

# Elevated levels of NR2A and PSD-95 in the lateral amygdala in depression



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## Abstract

Compelling evidence suggests that major depression is associated with dysfunction of the brain glutamatergic transmission, and that the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor plays a role in antidepressant activity. Recent post-mortem studies demonstrate that depression is associated with altered concentrations of proteins associated with NMDA receptor signalling in the brain. The present study investigated glutamate signalling proteins in the amygdala from depressed subjects, given strong evidence for amygdala pathology in depression. Lateral amygdala samples were obtained from 13–14 pairs of age- sex-, and post-mortem-interval-matched depressed and psychiatrically healthy control subjects. Concentrations of NR1 and NR2A subunits of the NMDA receptor, as well as NMDA receptor-associated proteins such as post-synaptic density protein-95 (PSD-95) and neuronal nitric oxide synthase (nNOS) were measured by Western immunoblotting. Additionally, levels of enzymes involved in glutamate metabolism, including glutamine synthetase and glutamic acid decarboxylase (GAD-67), were measured in the same amygdala samples. NR2A protein levels were markedly and significantly elevated (+115%,  $p=0.03$ ) in depressed subjects compared to controls. Interestingly, PSD-95 levels were also highly elevated (+128%,  $p=0.01$ ) in the same depressed subjects relative to controls. Amounts of NR1, nNOS, glutamine synthetase, and GAD-67 were unchanged. Increased levels of NR2A and PSD-95 suggest that glutamate signalling at the NMDA receptor in the amygdala is disrupted in depression.

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**Key words:** Amygdala, glutamate metabolizing enzymes, neuronal nitric oxide synthase, NMDA receptor, post-synaptic density protein-95.

## Introduction

A rapidly growing body of research studies provides evidence that major depressive disorder (MDD) is associated with a dysfunction of glutamate signalling. Numerous clinical studies demonstrate altered brain indices of glutamate transmission in depressed subjects. Altered brain glutamate or glutamate/glutamine levels have been observed in magnetic resonance spectroscopy (MRS) studies in depressed patients (Auer et al., 2000; Hasler et al., 2007; Michael et al., 2003a,b; Mirza et al., 2004; Pfeleiderer et al., 2003;

Sanacora et al., 2004) and in serum or plasma of medicated depressed subjects (Altamura et al., 1993; Kim et al., 1982; Mauri et al., 1998). Post-mortem brain findings demonstrate a depression-associated reduction in the expression of genes for glutamate transporters (EAAT-1, EAAT-2) and the glutamate synthesizing enzyme, glutamine synthetase, in the temporal lobe (Choudary et al., 2005). A role of glutamate in depression is also supported by numerous preclinical studies demonstrating that drugs that alter glutamate signalling have antidepressant activity in laboratory animals, and demonstration of antidepressant-like behaviour in transgenic animals lacking specific glutamate receptors (see Pittenger et al., 2007; Witkin et al., 2007 for reviews).

Of multiple receptors for glutamate, the *N*-methyl-D-aspartate (NMDA) receptor has received the most

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attention with respect to the biology of depression and its treatment (Kugaya and Sanacora, 2005; Pittenger et al., 2007). The NMDA receptor is a heterooligomeric structure composed of the NR1 subunit, NR2(A–D) subunits, and less common NR3(A, B) subunits. NMDA receptors are embedded in the post-synaptic density (PSD) of the post-synaptic membrane where they are anchored by PSD-95 and coupled to cytoplasmic signalling molecules, including neuronal nitric oxide synthase (nNOS; Kornau et al., 1995; Sheng and Kim, 2002). Activation of NMDA receptors results in  $\text{Ca}^{2+}$  entry and subsequent activation of nNOS. Human post-mortem studies provide evidence that radioligand binding to the NMDA receptor is altered in cerebral cortex from suicide victims and from depressed subjects (Beneyto et al., 2007; Nowak et al., 1995; Nudmamud-Thanoi and Reynolds, 2004). In addition, low levels of nNOS and elevated levels of the NR2C subunit of the NMDA receptor have been reported in the noradrenergic locus coeruleus in MDD (Karolewicz et al., 2004, 2005b). Interestingly, several antagonists of NMDA receptor activation demonstrate antidepressant activity in behavioural models in laboratory animals (Kugaya and Sanacora, 2005). Of particular relevance is the demonstration that ketamine, a NMDA receptor antagonist, exhibits rapid and sustained antidepressant activity in humans (Berman et al., 2000; Zarate et al., 2006). Together, these findings demonstrate compelling evidence that glutamate signalling through the NMDA receptor is disrupted in depression.

