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ARTICLE

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Reduced level of glutamic acid decarboxylase-67 kDa in the prefrontal cortex in major depression

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Abstract

Accumulating evidence suggests dysfunction of the gamma-aminobutyric acid (GABA) system in major depressive disorder (MDD). Neuroimaging studies consistently report reductions of cortical GABA in depressed patients. Our post-mortem analyses demonstrate a reduction in the density and size of GABAergic interneurons in the dorsolateral prefrontal cortex (DLPFC) in MDD. The goal of this study was to test whether the level of glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, will also be reduced in the same cortical region in MDD. Levels of GAD-65 and GAD-67 proteins were investigated by Western blotting in samples from the DLPFC (BA 9) in 13 medication-free subjects with MDD, and 13 psychiatrically healthy controls. The overall amount of GAD-67 was significantly reduced (−34%) in depressed subjects compared to matched controls. Since recent neuroimaging studies have demonstrated that antidepressants modulate GABA levels, additional experiments were performed to examine the levels of GAD in eight depressed subjects treated with antidepressant medications. Levels of GAD-67 were unchanged in these depressed subjects compared to their respective controls ($n=8$). The overall amounts of GAD-65 were similar in depressed subjects compared to matched controls, regardless of antidepressant medication. Reduced levels of GAD-67, which is localized to somata of GABA neurons, further support our observation of a decreased density of GABAergic neurons in the PFC in depression. It is likely that a decrease in GAD-67 accounts for the reduction in GABA levels revealed by neuroimaging studies. Moreover, our data support previous neuroimaging observations that antidepressant medication normalizes GABA deficits in depression.

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Key words: Antidepressants, dorsolateral prefrontal cortex, GAD, GABA, major depressive disorder, post-mortem.

Introduction

Several lines of evidence indicate that major depressive disorder (MDD) is associated with abnormalities in the gamma-aminobutyric acid (GABA) system (for review see Sanacora & Saricicek, 2007). Recent neuroimaging studies report reductions in GABA levels in the prefrontal and occipital cortex in depressed patients (Hasler *et al*. 2007; Sanacora *et al*. 1999, 2004). Reduced GABA concentrations have also been demonstrated in the plasma and cerebrospinal fluid in depression (Brambilla *et al*. 2003; Gerner & Hare, 1981; Kasa *et al*. 1982; Petty *et al*. 1992). Moreover, a metabolomic analysis demonstrates reductions in the level of GABA as well as several fatty acids and glycerol in blood plasma of older depressed patients (Paige *et al*. 2007). Recent post-mortem morphometric analyses in MDD demonstrate a reduction in the density and size of GABAergic interneurons immunoreactive for calbindin protein in the dorsolateral prefrontal cortex (DLPFC; Rajkowska *et al*. 2007) suggesting GABAergic system dysfunction in depression.

GABA is synthesized from glutamate in GABAergic neurons by glutamic acid decarboxylase (GAD), the pyridoxal phosphate (PLP)-dependent enzyme

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(Martin *et al*. 1991). GAD exists in two isoforms, GAD-65 and GAD-67, which are the products of two independent genes (Erlander *et al*. 1991; Kaufman *et al*. 1991). Gene knockout studies in mice have helped define distinct roles for each isoform. Mice lacking GAD-67 have significantly reduced GABA levels and die at birth of a severe cleft palate (Asada *et al*. 1997). In contrast, GAD-65 knockout mice have normal basal levels of GABA and appear normal at birth, but develop fatal seizures and anxiety phenotypes (Asada *et al*. 1996). It has been observed that GAD-65 is more abundant in the nerve terminals, whereas GAD-67 is more concentrated in the neuronal cell bodies (Erlander *et al*. 1991; Erlander & Tobin, 1991; Kaufman *et al*. 1991). Thus, based on the different neuronal distributions of GAD isoforms, GAD-67 may be involved in the synthesis of GABA for general metabolic activity, whereas GAD-65 may be predominantly involved in synthesizing GABA for neuronal transmission (Martin & Rimvall, 1993).

