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ORIGINAL PAPER

Functional coupling of M_1 muscarinic acetylcholine receptor to $G\alpha_{q/11}$ in dorsolateral prefrontal cortex from patients with psychiatric disorders: a postmortem study

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Abstract

Accumulating studies have implicated intracellular signaling through muscarinic acetylcholine receptors (mAChRs) in psychiatric illness. In the present study, carbamylcholine chloride (carbachol)-induced $G\alpha_{i/o}$ and $G\alpha_{q/11}$ activation was identified in postmortem human prefrontal cortical membranes. The following two sample cohorts were used: subjects [1], consisting of 40 controls without neuropsychiatric disorders, and subjects [2], consisting of 20 with bipolar disorder (BP), 20 major depressive disorder (MDD), 20 schizophrenia, and 20 controls, strictly sex- and age-matched. Carbachol-stimulated [^{35}S]GTPγS binding to human brain membranes was assessed by the two methods, i.e., conventional method using filtration techniques ($G\alpha_{i/o}$ activation coupled to M_2/M_4 mAChRs) applied to subjects [1], and [^{35}S]GTPγS binding/immuno precipitation assay ($G\alpha_{q/11}$ activation coupled to M_1 mAChR) applied to subjects [1] and [2]. The concentration eliciting the half-maximal effect (EC_{50}), maximum percent increase ($\%E_{max}$), and slope factor were obtained from concentration–response curve of carbachol-induced $G\alpha_{i/o}$ and $G\alpha_{q/11}$ activation. The pEC_{50} values of both carbachol-induced $G\alpha_{i/o}$ and $G\alpha_{q/11}$ activations in subjects [1] were significantly correlated, though its implications or underlying molecular processes are unclear. The results of M_1 mAChR-mediated $G\alpha_{q/11}$ activation in subjects [2] indicated no significant disorder-specific alterations. However, the distribution patterns of the pEC_{50} values showed unequal variances among the groups. There was a significant inverse correlation between the $\%E_{max}$ values and the pEC_{50} values in subjects with schizophrenia, but not in those with BP or MDD, or controls. These data support the notion that schizophrenia patients consist of biologically heterogeneous subgroups with respect to M_1 mAChR-mediated signaling pathways.

Keywords G-protein · Muscarinic acetylcholine receptor (mAChR) · Bipolar disorder · Major depressive disorder · Schizophrenia · Dorsolateral prefrontal cortex

Introduction

Acetylcholine (ACh) plays an important role as a neurotransmitter and neuromodulator in the central and peripheral nervous systems. ACh binds to two classes of receptors: metabotropic muscarinic receptors and ionotropic nicotinic receptors. In the central nervous system (CNS), there is evidence that muscarinic acetylcholine receptors (mAChRs) are involved in motor control, temperature regulation, cardiovascular regulation, and higher brain functions such as learning and memory. mAChRs are a family of seven-transmembrane domain receptors, consisting of five receptor subtypes (M_1–M_5) [1, 2]. As members of the G-protein coupled receptor (GPCR) superfamily, they associate with heterotrimeric G-proteins to translate extracellular signals into intracellular signals via transduction cascades. In general, M_1, M_3, and

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M_5 mAChRs are coupled to the $G_{q/11}$ class of G-proteins, leading to activation of phospholipase C-β (PLC-β), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). By contrast, M_2 and M_4 mAChRs are canonically coupled $G_{i/o}$ class proteins, resulting in inhibition of adenylate cyclase, which is responsible for catalyzing the conversion of ATP to cyclic AMP [3].

A growing number of studies have implicated the ACh muscarinic system in psychiatric disorders and degenerative illnesses associated with dementia, such as Alzheimer's disease [4]. Among the five mAChRs subtypes, M_1 mAChR has been most strongly associated with cognition and psychotic disorders such as schizophrenia [5], possibly because the M_1 subtype is most abundantly expressed in all major forebrain areas (including the cerebral cortex, hippocampus, and striatum), and has lower expression in the peripheral nervous system [6]. Additionally, there is some evidence to suggest that mAChRs are also involved in the pathological processes underlying mood disorders such as bipolar disorder (BP) and major depressive disorder (MDD) [4, 7, 8].

Initial investigations regarding mAChRs in postmortem human brain tissue from patients with mental disorders were performed using receptor binding assays with a non-selective radioligand, [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), and showed inconsistent results [9–11]. Since then, radioligand binding studies have been conducted with more selective radioligands such as [^{3}H]pirenzepine [12–21], [^{3}H]AF-DX384 [16–18, 22–24], and [^{3}H]4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) ([^{3}H]4-DAMP) [17, 18, 25].

By using [^{3}H]pirenzepine as a radioligand for an autoradiographic study, Scarr et al. [20] have shown that a decrease in cortical M_1 mAChRs is restricted to a subgroup of patients diagnosed with schizophrenia. The subgroup comprised approximately 25% of the schizophrenia group, and had on average 75% less M_1 mAChRs than did control subjects or other subjects with schizophrenia.

A subsequent study using a guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding assay combined with an immunocapture method revealed that activation of $G\alpha_{q/11}$ coupled to M_1 mAChR was also altered in a subgroup of schizophrenia patients, in whom M_1 mAChRs were decreased in Brodmann's area 9 compared to that in controls [26], suggesting biological heterogeneity of this disorder. This study used a method in which human brain tissues were pre-incubated with *N*-ethylmaleimide (NEM), an irreversible alkylating reagent, to reduce basal [^{35}S]GTPγS binding [27].

Recently, we succeeded in establishing a method of [^{35}S]GTPγS/immunoprecipitation to assess functional coupling between M_1 mAChRs and $G\alpha_{q/11}$ proteins in postmortem human brains [28]. This assay is a revised version of conventional immunoprecipitation techniques, the detailed technical notes of which have been described elsewhere [29]. This method allowed for the use of native brain membranes, with signal/noise ratio equivalent to that reported by Salah-Uddin et al. [26, 27]. Absence of NEM pre-treatment may avoid possible damage to membranes. In the present study, this method was applied to dorsolateral prefrontal cortical membranes prepared from control and psychiatric subjects, consisting of BP, MDD, and schizophrenia.