In the present study, levels of several proteins involved in glutamate signalling through the NMDA receptor were measured in brain tissue from subjects who had clinically defined depressed mood at the time of death, and psychiatrically normal control subjects. The NMDA receptor signalling proteins chosen for study were relevant subunits of the NMDA receptor (NR1 and NR2A), a major anchoring and scaffolding protein associated with the receptor (PSD-95), and an intracellular signalling protein (nNOS) linked to the receptor. In addition two major glutamate metabolic enzymes glutamine synthetase (GS) and glutamic acid decarboxylase (GAD-67) were investigated. The area of the brain chosen for study was the lateral nucleus of amygdala. The amygdala has long been implicated in the pathobiology of depressive disorders and suicide (Bowley et al., 2002; Cheetham et al., 1989; Drevets et al., 2002; Klimek et al., 2002; Sheline et al., 1998). Glutamatergic inputs from the prefrontal cortex and thalamus enter amygdala at the lateral level (Carmichael and Price, 1995; McDonald, 1998), making this amygdala subregion a key brain region in

receiving and processing cortical and thalamic information transmitted via glutamatergic transmission. The NR1 and NR2A subunits were specifically chosen for study because the NMDA receptor in the mature lateral amygdala is largely composed of these two subunits (Lopez de Armentia and Sah, 2003; Radley et al., 2007).

## Materials and methods

### *Psychiatric autopsy and tissue collection*

Post-mortem brain samples were collected at autopsy from a total of 28 subjects at the Cuyahoga County Coroner's Office in Cleveland, OH, USA. Informed written consent was obtained 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 Case Western Reserve University and the University of Mississippi Medical Center. A trained interviewer administered the Schedule for Affective Disorders and Schizophrenia: lifetime version (SADS-L) and/or the Structured Clinical Interview for DSM-IV Psychiatric Disorders (SCID-IV) to knowledgeable next-of-kin of subjects in the study.

A total of 14 subjects were studied that had the common feature of depressed mood: 11 subjects met clinical criteria for MDD and three subjects met criteria for adjustment disorder with depressed mood based on DSM-IV-R criteria. One MDD subject had a co-existing diagnosis of dysthymic disorder, and one MDD subject had a diagnosis of alcohol abuse (Table 1).

Fourteen subjects did not meet criteria for Axis I disorder (termed psychiatrically healthy controls) except for nicotine dependence as noted (see Table 1). Among 14 depressed individuals, 12 were suicide victims, and two died due to the other causes such as a homicide or pulmonary embolism (Table 1). Blood and urine samples from all subjects were examined by the coroner's office for psychotropic medications and substances of abuse, including ethanol (Table 1). No antidepressant drugs were detected in the post-mortem toxicology screening of subjects in the present study (Table 1). Fourteen depressed subjects and 14 psychiatrically healthy controls were arranged into 14 pairs, matched as closely as possible for age, gender, post-mortem interval (PMI), tissue pH and smoking history. As specified above, one MDD subject had a diagnosis of alcohol abuse; this subject was matched with an alcohol-dependent control subject with no other psychiatric disorders.

