synuclein and several bands of variable molecular weight consistent with α-synuclein-immunoreactive aggregates (3, 33). In addition, α-synuclein is nitrated (17, 23), and residue Ser^{129} of α-synuclein is selectively and extensively phosphorylated in PD and DLBD (22, 33). Moreover, phosphorylation of α-synuclein at Ser^{129} promotes fibril formation (22). Finally, altered conformation of α-synuclein and direct intermolecular interaction between the N-terminus of α-synuclein and ubiquitin occurs in Lewy bodies when compared with the neuropil (68). Yet immunohistochemical studies have ruled out PLCβ$_1$ immunoreactivity in Lewy bodies and Lewy neurites.

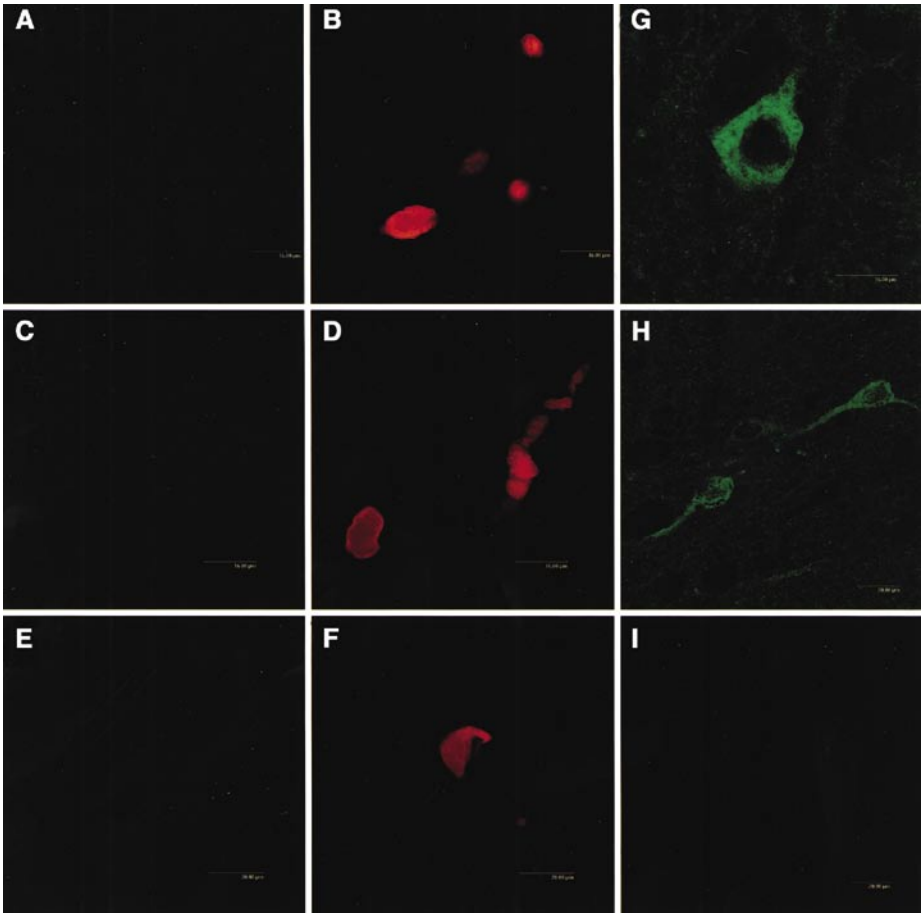


Figure 11. *Double-labeling α-synuclein and PLCβ$_1$.* Confocal microscopy of double-immunostained sections with PLCβ$_1$ and α-synuclein antibodies are visualized in green and red, respectively. Note that PLCβ$_1$ does not co-localize with α-synuclein in Lewy neurites (**A, B, C, D, E, F**). Yet PLCβ$_1$ immunoreactivity is shown in neurons as internal positive controls (**G, H**). A negative control, incubated only with the secondary antibodies, is shown in the inner right panel (**I**).

Previous studies have shown that synaptic vesicle trafficking is probably impaired in DLBD as a result of abnormal interactions of α-synuclein with members of the rab protein family, including rab3a and its associated protein rabphilin, in the cerebral cortex of patients with DLBD (14). Similar abnormal α-synuclein/rab interactions occur in the brains of transgenic mice expressing high levels of mutant A30P α-synuclein (15).

The present results have shown multiple PLCβ$_1$ bands from 45 kDa to about 150 kDa in the deoxycholate-soluble and SDS-soluble fractions in DLBD, thus indicating the presence of probable truncated forms of PLCβ$_1$, abnormal PLCβ$_1$ solubility in PBS, deoxycholate and urea, and abnormal solubility of PLCβ$_1$ in sucrose gradients in DLBD. These abnormalities appear to be particular of PLCβ$_1$ since no similar differences in solubility are observed for

PLCγ. Previous studies have demonstrated α-synuclein interaction with phospholipase D isozymes and α-synuclein inhibition of pervanadate-induced phospholipase D activation in embryonic kidney cell lines (1). The present immunoprecipitation studies have shown α-synuclein interaction with $PLC\beta_1$ in cortical brain homogenates from control subjects, and decreased $PLC\beta_1$/α-synuclein binding in DLBD. Interestingly, phosphorylation of α-synuclein at Ser129, as occurs in LBDs, also inhibits the interaction of α-synuclein with phospholipase D (64). Together, these findings indicate that expression and activity of $PLC\beta_1$, a crucial protein in the signaling pathway of group I mGluRs, is abnormal in DLBD, and that this abnormal activity is associated with abnormal $PLC\beta_1$ solubility and with an anomalous interaction of this protein with α-synuclein resulting in decreased $PLC\beta_1$/α-synuclein binding in DLBD.

mGluRs modulate excitability and synaptic transmission. Group I mGluRs increase neural excitability through inhibition of potassium conductances and activation of non-selective cation currents. Synaptic transmission is also regulated presynaptically and postsynaptically by mGluRs. Presynaptically, mGluRs depress glutamate release from presynaptic nerve terminals through calcium channel inhibition, and this capacity is ubiquitous. In addition, postsynaptic modulation of mGluRs is much more specific. For example, agonists of group I mGluRs potentiate N-methyl-D-aspartate acid (NMDA) receptor currents, but not α-amino-3-hydroxy-5-methyl-4-isoxalone-propionate acid (AMPA) receptor currents, in the hippocampus. Considering these properties, it is conceivable that major cortical functional deficits may be attributed to abnormal mGluR processing and $PLC\beta_1$ signaling. In addition, observations in $PLC\beta_1$-null mice have shown that $PLC\beta_1$ is involved in the development and control of brain inhibitory pathways (4, 40, 65). Finally, group I mGluRs accelerate the processing of amyloid precursor protein (APP) into non-amyloidogenic APPs (29, 37, 45, 47). Therefore, reduced group I mGluR activity may facilitate amyloidogenic APP processing, thus contributing to the formation and deposition of βA4-amyloid, in DLBD. Alternatively, increased amyloid deposition may result in reduced mGluR expression and activity. However, observations in Tg2576 mice expressing human APP with the Swedish?? double mutation K670N-M671L have shown no significant alteration in group I and II metabotropic glutamate receptors (9).

Blockade of group I mGluRs inhibits akinetic deficits in a rat model of parkinsonism (5, 56), and activation of group II mGluRs inhibits synaptic excitation of the substantia nigra *pars reticulata* (6). These and other activities have served to implicate mGluRs as putative pharmacological targets in controlling motor deficits in PD (20, 63). The present findings further support a rationale for considering mGluRs as additional possible pharmacological targets in the control of cognitive deficits in DLBD.

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