Methods

Postmortem human brain samples

Postmortem human brain samples were obtained at autopsy in the Basque Institute of Legal Medicine (Bilbao, Spain). In typical conditions, the corpse is refrigerated at 4℃ within 3–5 h of death, until autopsy. Samples from the dorsolateral prefrontal cortex (Brodmann's area 9) were dissected at the time of autopsy and immediately stored at −70 ℃. The screening of neuropathological disorders was performed in all the cases used in this study by expert pathologists. The presence of neuropathological or neurodegenerative alterations was an exclusion criteria in this study. Collection procedures were performed in accordance with the protocol for postmortem human brain research of the Basque Institute of Legal Medicine. The study was approved by the Research and Ethics Boards of both the University of the Basque Country, Spain and the Faculty of Medicine, Saitama Medical University, Japan.

In the present study, the following samples were used: subjects [1]; 40 subjects (24 males and 16 females, aged 16 to 80 years old) with no known history of neurological or psychiatric disorders, and subject [2]; 80 subjects comprising 4 subgroups: BP, MDD, schizophrenia and controls. Four samples (BP, MDD, schizophrenia, and control) included in one experimental procedure were strictly matched based on sex (15 males and 5 females for each cohort) and age (≤ 6 years difference). The means ± SD age for each cohort were 52.0 ± 11.8, 52.0 ± 10.8, 51.8 ± 11.6, and 51.8 ± 11.3 years for BP, MDD, schizophrenia, and control, respectively. It is especially important to match age, because we previously showed that carbamylcholine chloride (carbachol)-stimulated [^{35}S]GTPγS binding to $G\alpha_{q/11}$ is decreased with aging, particularly in females [28]. The postmortem delay (PMD) could not be matched as strictly, but the resultant means ± SD (17.2 ± 9.5, 18.0 ± 7.6, 16.4 ± 7.7, and 20.9 ± 9.8 h for BP, MDD, schizophrenia, and control, respectively) were not significantly different. Detailed information concerning cause of death and drugs detected in blood in subjects [1] is described in Table 1. The demographic data, cause of death, and drugs detected in blood in subjects [2] are

Table 1 Demographic data and characteristics for the subjects [1]

Sex	Age (years)	PMD (h)	Cause of death	pH	Drugs in bood
M	16	30	Accident	–	(–)
F	20	53	Accident	–	(–)
M	19	29	Accident	–	Ethanol (1.15)
M	80	48	Accident	–	(–)
M	62	41	Accident	–	Ethanol (0.29)
M	73	19	Accident	–	(–)
M	62	47	Accident	–	(–)
F	23	15	Accident	–	Ethanol (0.72)
F	35	17	Accident	–	(–)
F	20	39	Accident	–	(–)
F	52	64	Accident	–	(–)
M	56	38	Accident	–	Ethanol (2.2)
F	49	40	Accident	6.2	Ethanol (1.4)
M	23	3	Accident	–	Ethanol (1.5)
F	18	34	Accident	5.8	(–)
M	26	4	Accident	6.5	Ethanol (2.8)/Cocaine (0.08)/Amphetamine (0.007)
F	30	18	Accident	6.6	(–)
F	55	32	Accident	6.4	Ethanol (2.2)
M	22	5	Accident	6.3	(–)
M	49	20	Accident	6.7	(–)
M	20	21	Accident	6.4	Benzoylecgonine (0.58)
F	41	23	Accident	6.7	(–)
M	51	19	Accident	6.2	(-)
M	22	7	Accident	6.5	(–)
M	25	18	Accident	6.5	Ethanol (1.8)
M	32	4	Accident	6.7	(–)
M	34	17	Accident	6.7	(–)
M	47	17	Heart failure	–	(–)
M	71	21	Heart failure	–	(–)
F	64	21	Heart failure	–	(–)
M	47	7	Thrombosis	–	(–)
F	60	7	Heart failure	–	Furosemide
F	45	12	Heart failure	6.4	(–)
M	55	22	Heart failure	6.6	(–)
M	20	14	Myocardiopathy	6.6	(–)
M	41	14	Heart failure	6.8	(–)
M	43	20	Heart failure	6.5	(–)
M	48	6	Heart failure	6.1	(–)
M	60	20	Heart failure	6.4	Amiodarone
F	37	6	Hemorrhage	6.4	(–)

Drug concentration is indicated in parentheses (mg/mL for ethanol and µg/mL for other drugs)

listed in Table 2. The [^{35}S]GTPγS/immunoprecipitation assays were performed using four samples from each experimental group in parallel, in order to minimize inter-assay errors.

Membrane preparation

Membrane preparation was performed as previously described [28, 30]. Briefly, postmortem human brain tissues

Table 2 Demographic data and characteristics for the subjects [2]