Interestingly, it has been demonstrated that antidepressant therapies induce marked changes in GABAergic function. For example, GABA levels in the occipital cortex were increased in depressed patients after antidepressant treatments such as electroconvulsive therapy (ECT) or selective serotonin reuptake inhibitors (SSRIs; Sanacora *et al*. 2002, 2003) but not after cognitive behavioural therapy (Sanacora *et al*. 2006). Moreover, a number of earlier animal studies reveal that administration of tricyclic antidepressant drugs, inhibitors of monoamine oxidase, or electroconvulsive shock elevates GABA levels or increases its release (Bowdler *et al*. 1983; Korf & Venema, 1983; Patel *et al*. 1975; Perry & Hansen, 1973; Popov & Matthies, 1969). Collectively, these data clearly indicate a relationship between antidepressant medication and regulation of GABAergic transmission.

GABA is a major component of neuronal circuitry in the PFC and cortical GABAergic interneurons can be divided into non-overlapping subpopulations based on the calcium-binding protein, calbindin, parvalbumin or calretinin they co-express (Conde *et al*. 1994; Lund & Lewis, 1993). Since a population of calbindin-immunoreactive interneurons was selectively reduced in the DLPFC in depression (Rajkowska *et al*. 2007), the aim of the present study was to investigate whether GAD-65 or GAD-67 protein would also be reduced in the DLPFC in depression. The levels of GAD-65 and GAD-67 were measured in medication-free subjects with MDD and their individually matched control cases. Additionally, the levels of GAD-65 and GAD-67 were also determined in medicated subjects with MDD and their corresponding controls. Medication-free refers to subjects in whom antidepressant drugs were not detected in post-mortem blood samples; medicated subjects are those in which antidepressant drugs were detected in post-mortem blood samples. The levels of GAD-67 and GAD-65 were measured in homogenates from the grey matter of left DLPFC [Brodmann's Area (BA) 9] using Western blot method.

Material and methods

Human subjects

Post-mortem brain samples were collected at autopsy from the total of 34 subjects at the Cuyahoga County Coroner's Office in Cleveland, OH. Informed written consent was collected from the legal next-of-kin of all subjects. Next-of-kin were interviewed and retrospective psychiatric assessments were conducted in accordance with Institutional Review Board policies at University Hospitals of Cleveland and the University of Mississippi Medical Center. As previously described, a trained interviewer administered the Schedule for Affective Disorders and Schizophrenia: lifetime version (SADS-L; Endicott & Spitzer, 1978) and/or the Structured Clinical Interview for DSM-IV Psychiatric Disorders (SCID-IV) to knowledgeable next-of-kin of subjects about 3 months after the death to determine current and lifetime Axis I psychopathology. Diagnoses for Axis I major mental disorders were independently assessed by a clinical psychologist and a psychiatrist, and consensus diagnosis was reached in conference using information from the knowledgeable informants, the coroner's office, and available in-patient and outpatient records. Twenty-one subjects met DSM-IV criteria for MDD (APA, 1994). None of the 21 control subjects met criteria for depression (see Tables 1 and 2). Of 21 depressed individuals, 16 died by suicide. Eleven out of 21 depressed subjects were hospitalized (1–35 times) due to their depression. Blood and urine samples from all subjects were examined by the coroner's office for psychotropic medications and substances of abuse, including antidepressants and ethanol (Tables 1, 2). Thirteen subjects did not have antidepressants present in their post-mortem toxicology screening, thus they are referred to as MDD 'medication-free'. In fact, nine of these 13 'medication-free' MDD subjects did not have a prescription for antidepressants in the last month of life (for further details see Table 1).