**Table 1.** Demographic characteristics of subjects

Parameter	Controls ( <i>n</i> = 14)	Depressed ( <i>n</i> = 14)
Age (yr) (mean ± S.E.M.)	53 ± 4	54 ± 4
Age range	27–74	30–73
PMI (h) (mean ± S.E.M.)	18 ± 1	19 ± 2
PMI range	6–26	5–33
PH (mean ± S.E.M.)	6.68 ± 0.07	6.55 ± 0.07
PH range	5.99–6.96	6.15–6.91
Time in freezer ( <i>T<sub>F</sub></i> ) (months) (mean ± S.E.M.)	121 ± 5	132 ± 8
<i>T<sub>F</sub></i> range	81–158	61–181
Gender (F/M)	1/13	1/13
Medication history <sup>a</sup>	0/14	2/14 (sertraline)
Toxicology	Clean ( <i>n</i> = 7) Drugs detected ( <i>n</i> = 5) (brompheniramine, lidocaine, diazepam, chlorpheniramine) Ethanol ( <i>n</i> = 1) CO ( <i>n</i> = 1)	Clean ( <i>n</i> = 8) Drugs detected ( <i>n</i> = 3) (codeine, phenobarbital, phenytoin) Ethanol ( <i>n</i> = 1) CO ( <i>n</i> = 2)
Cause of death	Heart disease ( <i>n</i> = 9) Hypertension ( <i>n</i> = 1)  Aneurysm ( <i>n</i> = 1) Accidental CO poisoning ( <i>n</i> = 1) Homicide ( <i>n</i> = 1) Thrombophlebitis ( <i>n</i> = 1)	Suicide ( <i>n</i> = 12) (shotgun, <i>n</i> = 5; CO poisoning, <i>n</i> = 3; hanging, <i>n</i> = 2; drowning, <i>n</i> = 1; slit wrists, <i>n</i> = 1) Other causes ( <i>n</i> = 2) (pulmonary embolism, homicide)
Diagnosis	Alcohol dependence ( <i>n</i> = 1) Phobia (heights) ( <i>n</i> = 1)	MDD ( <i>n</i> = 9) MDD and dysthymic disorder ( <i>n</i> = 1) MDD and alcohol abuse ( <i>n</i> = 1) Adjustment disorder with depressed mood ( <i>n</i> = 3)
Smoking	Smokers ( <i>n</i> = 6) History of smoking ( <i>n</i> = 3)	Smokers ( <i>n</i> = 6) History of smoking ( <i>n</i> = 3)

PMI, Post-mortem interval; CO, carbon monoxide; MDD, major depressive disorder.

<sup>a</sup> Treatment with antidepressants within 4 wk of time of death. The average ages, PMI, pH and *T<sub>F</sub>* of depressed and control subjects were not statistically different.

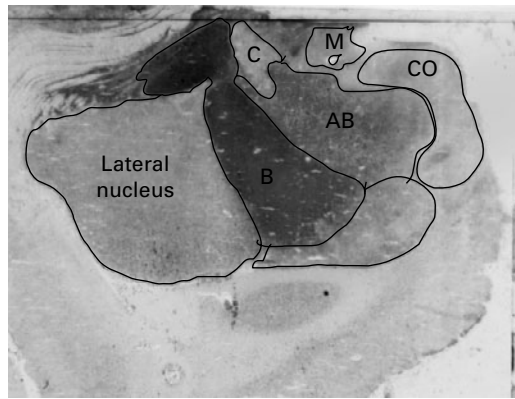
### Dissection and anatomical positioning of measurements

Tissue was collected from frozen blocks containing the right amygdaloid complex. Frozen blocks were cut into 50- $\mu$ m-thick sections for collecting tissue for Western blotting and 20- $\mu$ m-thick sections for histology. The 20- $\mu$ m-thick tissue sections collected at 1-mm intervals along the anterior–posterior extent of the amygdala were processed for acetylcholinesterase. Acetylcholinesterase histology (as described previously by Biegón and Wolff, 1986 and Karolewicz et al., 2005a) was used to create anatomical templates for anatomical positioning and as a guide for the dissection of the lateral nucleus. For all subjects, the anatomical position for collection of tissue punches of

the lateral amygdala for Western blotting was established approximately midway along the anterior–posterior extent of the amygdala (Figure 1). Tissue punches (diameter 5 mm) containing the lateral nucleus were collected in centrifuge tubes and stored at  $-80^{\circ}\text{C}$  until assayed.