Experimental no.	Diagnosis	Sex	Age (years)	PMD (h)	Cause of death	Drugs in bood
1	Control	M	64	29	Natural/CRF	(–)
	BP	M	64	23	Suicide/Jumping	Venlafaxine, BZD
	MDD	M	61	5	Natural/Aneurism	Fluoxetine
	Schizophrenia	M	61	21	Natural/CRF	Clozapine, Fluoxetine
2	Control	M	51	22	Natural/CRF	(–)
	BP	M	50	6	Suicide/Jumping	BZD
	MDD	M	50	24	Suicide/Gun	(–)
	Schizophrenia	M	50	3	Suicide/Drowning	BZD
3	Control	M	63	23	Accident/Car	Ethanol (0.44)
	BP	M	63	7	Suicide/Jumping	BZD
	MDD	M	64	16	Suicide/Gun	Citalopram
	Schizophrenia	M	62	10	Natural/CRF	Thioridazine
4	Control	F	66	16	Accident/Car	(–)
	BP	F	64	22	Natural/CRF	(–)
	MDD	F	66	19	Suicide/Hanging	BZD
	Schizophrenia	F	67	21	Natural/CRF	(–)
5	Control	M	27	20	Accident/Drowning	(–)
	BP	M	27	10	Suicide/Jumping	(–)
	MDD	M	31	23	Suicide/Hanging	(–)
	Schizophrenia	M	28	13	Suicide/Jumping	(–)
6	Control	M	56	16	Accident/Car	(–)
	BP	M	57	22	Natural/CRF	(–)
	MDD	M	56	22	Suicide/Gun	(–)
	Schizophrenia	M	57	19	Suicide/Train	Quetiapine, Phenobarbital
7	Control	M	43	4	Natural/AMI	(–)
	BP	M	40	17	Suicide/Jumping	BZD
	MDD	M	43	15	Suicide/Train	BZD
	Schizophrenia	M	43	17	Natural/CRF	BZD
8	Control	F	54	28	Natural/AMI	Ethanol (0.06)
	BP	F	55	16	Suicide/Hanging	Sertraline, BZD, Ethanol (1.64)
	MDD	F	54	18	Suicide/Hanging	Venlafaxine, BZD
	Schizophrenia	F	56	13	Natural/CRF	Clozapine
9	Control	F	36	38	Accident/Asphixia	(–)
	BP	F	39	46	Suicide/Hanging	(–)
	MDD	F	42	14	Suicide/Drugs	Citalopram, Clomipramine, BZD, Propranolol
	Schizophrenia	F	38	23	Suicide/Jumping	(–)
10	Control	M	58	16	Accident/Car	Ethanol (0.77)
	BP	M	58	10	Suicide/Jumping	Olanzapine, Lamotrigine
	MDD	M	56	4	Natural/Embolism	(–)
	Schizophrenia	M	56	8	Suicide/Jumping	Quetiapine, BZD
11	Control	M	60	38	Natural/AMI	(–)
	BP	M	63	31	Natural/Hemorrhage	(–)
	MDD	M	59	29	Suicide/Hanging	Citalopram, BZD
	Schizophrenia	M	62	28	Suicide/Jumping	(–)
12	Control	M	55	36	Accident/Car	(–)
	BP	M	54	16	Suicide/Hanging	Carbamazepine, Lamotrigine, Olanzapine, BZD
	MDD	M	56	16	Natural/CRF	Mirtazapine
	Schizophrenia	M	56	23	Natural/AMI	Citalopram

Table 2 (continued)

Experimental no.	Diagnosis	Sex	Age (years)	PMD (h)	Cause of death	Drugs in bood
13	Control	M	58	27	Accident/Work	(–)
	BP	M	58	11	Suicide/Gun	(–)
	MDD	M	59	31	Suicide/Hanging	(–)
	Schizophrenia	M	60	7	Natural/CRF	Oxcarbazepine
14	Control	M	47	7	Natural/AMI	(–)
	BP	M	47	16	Natural/CRF	(–)
	MDD	M	47	18	Suicide/Hanging	BZD, Ethanol (0.73)
	Schizophrenia	M	49	19	Accident/Fall from a height	(–)
15	Control	M	70	14	Accident/Car	Ethanol (0.29)
	BP	M	72	16	Suicide/Jumping	Citalopram, Mirtazapine
	MDD	M	70	16	Suicide/Hanging	Venlafaxine, Mirtazapine
	Schizophrenia	M	72	14	Natural/CRF	Periciazine, BZD
16	Control	F	59	25	Accident/Car	(–)
	BP	F	62	20	Suicide/Drugs	Duloxetine, Lamotrigine
	MDD	F	61	15	Suicide/Jumping	Venlafaxine
	Schizophrenia	F	57	35	Suicide/Drowning	(–)
17	Control	M	48	10	Natural/AMI	THC, Cocaine
	BP	M	48	15	Natural/CRF	Venlafaxine, Trazodone
	MDD	M	48	32	Suicide/Hanging	(–)
	Schizophrenia	M	47	15	Natural/Peritonitis	Clozapine
18	Control	M	37	17	Accident/Fall from a height	(–)
	BP	M	35	23	Suicide/Drugs	Olanzapine, Trazodone, BZD
	MDD	M	35	20	Suicide/Gun	Fluoxetine,BZD
	Schizophrenia	M	37	11	Suicide/Jumping	Olanzapine, BZD
19	Control	F	43	12	Natural/CRF	(–)
	BP	F	43	4	Suicide/Jumping	(–)
	MDD	F	41	15	Suicide/Hanging	BZD
	Schizophrenia	F	38	19	Suicide/Drowning	Clotiapin, Zuclopenthixol, BZD
20	Control	M	41	19	Accident/Car	(–)
	BP	M	40	12	Natural/CRF	Risperidone, Valproic acid, BZD
	MDD	M	40	8	Suicide/Knife	Paroxetine, Quetiapine, BZD
	Schizophrenia	M	39	9	Suicide/Jumping	Citalopram, Oxcarbazepine

CRF, cardiorespiratory failure; AMI, acute myocaridial infarction; BZD, benzodiazepine; THC, Δ9-tetrahydrocannabinol

Ethanol concentration is indicated in parentheses (mg/mL)

were homogenized in ice-cold TED buffer [5 mM Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol (DTT); pH 7.4] containing 10% (w/v) sucrose. The nuclear fraction was removed by low-speed centrifugation, and membranes were prepared by sequential centrifugation and resuspension in buffer. Final aliquots were quickly frozen and stored at −80 ℃ until the assay was performed.

Carbachol-stimulated [^{35}S]GTPγS binding to $G\alpha_{i/o}$

The [^{35}S]GTPγS binding assay using conventional filtration techniques was performed as previously described [31]. Carbachol-stimulated [^{35}S]GTPγS binding determined by this method is derived from $G\alpha_{i/o}$ proteins functionally coupled to M_2/M_4 subtypes of mAChRs. Briefly, thawed human brain membranes equivalent to 60 µg protein per tube were incubated at 30 ºC for 60 min in 500 µl of 50 mM Tris–HCl buffer (pH 7.4) containing 0.2 nM [^{35}S]GTPγS, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 0.2 mM ethylene glycolbis(2-aminoethylether)-*N*,*N*,*N*,*N*-tetraacetic acid (EGTA), 0.2 mM dithiothreitol, 100 mM NaCl, 50 µM GDP, and carbachol at the indicated concentrations. After the incubation period, the homogenates were filtered under vacuum through glass fiber filters (GF/B; Whatman

International, Maidstone, UK) using a Brandel cell harvester with 2 × 5 ml washes with ice-cold 50 mM Tris–HCl buffer (pH 7.4). Nonspecific binding was determined in the presence of 100 µM unlabeled GTPγS. Radioactivity of [^{35}S]GTPγS bound to the G-proteins retained on the filters was counted using a liquid scintillation spectrometer in 8 ml of Emulsifier Scintillator Plus cocktail (PerkinElmer, Waltham, MA, USA).