Eight depressed subjects tested positively for different antidepressants in their toxicology screening, and thus they are referred to as MDD 'medicated'

Table 1. Demographic characteristics of medication-free MDD subjects and controls

Parameter	Controls ($n=13$)	MDD ($n=13$)
Age (mean ± S.E.M.)	51 ± 5 yr	51 ± 4 yr
Age range	30–80 yr	30–78 yr
PMI (mean ± S.E.M)	21 ± 2 h	24 ± 2 h
PMI range	9–32 h	11–44 h
pH (mean ± S.E.M)	6.72 ± 0.07	6.58 ± 0.08
pH range	6.3–7.14	6.06–6.97
Time in freezer (mean ± S.E.M)	105 ± 8 mos.	114 ± 10 mos.
Time in freezer range	42–146 mos.	36–152 mos.
Gender (F:M)	6:7	5:8
Medication history[a]	None	$n=4$
Toxicology		
• Clean	$n=9$	$n=7$
• Antidepressant drugs	None	None
• Other	$n=4$ (brompheniramine, $n=1$; CO, $n=1$; brompheniramine, orphenadrine, acetaminophen, $n=1$; caffeine, $n=1$)	$n=6$ (CO, alprazolam, $n=1$; ethanol, $n=1$; propoxyphene, acetaminophen, $n=1$; CO, phenobarbital, phenytoin, $n=1$; diazepam, acetaminophen, $n=1$; diphenhydramine, $n=1$)
Cause of death	Cardiovascular disease, $n=12$ Acute haemorrhagic pancreatitis, $n=1$	Suicide, $n=9$ (shotgun, $n=3$; CO poisoning, $n=2$; hanging, $n=2$; drug overdose, $n=1$; jumper, $n=1$) Other causes, $n=4$ (all cardiovascular disease)
Diagnosis	None, $n=10$ History of alcohol abuse, $n=2$ Specific phobia-situational type (heights), $n=1$	MDD, $n=10$ MDD and history of alcohol abuse, $n=1$ MDD and history of alcohol dependence, $n=1$ MDD, bulimia nervosa and drug abuse, $n=1$
Smoking	Smokers, $n=6$ History of smoking, $n=2$	Smokers, $n=6$ History of smoking, $n=2$

PMI, Post-mortem interval; CO, carbon monoxide; MDD, major depressive disorder; mos., months.
[a] Treatment with antidepressants within 4 wk of time of death. The average ages, PMI, pH and time in freezer of depressed and control subjects were not statistically different.

(Table 2). All of these 'medicated' MDD subjects had prescriptions for antidepressants in the last month of life. Depressed subjects and psychiatrically healthy controls were matched as close as possible for age, gender, post-mortem interval (PMI), tissue pH, and storage time in freezer. The duration of depression for antidepressant-free depressed subjects ranged from 0.2 to 50 yr (13.8 ± 4.1 yr) and for medicated MDD subjects ranged from 0.25 to 30 years (7.4 ± 3.5 yr).

Immunoblotting

Frozen sections from the blocks of tissue containing DLPFC were cut on a cryostat at 50 μm. The first two sections collected were stained for Nissl substance to distinguish BA 9 (Rajkowska & Goldman-Rakic, 1995). The immediately adjacent sections were used to collect punches of tissue. The diameter of the punches was adjusted to include all six cortical layers of the grey matter of BA 9, and the underlying white matter was excluded.

Tissue samples were prepared according to a method published previously (Karolewicz *et al*. 2008). Samples were homogenized in ice-cold TE buffer (10 mM Tris–HCl and 1 mM ethylene-diamine-tetraacetate; EDTA) containing protease inhibitors (protease inhibitor cocktail tablets – Complete™, Boehringer-Mannheim GmbH, Germany). The homogenized tissue was centrifuged at 900 *g* for 10 min. Total protein concentration was determined in the resulting supernatant using the bicinchoninic acid (BCA) method (Pierce Biotechnology Inc., USA). Samples were mixed with sample buffer [125 mM Tris base, 20% glycerol, 4% SDS, 10% mercaptoethanol,