### Western blotting

Tissue samples were prepared as published previously (Karolewicz et al., 2005b). Samples were homogenized in ice-cold TE buffer (10 mM Tris–HCl and 1 mM ethylene-diaminetetraacetate; EDTA) containing protease inhibitors (Protease Inhibitor Cocktail Tablets – Complete™, Boehringer Mannheim GmbH, Mannheim, Germany). Total protein concentrations



**Figure 1.** Digital image of a coronal section of the amygdala stained histochemically for acetylcholinesterase. The acetylcholinesterase staining preparation was used to visualize the lateral amygdala and to distinguish it from neighbouring nuclei: basal (B), accessory basal (AB), central (C), medial (M), cortical (CO).

for all samples were determined using the bicinchoninic acid (BCA) method (Pierce Biotechnology Inc., Rockford, IL, USA). Samples were mixed with sample buffer [125 mM Tris base, 20% glycerol, 4% SDS, 10% mercaptoethanol, 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, Hercules, CA, USA) and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Piscataway, NJ, USA). Membrane 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<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)] for 2 h, then incubated (overnight at 4 °C) with primary antibody (see Antibodies section below). Membranes were washed twice for 15 min in TBS (or PBST) buffer and incubated with secondary anti-mouse antibody for NR1, PSD-95, nNOS, GS, actin (diluted 1:2000; Amersham Biosciences, no. NA931) and anti-rabbit secondary antibody for NR2A and β-tubulin (diluted 1:5000; Amersham Biosciences, no. NA934). After incubation, blots were washed three times for 15 min and developed using enhanced chemiluminescence detection (ECL; PerkinElmer Life Sciences Inc., Boston, MA, USA) and immediately exposed to film (Hyperfilm ECL, Amersham Biosciences).

#### Antibodies

All antibodies used in this study were commercially available and have been used previously to detect NR1, NR2A, PSD-95, nNOS, GS, and GAD-67 in

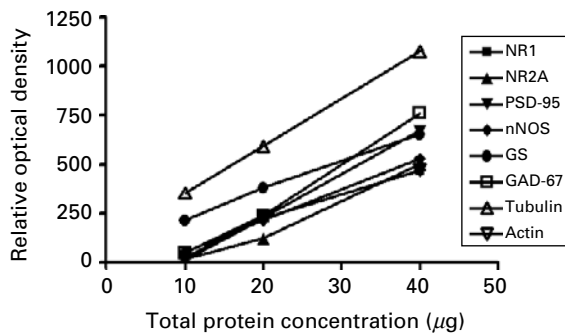
numerous regions of the human and rat brain as well as in-vitro studies (Gerlach et al., 2001; Karolewicz et al., 2004, 2005b, 2008; Kronfeld et al., 2000; Kristiansen et al., 2006; Ling et al., 2005). Mouse monoclonal antibodies were used to label NR1 subunit (1:1000; BD Biosciences/Pharmingen, San Diego, CA, USA; no. 556308), PSD-95 (1:1000; Upstate Biotechnology, Lake Placid, NY, USA; no. 05-494), nNOS (1:1000; BD Biosciences/Transduction Laboratories, Franklin Lakes, NJ, USA; no. 610309), GS (1:1000; BD Biosciences/Transduction Laboratories; no. 610518), and GAD-67 (1:1000; Chemicon, Temecula, CA, USA, no. MAB5406). NR2A subunit was labelled using rabbit polyclonal antibodies diluted 1:500 (Novus Biologicals, Littleton, CO, USA; no. NB 300-105). As a control for transfer and loading, actin or β-tubulin was detected on blots using an anti-actin (Chemicon, no. MAB1501) or anti-tubulin (Abcam Inc., Cambridge, MA, USA; no. ab6046) antibody.