Carbachol-stimulated [^{35}S]GTPγS binding to $G\alpha_{q/11}$

The [^{35}S]GTPγS binding/immunoprecipitation assay was performed as previously described [28, 29]. Thawed human brain membranes equivalent to 80 µg protein per tube were incubated at room temperature for 60 min in 200 µl of 50 mM Tris–HCl buffer (pH 7.4) containing 2.0 nM [^{35}S]GTPγS, 20 mM $MgCl_2$, 0.2 mM EGTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10 nM GDP, and carbachol at the indicated concentrations. The tubes were incubated for 30 min after addition of Nonidet P40 substitute (0.3%). Finally, 25 µl of Dynabeads Protein A suspension coated with anti-$G\alpha_{q/11}$ antibody (0.25 µg and 0.125 µg for Subjects [1] and [2], respectively), was added, and incubated for 60 min at room temperature with gentle occasional mixing. The Dynabeads Protein A were washed thoroughly with wash buffer (100 mM phosphate buffer containing 0.05% Tween 20, pH 7.4), and resuspended in 100 µl of wash buffer. The suspension was transferred into a scintillation mini vial, to which 4 ml of Emulsifier Scintillator Plus cocktail was added. The radioactivity of [^{35}S]GTPγS bound to the $G\alpha_{q/11}$ proteins, immunoprecipitated by Dynabeads Protein A coated with anti-$G\alpha_{q/11}$ antibody, was determined with a liquid scintillation spectrometer. Non-specific binding was determined in the presence of 1 mM unlabeled GTPγS. As demonstrated previously [28], carbachol-stimulated [^{35}S]GTPγS binding determined by this method is derived from $G\alpha_{q/11}$ proteins functionally coupled to M_1 mAChRs.

Data analysis

Data are presented as mean ± SEM of the indicated number of independent experiments, each performed in duplicate. The concentration-dependent increase in specific binding of [^{35}S]GTPγS elicited by carbachol was expressed as a percentage of the basal unstimulated value, and analyzed using nonlinear regression with GraphPad Prism (GraphPad Software; LaJolla, CA, USA), to determine the concentration eliciting the half-maximal effect (EC_{50}), maximum percent increase ($\%E_{max}$), and slope factor. The EC_{50} values were transformed into pEC_{50} ($-\log EC_{50}$) to be analyzed. Pharmacological parameters among the four groups were analyzed using one-way analyses of variance (ANOVA) or Kruskal–Wallis tests according to the results of Bartlett's test for equal variances. Post-hoc comparisons were performed using Tukey's test and Dunn's multiple comparison test, respectively. Linear regressions were calculated using the method of least squares and Pearson's coefficient for simple correlation was calculated to test for possible associations between variables.

Materials

[^{35}S]GTPγS (NEG030H, 1250 Ci/mmol) was purchased from PerkinElmer (Waltham, MA, USA). Carbachol, GDP, GTPγS, and Tween 20 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dynabeads Protein A were purchased from Life Technologies (Carlsbad, CA, USA). Anti-$G\alpha_{q/11}$ rabbit polyclonal antibody sc-393 (E-17) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Non-ionic detergent Nonidet P40 substitute was obtained from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals used in this study were obtained from standard sources and were of the highest commercially available purity.

Results

Interrelationship between carbachol-stimulated $G\alpha_{i/o}$ and $G\alpha_{q/11}$ functionality

The individual concentration–response curves of carbachol-induced $G\alpha_{i/o}$ activation determined in 38 samples from subjects [1] are depicted in Fig. 1a. The data for two subjects were lacking due to low sample volume. The $\%E_{max}$, pEC_{50}, and slope factors varied substantially, with a range of 11.6–72.9%, 3.77–5.24, and 0.46–1.24, respectively.

The individual concentration–response curves of carbachol-induced $G\alpha_{q/11}$ activation in 40 samples from subjects [1] have been reported elsewhere [28], which are replicatively presented in Fig. 1b.

There was no significant correlation with the $\%E_{max}$ values (Fig. 2a) or slope factors (not shown) determined by the two different assays. Interestingly, the pEC_{50} values determined for $G\alpha_{q/11}$ were significantly correlated with those determined for $G\alpha_{i/o}$, as presented in Fig. 2b ($r = -0.38$, $p < 0.05$).

Carbachol-stimulated $G\alpha_{q/11}$ functionality in subjects with psychiatric disorders

Carbachol-stimulated increase in specific [^{35}S]GTPγS binding to $G\alpha_{q/11}$ was determined in 80 individuals belonging to one of the four subgroups of subjects [2]. As shown in Fig. 3a, the $\%E_{max}$ values were not significantly different between groups, determined by one-way ANOVA

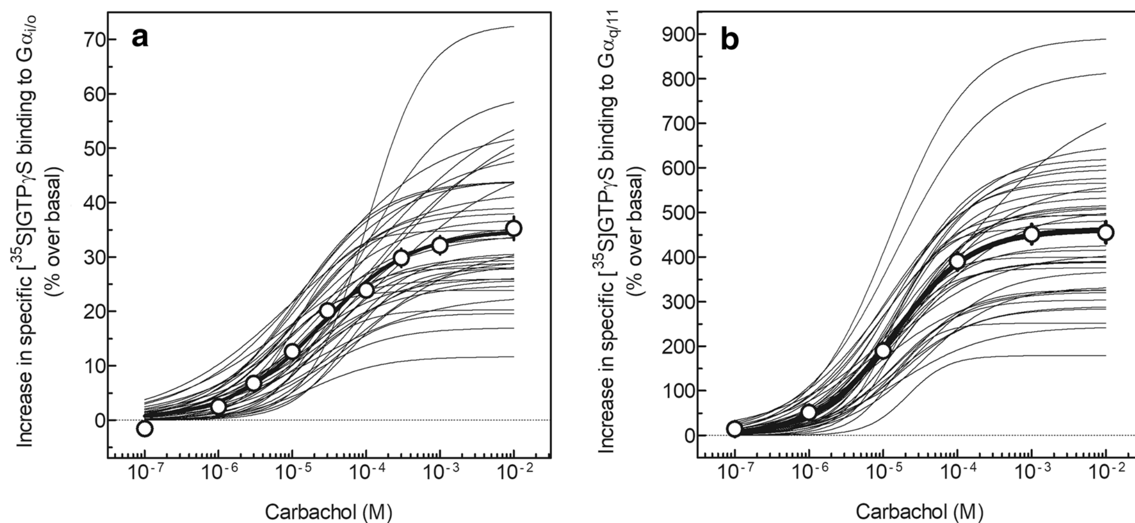

Fig. 1 Stimulatory effects of carbachol on specific [^{35}S]GTPγS binding to $G\alpha_{i/o}$ (**a**) and $G\alpha_{q/11}$ (**b**) in postmortem human prefrontal cortical membranes. The thin lines represent individual concentration–response curves for 38 (**a**) and 40 (**b**) subjects without any neuropsychiatric disorder, expressed as a percentage increase over the basal, unstimulated specific binding. Open symbols represent the mean ± SEM of all samples, and the bold line depicts the concentration–response curve derived from these values. Figure 1b is replicated from [28]