Table 2. Demographic characteristics of medicated MDD subjects and controls

Parameter	Controls ($n=8$)	MDD ($n=8$)
Age (mean ± S.E.M.)	51 ± 6 yr	51 ± 7 yr
Age range	27–78 yr	20–77 yr
PMI (mean ± S.E.M.)	21 ± 2 h	21 ± 3 h
PMI range	10–29 h	10–29 h
pH (mean ± S.E.M.)	6.83 ± 0.04	6.57 ± 0.07
pH range	6.71–7.09	6.21–6.79
Time in freezer (mean ± S.E.M.)	100 ± 17 mos.	105 ± 15 mos.
Time in freezer range	29–149 mos.	28–149 mos.
Gender (F:M)	2:6	2:6
Medication history[a]	None	$n=8$
Toxicology		
• Clean	$n=7$	None
• Antidepressant drugs	None	$n=8$ (amitriptyline, $n=1$; sertraline, norsertraline, $n=2$; bupropion, venlafaxine, $n=1$; venlafaxine, $n=1$; nortriptyline, $n=1$; paroxetine, $n=1$; sertraline, $n=1$)
• Other	$n=1$ (CO)	$n=5$ (chlorpromazine, amantadine, lidocaine, $n=1$; diphenhydramine, $n=1$; diphenhydramine, hydrocodone, $n=1$; cannabinoids, $n=1$; lidocaine, $n=1$)
Cause of death	Cardiovascular disease, $n=7$ Asphyxiation due to CO, $n=1$	Suicide, $n=7$ (shotgun, $n=2$; CO poisoning, $n=1$; hanging, $n=2$; drowning, $n=1$; overdose-hyperkalemia, $n=1$) Other causes, $n=1$ (pulmonary thromboembolism)
Diagnosis	None, $n=6$ History of alcohol abuse, $n=1$ History of alcohol dependence (32 yr prior to death), $n=1$	MDD, $n=6$ MDD, hypochondriasis, polysubstance dependence, and alcohol abuse, $n=1$ MDD, alcohol abuse and Marijuana abuse, $n=1$
Smoking	Smokers, $n=5$ History of smoking, $n=1$	Smokers, $n=3$ History of smoking, $n=1$

PMI, Post-mortem interval; CO, carbon monoxide; MDD, major depressive disorder; mos., months.
[a] Treatment with antidepressants within 4 weeks of time of death. The average ages, PMI, pH and time in freezer of depressed and control subjects were not statistically different.

0.05% Bromophenol Blue (pH 6.8)] and heated at 95 °C for 8 min. Solubilized protein (20 μg per lane) was subjected to 10% Criterion Precast Tris–HCl gel electrophoresis (Bio-Rad Laboratories, USA) and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, USA). After transfer, blots were blocked in 5% non-fat milk/TBS [20 mM Tris base and 0.5 M NaCl (pH 7.5)] or 5% non-fat milk/PBS [137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and KH_2PO_4 (pH 7.4)] for 2 h, then incubated (overnight at 4 °C) with mouse anti-GAD-67 monoclonal antibodies (diluted 1:1000; Chemicon, USA; no. MAB5406). GAD-65 was labelled using rabbit polyclonal antibodies diluted 1:1000 (Chemicon, no. AB5082). As a control for transfer and loading β-tubulin was detected on each blot using anti-tubulin (Abcam Inc., USA; no. ab6046) antibody, diluted 1:10 000. Membranes were washed two times for 15 min in TBS buffer and incubated with secondary anti-mouse antibody for GAD-67 (diluted 1:2000; Amersham Biosciences, no. NA931) or anti-rabbit secondary antibody for GAD-65 and β-tubulin (diluted 1:5000; Amersham Biosciences; no. NA934). After incubation, blots were washed 3 or 4 times for 15 min and developed using enhanced chemiluminescence detection (ECL; PerkinElmer Life Sciences Inc., USA) and immediately exposed to film (Hyperfilm-ECL, Amersham Biosciences).