#### Experimental design and data analysis

Immunoreactivities of glutamate-related proteins were investigated in 13–14 depressed subjects and 13–14 matched normal control subjects. Pairs of subjects were immunoblotted on the same gel (maximum four pairs) with duplicates on separate gels. In order to minimize inter-blot variability and to aid in quantifying blots, each gel was loaded with three concentrations of a cortical tissue standard consisting of 10, 20, and 40 µg total protein. The tissue standard was dissected from prefrontal cortex (area 10) of one control subject and the same cortical tissue standard was used for all experimental gels. Band densities were analysed using MCID Elite 7.0 (Imaging Research, St Catherine's, ON, Canada). Linear regression (GraphPad Prism 4.0; GraphPad Software Inc., San Diego, CA, USA) was used to plot a standard curve for each gel, from which relative optical density (ROD) values of samples were converted to cortical standard protein units for each experimental sample for each gel. In order to control for accuracy of tissue loading and efficiency of transfer, data were normalized to actin or tubulin detected on the same blots. The final data are expressed in cortical standard protein units and presented as a ratio of (protein of interest)/(actin or tubulin).

#### Statistical analyses

Data were analysed statistically using a two-tailed paired Student's *t* test (GraphPad Prism 4.0). Linear regression analysis was performed to test for associations between age, pH, PMI, time in freezer ( $T_F$ ) and



**Figure 2.** Relationship between the optical density values of Western-blotted protein immunoreactivities and total protein concentration for NR1, NR2A, PSD-95, nNOS, GS, GAD-67, actin, and tubulin. Wells were loaded with three concentrations of cortical standard (dissected from a healthy control subject) consisting of 10, 20, and 40  $\mu\text{g}$  total protein.

each protein investigated. If a significant correlation between protein immunoreactivities and ones of these variables was observed ( $p < 0.05$ ), analysis of covariance (ANCOVA) was used to correct the test for the effect of diagnosis on the protein expression. Summary statistics are reported as the mean  $\pm$  S.E.M. A  $p$  value  $< 0.05$  was considered significant.

## Results

### Relationship between optical density and total protein concentration

In order to determine the relationship between optical density values and the concentrations of protein immunoreactivities, 10, 20, and 40  $\mu\text{g}$  total cortical protein (standard) was immunoblotted with all antibodies used in this study. Analyses of blots revealed a linear relationship between optical density values and protein concentrations (Figure 2). All measurements of control and depressed samples fell within the linear range of these standard plots.

### NMDA receptor subunits

For NMDA receptor subunits, immunoreactive bands corresponding to molecular masses of 120 kDa and 180 kDa for NR1 and NR2A, respectively, were observed in homogenates of lateral amygdala (Figure 3a). Averaged NR1 immunoreactivities from depressive ( $1.22 \pm 0.15$ ) and control subjects ( $1.36 \pm 0.20$ ) were not statistically different ( $t = 1.1$ , d.f. = 13,  $p = 0.28$ ). There were no significant correlations between amounts of NR1 and pH, PMI, age or  $T_F$ . The amount of NR2A immunoreactivity from depressed subjects ( $1.38 \pm 0.36$ ) was significantly higher (+115%) than that from

control subjects ( $0.64 \pm 0.11$ ,  $t = 2.4$ , d.f. = 12,  $p = 0.03$ ). A significant negative correlation was found between  $T_F$  and amounts of NR2A immunoreactivity in depressed subjects ( $r^2 = 0.48$ ,  $p = 0.008$ ). Using ANCOVA with  $T_F$  as a covariate for NR2A, an effect of diagnosis on this subunit was still detected ( $p = 0.011$ ).

### NMDA receptor-interacting proteins

PSD-95 and nNOS immunoreactivities appeared as bands with molecular masses of 95 kDa and 160 kDa, respectively (Figure 3b). Averaged PSD-95 immunoreactivities from depressive ( $1.49 \pm 0.26$ ) and control subjects ( $0.65 \pm 0.12$ ) were statistically different ( $+128\%$ ,  $t = 2.9$ , d.f. = 13,  $p = 0.01$ ; Figure 4). Linear regression analysis showed negative correlations between PMI and amounts of PSD-95 immunoreactivity in controls ( $r^2 = 0.32$ ,  $p = 0.04$ ) and between  $T_F$  and PSD-95 immunoreactivity in depressed subjects ( $r^2 = 0.32$ ,  $p = 0.03$ ). Nevertheless, using ANCOVA with PMI and  $T_F$  as a covariates, a significant effect of depression for PSD-95 was still detected ( $p = 0.002$ ).