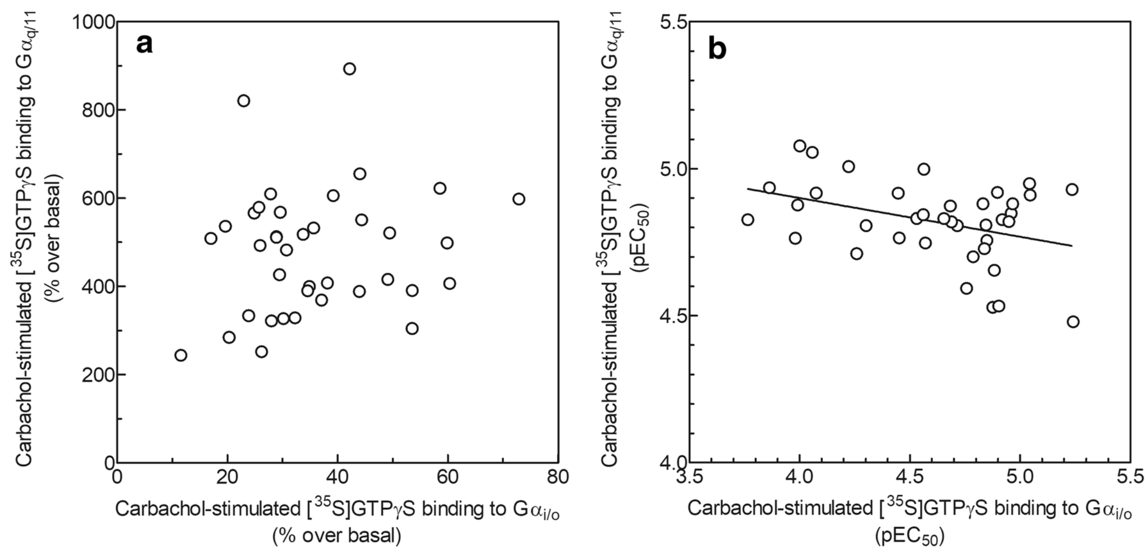

Fig. 2 Interrelationship between carbachol-stimulated $G\alpha_{i/o}$ and $G\alpha_{q/11}$ functionality in 38 subjects without any neuropsychiatric disorder. **a** The symbols represent individual subjects with a $\%E_{max}$ value determined for M_2/M_4 mAChR-mediated $G\alpha_{i/o}$ activation (abscissa) and for M_1 mAChR-mediated $G\alpha_{q/11}$ activation (ordinate). **b** The symbols represent individual subjects with a pEC_{50} value determined for M_2/M_4 mAChR-mediated $G\alpha_{i/o}$ activation (abscissa) and for M_1 mAChR-mediated $G\alpha_{q/11}$ activation (ordinate). The regression line indicates a significant correlation ($r = -0.38$, $p < 0.05$)

[$F(3,76) = 0.074$, N.S.]. The results of Bartlett's test for equal variances indicated unequal distributions of pEC_{50} ($p < 0.0001$) and slope ($p = 0.022$). There was no significant difference in pEC_{50} values (Fig. 3b) or slope factors (Fig. 3c) among the groups, as determined by Kruskal–Wallis analysis. Since the psychiatric patients included some suicide victims (14/20 in BP, 17/20 in MDD, and 10/20 in schizophrenia), each group was further subdivided into suicide and non-suicide subjects. No significant difference was detected by Kruskal–Wallis analysis with respect to $\%E_{max}$, pEC_{50}, or slope factor among the groups (not shown). Further, some patients had been taking a variety of psychotropic drugs, as revealed by the toxicological data (Table 2). However, it is difficult to evaluate the effect of pharmacotherapy