Immunoreactivities of GAD-67 and GAD-65 were investigated in two different experiments in pairs of depressed subjects and matched controls. Pairs of subjects were matched as closely as possible for age, gender, PMI, tissue pH, and storage time in freezer. A maximum five pairs of control/depressed subjects were run on the same gel with duplicates run on separate gels. There was a linear relationship between optical density values and protein concentrations for

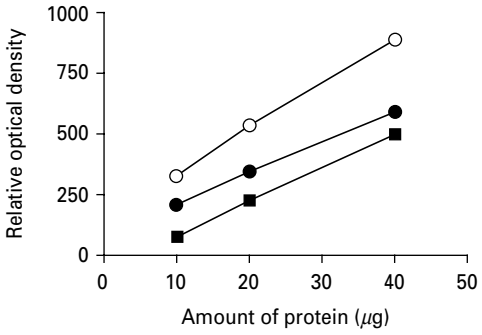

Fig. 1. Relationship between the optical density values of Western-blotted protein immunoreactivities and protein amounts for GAD-67 (–■–), GAD-65 (–○–), and tubulin (–●–). Wells were loaded with three concentrations of cortical tissue consisting of 10, 20, and 40 μg total protein.

all antibodies used (Fig. 1). In order to minimize inter-blot variability and to aid in quantifying blots, each gel was loaded with three concentrations of the same cortical tissue standard (dissected from a healthy control subject) consisting of 10, 20, and 40 μg total protein.

Data analysis

Band densities were analysed using MCID Elite 7.0 (Imaging Research, Canada). Relative optical density values were converted to arbitrary protein units using the standards. Data were then normalized to β-tubulin immunoreactivity detected on the same blot.

The levels of GAD-67 and GAD-65 immunoreactivity in depressed subjects were compared to their respective controls in two separate experiments using non-parametric Wilcoxon signed-ranks tests (SPSS version 16.0; SPSS Inc., USA). In the first experiment medication-free MDD subjects ($n=13$) were compared to their matched psychiatrically healthy control subjects ($n=13$), whereas, in the additional experiment, MDD subjects medicated at the time of death ($n=8$) were compared to their matched controls ($n=8$).

Pearson correlation analyses was used to assess the correlations between investigated proteins and potentially confounding factors such as age, pH, PMI, and storage time in freezer. In order to adjust for multiple comparisons (two GAD isoforms analysed per brain region) and to avoid a Type 1 error, the p value $\leqslant 0.025$ was considered as a threshold for significance.

Results

Medication-free subjects with MDD vs. controls

Nine out of thirteen medication-free depressed subjects had reduced amount of GAD-67 compared to their respective controls, two depressed subjects had unchanged levels of GAD, and two depressed subjects had slightly higher levels of GAD compared to matched control. The overall amount of GAD-67 immunoreactivity from medication-free MDD subjects was significantly lower (-34%) compared to control subjects (Wilcoxon signed ranks test, $Z=-2.341$, $p=0.019$; Fig. 2). Figure 3 shows GAD-67 immunoreactivity from individual medication-free MDD subjects expressed as percentages of values from paired control subjects. The amount of GAD-65 immunoreactivity from depressed subjects was unchanged compared to controls (Wilcoxon signed ranks test, $Z=-1.153$, $p=0.249$; Fig. 2). Pearson correlation analyses revealed no association between levels of GAD-67 or GAD-65 proteins and potentially confounding variables such as: age, PMI, brain pH, gender or storage time in freezer. The lack of influence of these variables on the level of GAD proteins was observed whether all subjects (MDD + controls) or each of the diagnostic groups were tested separately.

Medicated subjects with MDD vs. controls

In contrast to medication-free depressed subjects, depressed subjects that were medicated had unchanged levels of GAD-67 (Wilcoxon signed ranks test, $Z=-0.14$, $p=0.889$) and GAD-65 (Wilcoxon signed ranks test, $Z=-0.84$, $p=0.401$) proteins compared to their matched controls (Fig. 4).

The levels of GAD-67 and GAD-65 proteins in medicated MDD subjects was not significantly influenced by age, PMI, brain pH, gender or storage time in freezer. Similarly, in control subjects the level of GAD proteins was not correlated with any of these variables.

Discussion

We have found that the level of GAD-67, but not GAD-65, was significantly reduced in medication-free depressed subjects compared to their matched controls. In contrast, depressed subjects medicated with antidepressants at the time of death had unchanged levels of both GAD isoforms compared to corresponding control subjects. Together, these data raise the intriguing possibility that GAD-67 is involved in the pathophysiology of depression and antidepressants are able to normalize a GAD deficit in MDD.