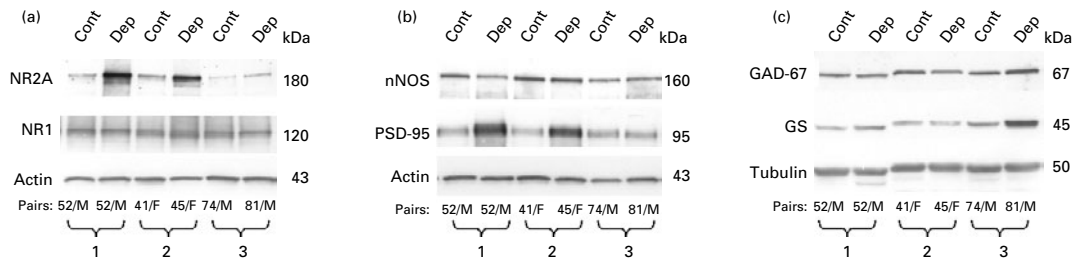
Amounts of nNOS immunoreactivity in depressives ( $1.75 \pm 0.16$ ) and control subjects ( $1.89 \pm 0.16$ ) were not significantly different ( $t = 0.68$ , d.f. = 13,  $p = 0.5$ ). There was a significant negative correlation between PMI and amounts of nNOS immunoreactivity in depressed subjects ( $r^2 = 0.45$ ,  $p = 0.008$ ). Using ANCOVA with pH as a covariate for nNOS did not change the statistical outcome of the analysis ( $p = 0.998$ ).

### Glutamate metabolizing enzymes

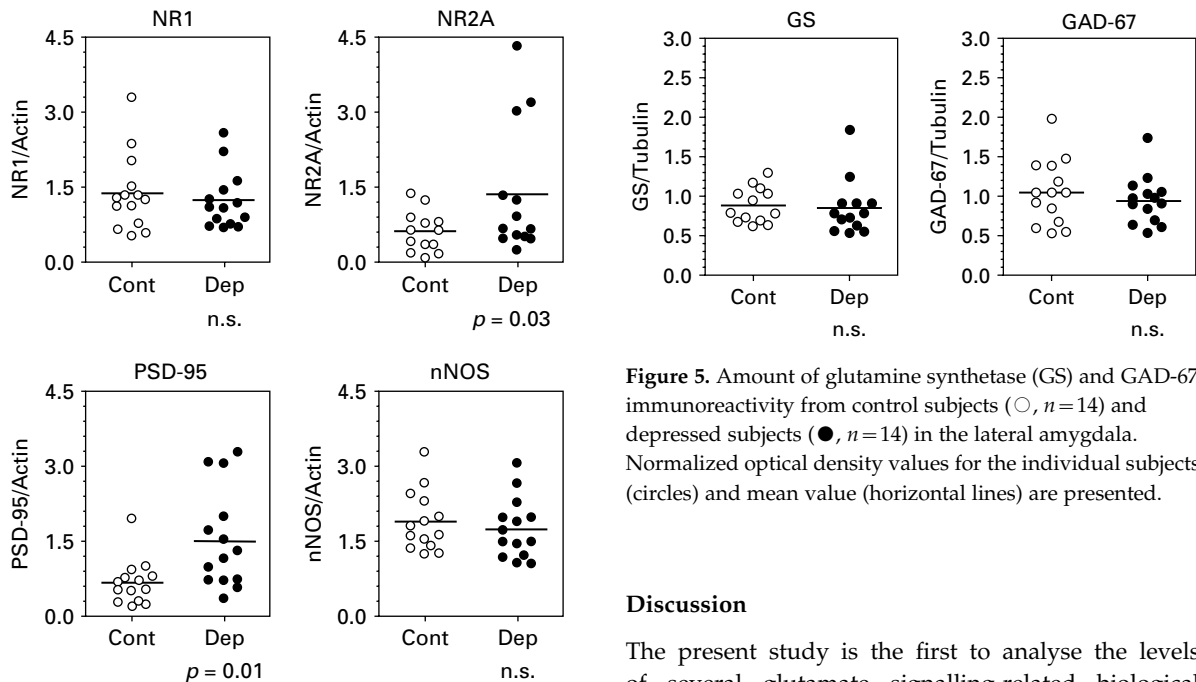
GS immunoreactivity appeared as a band at 45 kDa (Figure 3c) and amounts of GS immunoreactivity were unchanged in depressed ( $0.84 \pm 0.09$ ) compared to control subjects ( $0.89 \pm 0.06$ ,  $t = 0.43$ , d.f. = 13,  $p = 0.67$ ; Figure 5). There was a significant positive correlation between pH and amounts of GS immunoreactivity in control subjects ( $r^2 = 0.36$ ,  $p = 0.02$ ). Using ANCOVA with pH as a covariate for GS, a main effect for diagnosis on GS was not detected ( $p = 0.98$ ). GAD-67 was revealed as a single band at 67 kDa (Figure 3c). Average amounts of GAD-67 immunoreactivity in control subjects ( $1.05 \pm 0.11$ ) and in depressed subjects ( $0.95 \pm 0.08$ ) were not statistically different ( $t = 0.89$ , d.f. = 13,  $p = 0.38$ ; Figure 5). There were no significant correlations between amounts of GAD-67 and pH, age, PMI or  $T_F$ .