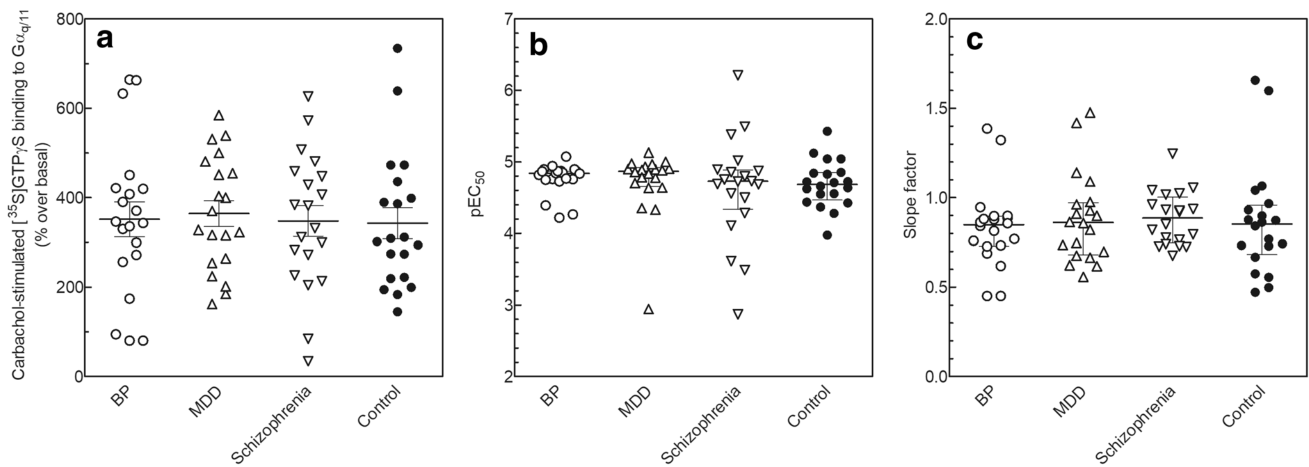

Fig. 3 Comparison of the stimulatory effects of carbachol on the specific [^{35}S]GTPγS binding to $G\alpha_{q/11}$ in postmortem human prefrontal cortical membranes among bipolar disorder (BP), major depressive disorder (MDD), schizophrenia, and control groups. **a** The symbols represent individual subjects with a $\%E_{max}$ value determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in BP (empty circle), MDD (upward triangle), schizophrenia (downward triangle), and control (filled circle) group. The horizontal line with error bars indicates the mean ± SEM. **b** The symbols represent individual subjects with a pEC_{50} value determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in BP (empty circle), MDD (upward triangle), schizophrenia (downward triangle), and control (filled circle) group. The horizontal line with error bars indicates the median ± interquartile range. **c** The symbols represent individual subjects with slope factor determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in BP (empty circle), MDD (upward triangle), schizophrenia (downward triangle), and control (filled circle) group. The horizontal line with error bars indicates the median ± interquartile range

with psychotropic drugs, considering the multiplicity of modes of action of the medications used, and the possibility that lack of drug detection in toxicological screening does not infer lack of psychotropic medication prior to death. Nonetheless, the possible effects of antipsychotic agents in the schizophrenia group were examined, since these drugs have pharmacological properties common to dopamine D_2 receptor antagonists. When divided into the two subgroups according to the toxicological data (antipsychotic (+) and antipsychotic (–)), the $\%E_{max}$ values were significantly different, as determined by one-way ANOVA [$F(2,37) = 4.81$, $p < 0.05$] (Fig. 4a). Post-hoc comparison using Tukey's test indicated significant differences between antipsychotic (+) and antipsychotic (−) subgroups. Likewise, Kruskal–Wallis analysis indicated significant differences between the groups in the pEC_{50} values ($p < 0.05$), which was also ascribed to the difference between antipsychotic (+) and antipsychotic (−) subgroups as revealed by Dunn's multiple comparison test ($p < 0.05$) (Fig. 4b). Slope factor was not significantly different between the antipsychotic (+) and antipsychotic (−) subgroup, or the controls (Kruskal–Wallis test) (not shown).

Interrelationship between $\%E_{max}$ and pEC_{50} of carbachol-induced $G\alpha_{q/11}$ activation

The correlations between the $\%E_{max}$ values and the pEC_{50} values determined for carbachol-induced [^{35}S]GTPγS binding to $G\alpha_{q/11}$ were investigated in each cohort. In subjects [1], there was no significant correlation between the two pharmacological parameters (Fig. 5a). Likewise, no significant correlations were detected in three of the cohorts (BP, MDD, and control) of subjects [2] (Fig. 5b). However, the $\%E_{max}$ values were significantly negatively correlated with the pEC_{50} values in the schizophrenia group ($r = -0.56$, $p < 0.05$) (Fig. 5c).

Discussion

The muscarinic component of the central cholinergic system has been implicated in the pathophysiology of a number of neurological and psychiatric disorders associated with cognitive dysfunction. Accumulating evidence has revealed that cognitive deficits are one of the core symptoms of schizophrenia [32, 33]. Further, cognitive dysfunction is now considered an important feature of mood disorders including BP and MDD [34]. The main purpose of the current study was to elucidate possible alterations in M_1 mAChR-mediated $G\alpha_{q/11}$ signaling in psychiatric patients diagnosed as having BP, MDD, or schizophrenia prior to death, by assessing [^{35}S]GTPγS binding/immunoprecipitation in prefrontal cortical membranes from postmortem brains. The method applied in this study was pharmacologically characterized previously [28]. That study showed the carbachol-stimulated increase in [^{35}S]GTPγS binding to $G\alpha_{q/11}$ in human prefrontal cortical membranes was potently inhibited by (±)-telenzepine, a selective M_1 mAChR antagonist, in a competitive manner, with a pA_2 value of 8.81. Although the involvement of other