GAD-67 is found abundantly in cell bodies and proximal dendrites, and GAD-65 is predominantly present in axon terminals (Kaufman *et al*. 1991). Hence, a selective decrease in GAD-67 protein suggests that

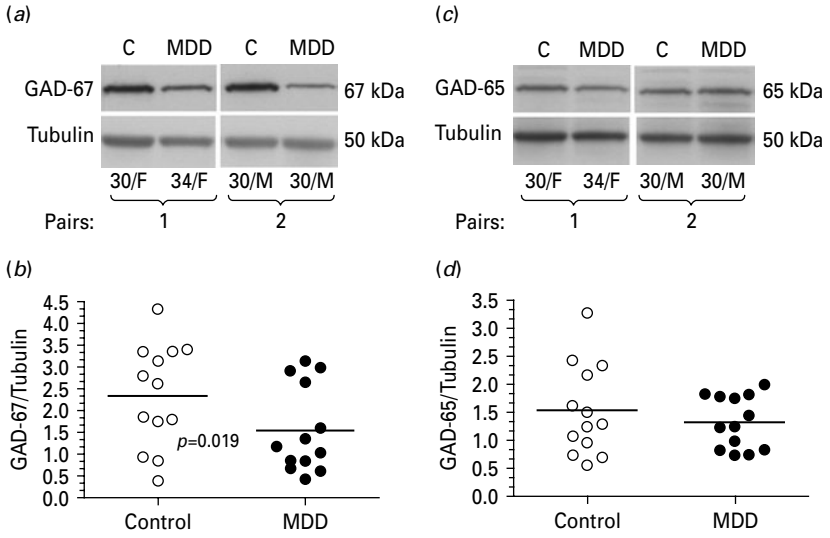

Fig. 2. Medication-free major depressive disorder (MDD) subjects *vs.* controls. Immunoblots of GAD-67 (*a*), GAD-65 (*c*) and tubulin from two representative pairs of control and medication-free MDD subjects used in the analysis; (*b*) significant decrease in GAD-67 immunoreactivity (−34%) was observed in depressed subjects ($n=13$) compared to controls ($n=13$); (*d*) amount of GAD-65 immunoreactivity from control ($n=13$) and depressed ($n=13$) subjects. Normalized optical density values for the individual subjects (circles) and mean values (horizontal lines) are presented.

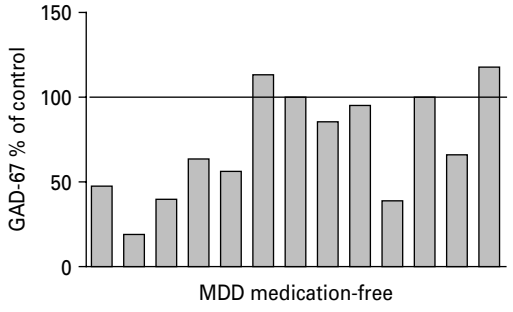

Fig. 3. GAD-67 immunoreactivity from individual medication-free major depressive disorder (MDD) subjects expressed as percentages of values from paired control subjects. Each bar is an average of duplicate comparisons.

somata and dendrites of specific populations of GABAergic neurons may be affected in depression. Low levels of GAD-67 protein immunoreactivity in depression may suggest either a loss of cell bodies and/or their dendritic processes or a decreased rate of GAD-67 protein synthesis per unchanged number of neurons. In fact, we have previously reported a selective reduction in the density of calbindin-positive GABAergic interneurons in the DLPFC (Rajkowska *et al*. 2007). Therefore, it is likely that reduced GAD-67 immunoreactivity in the DLPFC is due to a reduction in the density and size of calbindin-positive somata of GABAergic interneurons. Other post-mortem studies investigating GAD in depression found reductions in GAD-65/GAD-67 immunopositive structures in the DLPFC (Gos *et al*. 2008) and in GAD protein levels in the cerebellum (Fatemi *et al*. 2005).