### Correlations of immunoreactivities

The possibility that amounts of certain proteins in the NMDA receptor signalling cascade would correlate

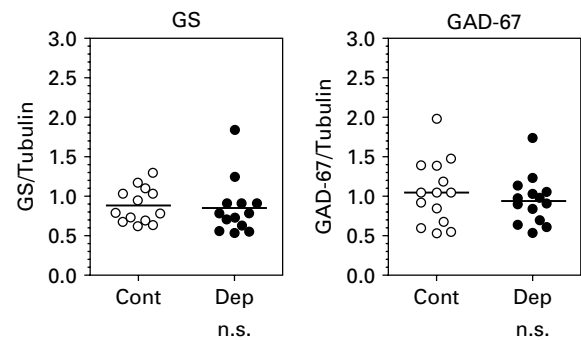


**Figure 3.** Immunoblots of (a) NR1, NR2A, and actin; (b) nNOS, PSD-95, and actin and (c) GS, GAD-67, and tubulin from three representative pairs of controls (Cont) and depressed subjects (Dep) used in the analysis. Each well was loaded with 20  $\mu$ g total protein.



**Figure 4.** Amounts of NR1, NR2A, PSD-95, and nNOS immunoreactivities in the lateral amygdala from control subjects ( $\circ$ ,  $n = 13-14$ ) and depressed subjects ( $\bullet$ ,  $n = 13-14$ ). Significant increases in the NR2A and PSD-95 immunoreactivities (+115% and +128%, respectively) were observed in depressed subjects as compared to controls. Normalized optical density values for the individual subjects (circles) and mean values (horizontal lines) are presented.

was investigated. Amounts of NR2A immunoreactivity were highly positively correlated with PSD-95 immunoreactivity only in depressed subjects ( $r^2 = 0.79$ ,  $p < 0.0001$ ). There were no significant correlations between NR2A and nNOS or PSD-95 and nNOS in both control and depressed subjects (data not shown).



**Figure 5.** Amount of glutamine synthetase (GS) and GAD-67 immunoreactivity from control subjects ( $\circ$ ,  $n = 14$ ) and depressed subjects ( $\bullet$ ,  $n = 14$ ) in the lateral amygdala. Normalized optical density values for the individual subjects (circles) and mean value (horizontal lines) are presented.

## Discussion

The present study is the first to analyse the levels of several glutamate signalling-related biological markers in post-mortem amygdala from depressed subjects. Evidence is provided that amounts of the NR2A subunit and its anchoring protein, PSD-95, are robustly elevated in the lateral amygdala of depressed subjects compared to psychiatrically healthy control subjects. Increased expression of NR2 subunits of the NMDA receptor may be a common pathology in depression since higher levels of the NR2C subunit were previously reported in the noradrenergic locus coeruleus in depressed subjects (Karolewicz et al., 2005b). Interestingly, recent research points to clinical efficacy of NMDA receptor antagonism in depression, i.e. recent clinical studies have shown that a single intravenous dose of ketamine, an NMDA receptor antagonist, produces robust, rapid, and relatively sustained antidepressant effects in treatment-resistant depression (Berman et al., 2000; Zarate et al., 2006).