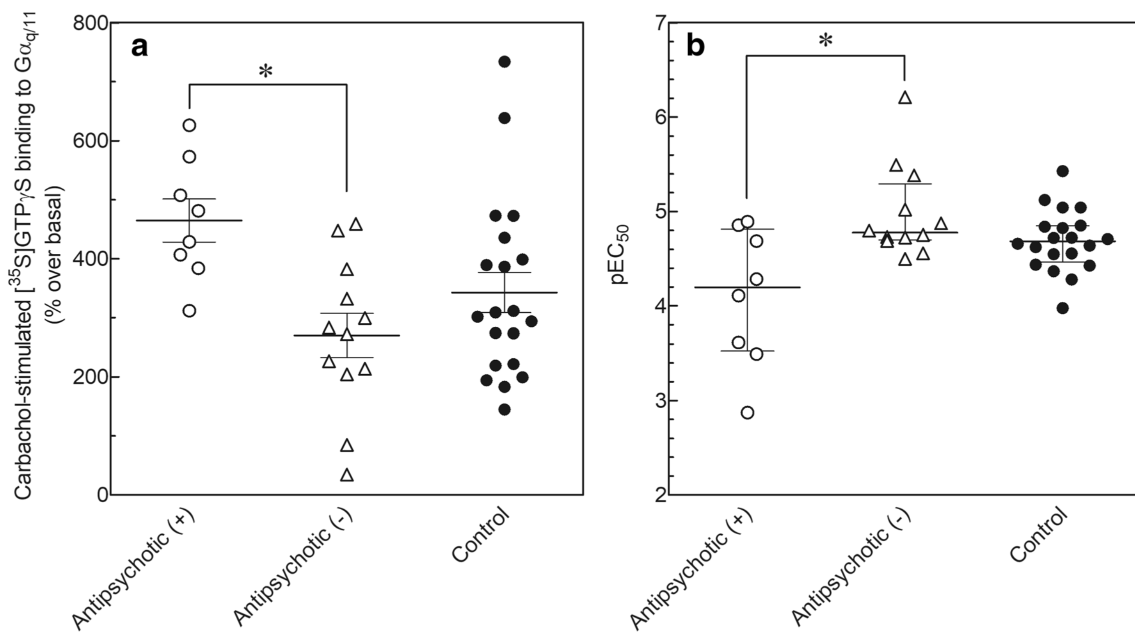

Fig. 4 Comparison of the stimulatory effects of carbachol on the specific [^{35}S]GTPγS binding to $G\alpha_{q/11}$ in postmortem human prefrontal cortical membranes among the two schizophrenia subgroups [antipsychotic (+) and antipsychotic (−), divided according to the toxicological data] and controls. **a** The symbols represent individual subjects with a $\%E_{max}$ value determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in antipsychotic (+) (empty circle), antipsychotic (−) (upward triangle), and control (filled circle) group. The horizontal line with error bars indicates the mean ± SEM. **b** The symbols represent individual subjects with a pEC_{50} value determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in antipsychotic (+) (empty circle), antipsychotic (−) (upward triangle), and control (filled circle) group. The horizontal line with error bars indicates the median ± interquartile range. Significant differences revealed by post-hoc tests are indicated with asterisks (*$p < 0.05$)

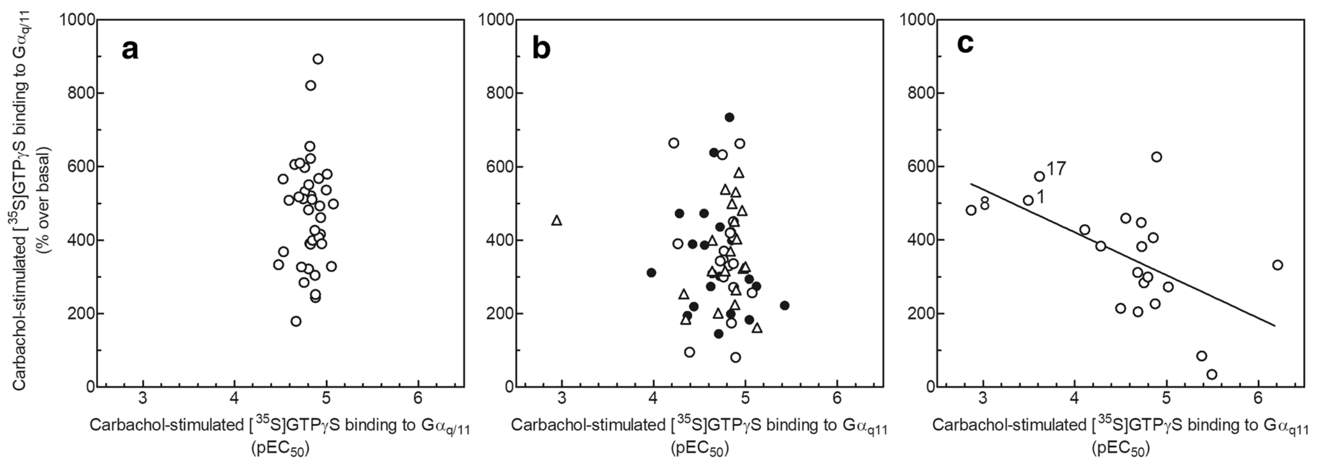

Fig. 5 Interrelationship between pEC_{50} and $\%E_{max}$ values determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in postmortem human prefrontal cortical membranes. **a** The symbols represent individual subjects with a pEC_{50} value (abscissa) and a $\%E_{max}$ value (ordinate) determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in 40 subjects without any neuropsychiatric disorder (subjects[1]). **b** The symbols represent individual subjects with a pEC_{50} value (abscissa) and a $\%E_{max}$ value (ordinate) determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in bipolar disorder (BP) (empty circle), major depressive disorder (MDD) (upward triangle), and control (filled circle) group. **c** The symbols represent individual subjects with a pEC_{50} value (abscissa) and a $\%E_{max}$ value (ordinate) determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation in the schizophrenia group. The regression line indicates a significant correlation ($r = -0.56$, $p < 0.05$). Three patients with extremely low pEC_{50} values are indicated with the experimental number (1, 8, and 17) (see Table 2)

receptor subtypes such as M_3 mAChR cannot be entirely excluded, the response appears to be derived mostly from $G\alpha_{q/11}$ coupled to M_1 mAChR, considering the relative density of mAChRs ($M_1 >> M_3$) in brain tissue [35, 36].

Although the pharmacological parameters of carbachol-stimulated $G\alpha_{q/11}$ functionality ($\%E_{max}$, pEC_{50}, and slope factor) were not significantly different in any psychiatric disorder cohort (BP, MDD, or schizophrenia) compared to the control group, some interesting findings were obtained in the present study. In subjects [1], which consisted of 40 individuals without any neuropsychiatric disorder, both M_1 mAChR-mediated $G\alpha_{q/11}$ and M_2/M_4 mAChR-mediated $G\alpha_{i/o}$ activation were determined in 38 individuals. Neither the $\%E_{max}$ values nor slope factors determined by the two biochemical measures were not correlated, whereas the pEC_{50} values for M_1 mAChR/$G\alpha_{q/11}$ coupling were significantly negatively correlated to those for M_2/M_4 mAChR/$G\alpha_{i/o}$ coupling. Although the exact implications of these findings are unclear, it has been reported that mAChRs are regulated directly by, or are a consequence of, multiple internal changes due to different types of stressful stimuli such as physical, chemical, psychological/social, and cardiovascular system-disturbing events [37]. Multiple and complex molecular mechanisms have been reported to underlie alterations in mAChR-mediated signaling pathways [38]. Interestingly, crosstalk between $G\alpha_i$- and $G\alpha_q$-coupled receptors mediated by Gβγ exchange has been indicated, as exemplified by adenosine A_1 and α_{2C} adrenoceptor ($G\alpha_i$-coupled), and bradykinin B_2 and UTP-preferring P2Y receptor ($G\alpha_q$-coupled) [39]. The data in the present study should be interpreted with caution, because the two determinations were obtained from the two independent experiments under different conditions, particularly regarding GDP concentrations (10 nM and 50 µM for M_1 mAChR/$G\alpha_{q/11}$ coupling and M_2/M_4 mAChR/$G\alpha_{i/o}$ coupling, respectively). Nevertheless, our results suggest there might also be some interactive regulatory processes between $G\alpha_i$-coupled M_2/M_4 mAChRs and $G\alpha_q$-coupled M_1 mAChR via molecular mechanisms not yet elucidated.