A reduction in GAD-67 in post-mortem DLPFC of medication-free MDD subjects reported herein supports a recent MRI spectroscopy observation of a reduced GABA level in the PFC in unmedicated living depressed subjects (Hasler *et al*. 2007). This and other neuroimaging studies which consistently report reduced GABA levels in MDD (Sanacora *et al*. 1999, 2004) measure total amount of GABA, consisting of metabolic and neurotransmitter pools in cellular and extracellular compartments. Given that the present study demonstrates a reduction in the level of GAD-67 isoform which is predominantly involved in the synthesis of metabolic or cytosolic GABA, it could be suggested that a reduced metabolic pool of GABA significantly contributes to the GABA deficits observed by neuroimaging studies in MDD.

Our findings of a reduction in GAD-67 levels in untreated depressed subjects and lack of a reduction in depressed subjects treated with antidepressants raise the possibility that antidepressants may normalize GAD-67 levels in depression. In the present study eight depressed subjects had antidepressants (sertraline, citalopram, paroxetine, venlafaxine, amitriptyline, and nortriptyline) present in their toxicology screenings (Table 2). Among these drugs are SSRIs, and inhibitors of reuptake of both serotonin and

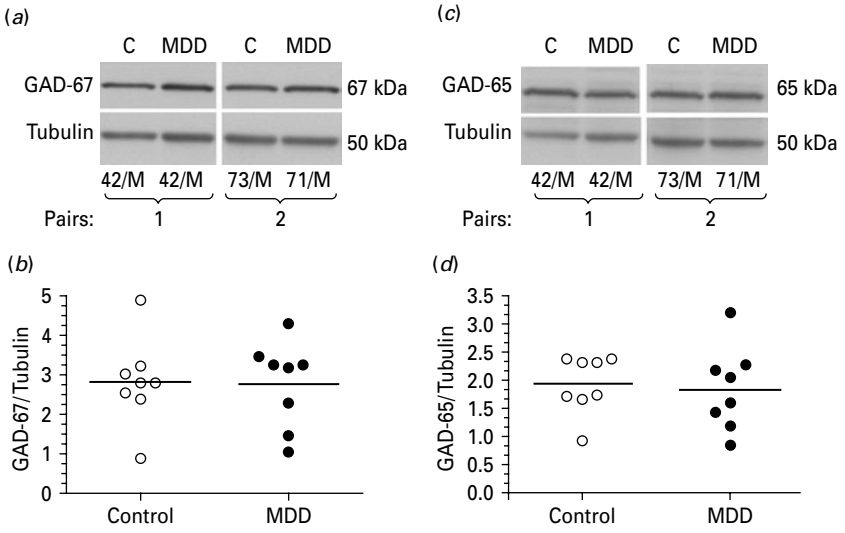

Fig. 4. Medicated major depressive disorder (MDD) subjects *vs.* controls. Immunoblots of GAD-67 (*a*), GAD-65 (*c*) and tubulin from two representative pairs of control and medicated MDD subjects used in the analysis; (*b*) amount of GAD-67 immunoreactivity from control ($n=8$) and depressed ($n=8$) subjects; (*d*) amount of GAD-65 immunoreactivity from control ($n=8$) and depressed ($n=8$) subjects. Normalized optical density values for the individual subjects (circles) and mean values (horizontal lines) are presented.

norepinephrine. Thus, the observed protective effect of antidepressants on the cortical GAD-67 level could be explained, to some extent, by the action of serotonin on GABAergic neurons.

More direct evidence for the influence of SSRIs on the GABA system is provided by Sanacora *et al*. (2002, 2003) demonstrating that chronic treatment with fluoxetine and citalopram as well as ECT results in higher levels of GABA compared to levels recorded before treatment. Moreover, acute administration of the SSRI citalopram increased total occipital GABA levels in healthy subjects (Bhagwagar *et al*. 2004) with a magnitude similar to that observed after chronic (on average 8 wk) treatment with SSRIs. The ability of serotoninergic antidepressant drugs to normalize GABA levels in depression and even increase it in healthy subjects indicates close interactions between these two systems and suggests that GABAergic mechanism may indirectly contribute to the mechanism of action of clinically active antidepressants.