Secondly, a significant inverse correlation between $\%E_{max}$ values and pEC_{50} values determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation was detected only in the schizophrenia group, but not in BP, MDD, or controls. The lack of a significant correlation was replicated in subjects [1], in which all subjects were free of neuropsychiatric illness. The pEC_{50} values for M_1 mAChR-mediated $G\alpha_{q/11}$ activation were distributed within a relatively narrow range in controls (4.48–5.08 in subjects [1], and 3.98–5.43 in subjects [2]), BP (4.22–5.07), and MDD (4.33–5.13, except for one subject with a particularly low value of 2.94). In contrast, the pEC_{50} values in the schizophrenia group ranged from 2.81 to 6.21, more than three orders of magnitude. The unequal distributions of pEC_{50} values were detected using Bartlett's test for equal variances in subjects [2] ($p < 0.0001$), and the unique pattern of pEC_{50} distribution in the schizophrenia group appears to contribute to the significant negative correlation between pEC_{50} and $\%E_{max}$ values in this group.

The M_1 mAChR-mediated $G\alpha_{q/11}$ activation in postmortem human brains from schizophrenia patients has been reported by Salah-Uddin et al. [26]. They divided the schizophrenia subjects into two sub-populations based on [^{3}H]pirenzepine binding, termed "muscarinic receptor-deficit schizophrenia (MRDS)" and "non-MRDS". In addition to the lower M_1 mAChR binding, the pEC_{50} values and maximal increases in oxotremorine-M-stimulated $G\alpha_{q/11}$ determined in the MRDS group were significantly decreased and increased, respectively, compared to the control group. These findings suggest biological heterogeneity in schizophrenia, and the results of the present study support this hypothesis. To ascertain this, it is important to assess M_1 mAChR expression levels in schizophrenic patients by radioligand binding assay and/or western blot in future studies.

Of 20 subjects in the schizophrenia group, three patients demonstrated extremely reduced potency of carbachol (Fig. 5c; 1, 8, and 17). These three patients had relatively high $\%E_{max}$ values, and the characteristics of these subjects underlie the significant negative correlation between pEC_{50} and $\%E_{max}$ values in this group. Interestingly, all of these patients belong to the antipsychotic (+) subgroup. Significant differences in $\%E_{max}$ and pEC_{50} values between antipsychotic (+) and antipsychotic (−) subjects may indicate that altered M_1 mAChR/$G\alpha_{q/11}$ coupling in schizophrenia patients is ascribed, at least in part, to pharmacotherapy with antipsychotics.

This significant negative correlation was restricted to the schizophrenia group, suggesting disorder-specific alterations in mAChR signaling. There is relatively little direct evidence to show perturbed central cholinergic activity in mood disorders [4, 7, 8]. Levels of M_1 mAChRs in postmortem brains from patients with mood disorders have been reported similar to controls [17, 21, 25]. Our data are in line with these previous reports using radioligand binding techniques. On the other hand, several reports have indicated that $G\alpha_{i/o}$-coupled mAChRs (M_2 and/or M_4 mAChR) are implicated in the pathogenesis of mood disorders [17, 23, 40]. As such, it is of interest to investigate possible alterations in mAChR-mediated $G\alpha_{i/o}$ activation in mood disorder patients. This work is ongoing.

G-proteins play a pivotal role in receptor-mediated signal transduction pathways. Enhanced receptor/G-protein coupling has been reported in frontal cortical membranes obtained from postmortem brains of BP patients, compared to age-, sex-, and postmortem interval-matched controls [41]. Enhanced receptor/G-protein coupling in BP subjects has been detected between several receptors and Gα subtypes (i.e., isoproterenol-stimulated $G\alpha_s$;

carbachol-stimulated $G\alpha_i$, $G\alpha_o$, and $G\alpha_q$; 5-HT-stimulated $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_q$). This universal hypersensitivity of receptor-coupled G-protein function in BP patients, along with an epoch-making finding that the anti-bipolar agent lithium inhibits adrenergic and cholinergic increases in GTP binding [42], has led to the so-called "G-protein hypothesis of mood disorders" [43–45]. However, such an oversimplified hypothesis appears insufficient, considering controversial results on quantitative and functional status of the heterotrimeric G-proteins and G-protein-mediated signaling in various experimental designs implicated in the pathogenesis and treatment of mood disorders [46, 47]. Our previous efforts aiming to replicate the report of Avissar et al. [42] failed to identify interfering effects of lithium on receptor/G-protein coupling, at least in the case of $G_{i/o}$ proteins coupled with various neurotransmitter receptors [48]. Further investigation is needed to evaluate the implications of G-protein and G-protein-linked molecular machinery in the pathophysiology and treatment of mood disorders.

In conclusion, we examined mAChR-mediated G-protein activation in postmortem human prefrontal cortical membranes. Although there was no significant correlation in $\%E_{max}$ values or slope factors between M_2/M_4 mAChR-mediated $G\alpha_{i/o}$ activation and M_1 mAChR-mediated $G\alpha_{q/11}$ activation, pEC_{50} values were significantly correlated with each other, indicating some interactive molecular processes between these two mAChR-mediated signaling pathways. The results of M_1 mAChR-mediated $G\alpha_{q/11}$ activation in the BP, MDD, schizophrenia, and control subjects, indicated no significant disorder-specific differences in each pharmacological parameters. However, the distribution patterns of the pEC_{50} values determined for M_1 mAChR-mediated $G\alpha_{q/11}$ activation showed unequal variances among the groups, and there was a significant inverse correlation between the $\%E_{max}$ values and the pEC_{50} values restricted to the schizophrenia cohort, but not in the BP, MDD, or controls. The lack of direct evidence indicating heterogeneous subgroups in schizophrenia patients is a major limitation of this study. Furthermore, the densities of M_1 mAChRs as well as $G\alpha_{q/11}$ proteins were not determined by radioligand binding assay or western blot. Nevertheless, the data may support the notion that schizophrenia patients consist of biologically heterogeneous groups, i.e., a small proportion with M_1 mAChR-mediated $G\alpha_{q/11}$ signaling deficits, and the majority without such deficit [20, 26].

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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