The evidence exists that facilitation of GABAergic transmission produces antidepressant effects in humans (Nielsen *et al*. 1990; Petty *et al*. 1995; Smith *et al*. 1998). Interestingly, agents acting at $GABA_A$ or $GABA_B$ receptors exhibit antidepressant-like activity in animal screening procedures (Lloyd *et al*. 1983; Nowak *et al*. 2006). If there is a deficit in GABA neurotransmission in depression, then adaptive changes in the density of GABA receptors might be predicted. Studies of $GABA_A$ receptor binding in post-mortem tissues from depressed and suicide subjects have not demonstrated consistent alterations as increases, decreases or no change were found in the benzodiazepine-binding sites (Cheetham *et al*. 1988; Kugaya *et al*. 2003; Pandey *et al*. 1997; Rochet *et al*. 1992; Zhu *et al*. 2006). Moreover, gene expression studies report reduced (Merali *et al*. 2004) or up-regulated (Choudary *et al*. 2005) transcripts encoding specific subunits of the $GABA_A$ receptor in depression and suicide. Collectively, these post-mortem studies of $GABA_A$ receptors do not provide consistent evidence for GABA receptor dysregulation as a pathological marker of depression. Differences between experimental techniques and subject characteristics (e.g. medication exposure, PMI, brain tissue pH) are key factors that may be associated with discrepancies between post-mortem studies.

One of the shortcomings of this study is that GAD protein immunoreactivity was measured in post-mortem tissue homogenates as opposed to measuring GAD immunoreactivity localized to specific subpopulations of cortical GABA interneurons. However, we have previously reported that calbindin-positive GABAergic interneurons were selectively reduced in the same brain region in MDD (Rajkowska *et al*. 2007); thus it is plausible to speculate that lower GAD-67 protein levels could reflect a reduction in interneurons expressing calbindin. However, among 21 MDD

subjects examined in the present study, only seven subjects who were antidepressant-free were the same as those analysed in our previous study on reductions in GABA-calbindin immunoreactive neurons (Rajkowska *et al*. 2007). There was no significant correlation between the amount of GAD-67 and the density or size of calbindin immunoreactive neurons. This discrepancy could be explained by the fact, that GAD-67 was measured in homogenates collected from the entire extent of available BA 9 and across all six cortical layers. In contrast, calbindin immunoreactive neurons were counted only in a very narrow strip of BA 9 and only within one cortical layer (layer II).

Another possible weakness of our study is that we classify the MDD subjects as 'medication-free' based on clean post-mortem toxicology screening. However, upon examination of the medical records we can determine that nine out of these 13 'medication-free' MDD subjects did not have a prescription for antidepressants in the last month of life (Table 1). Therefore, it is likely that a majority of our 'medication-free' MDD subjects were antidepressant-naive for at least 4 wk before their death. Further studies are needed to test the influence of antidepressants on the GAD protein level in the brain.

A further limitation of the present study is the relatively small sample size ($n=8$) of 'medicated' MDD subjects. Thus, our observation on unchanged levels of GAD-67 and GAD-65 in these subjects needs to be tested on a larger number of subjects treated with antidepressants.

In summary, to our knowledge, this is the first observation of selective reduction in GAD-67 immunoreactivity in the DLPFC of antidepressant-free MDD subjects and a lack of reduction in medicated MDD subjects compared to matched controls. The present findings are consistent with studies using other approaches to implicate the GABA system in the pathophysiology of depression. Moreover, indirect evidence is provided here of a regulatory effect of antidepressants on the GABAergic system. Additional studies of GABAergic markers will lead to a better understanding of the role of GABA in the pathology of depression and may lead to the development of more effective approaches for the treatment of depressive symptoms.

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Statement of interest

Dr Rajkowska served in 2008 as a consultant to Lilly Research Laboratories, a division of Eli Lilly and Company.

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