Preclinical research also suggests that inhibition of NMDA receptor, as well as inhibition of intracellular signalling cascades including nNOS that are linked to the NMDA receptor, exhibit antidepressant-like effects in animal screening procedures (Paul, 2001; Paul and Skolnick, 2003). Interestingly, NR2A knockout mice show antidepressant-like profiles in animal models, as compared to wild-type controls (Boyce-Rustay and Holmes, 2006). Moreover, a selective NR2B antagonist Ro25-6981 exhibits antidepressant-like properties in mice (Maeng et al., 2007). A preliminary clinical study has demonstrated the benefit of supplementation with zinc, a non-selective NMDA receptor antagonist, in antidepressant therapy (Nowak et al., 2003). Collectively, current research suggests that the NMDA receptor complex is abnormal in depression and regulation of NMDA receptor signalling in critical brain circuits may represent a strategy for the development of effective and rapid-acting antidepressants.

The abnormalities at the excitatory synapse, as revealed by our post-mortem research, may occur in response to abnormal levels of glutamate in depression. Several lines of evidence indicate altered glutamate or glutamate/glutamine levels in multiple brain regions in depressed patients (Auer et al., 2000; Hasler et al., 2007; Michael et al., 2003a,b; Mirza et al., 2004; Pfeleiderer et al., 2003; Sanacora et al., 2004). It has been well established that glia play a key role in glutamate neurotransmission, including glutamate metabolism and recycling, therefore depression-associated reductions in the density of these cells in prefrontal cortex (Miguel-Hidalgo et al., 2000; Ongur et al., 1998; Rajkowska et al., 1999; Si et al., 2004) and amygdala (Bowley et al., 2002; Hamidi et al., 2004) may also contribute to altered synaptic glutamate in depression. GS gene expression has been shown to be reduced in the prefrontal cortex in depression (Choudary et al., 2005), which could also contribute to altered glutamate levels and compensatory changes in glutamate receptors. Since GS is exclusively expressed in astrocytes and a reduced density of astrocytes has been consistently reported in depression (see above); we hypothesized that GS protein immunoreactivity would be reduced in the amygdala. However, the present findings demonstrate unaltered levels of GS in the lateral amygdala in depression. It is conceivable that unchanged levels of GS may indicate (1) unchanged density of astrocytes in the amygdala in depression, (2) a compensatory increase in GS protein expression in remaining astrocytes, or (3) GS expression may be turned on in cells other than astrocytes during depression. One other study has also reported

unchanged GS immunoreactivity in the prefrontal cortex in depression (Toro et al., 2006).

Glutamate is an immediate precursor for GABA, and reductions in GABA levels are consistently reported in neuroimaging studies in depression (Hasler et al., 2007; Sanacora et al., 1999, 2004). In addition, post-mortem analyses demonstrate a significant reduction in the density and size of GABAergic interneurons immunoreactive for calbindin in the dorso-lateral prefrontal cortex in depression (Rajkowska et al., 2007). Reduced levels of GAD-67 and GAD-65 were previously reported in cerebellum in MDD subjects (Fatemi et al., 2005). We expected to find lower GAD-67 protein levels in depression in the present study. However, GAD-67 levels in the lateral amygdala were unchanged in depressed subjects. Further studies aimed to investigate protein levels and/or activity of GAD-65, another isoforms of GAD (Bu et al., 1992) are needed to fully address the status of GABA synthesis in the amygdala in depression.

The NMDA receptor in the mature lateral amygdala is largely composed of the NR1 subunit, a mandatory subunit, and the regulatory NR2A subunit (Lopez de Armentia and Sah, 2003; Radley et al., 2007). To date, few studies have investigated NMDA receptors in post-mortem brain from depressed subjects. In this study, NR1 subunit immunoreactivity was unchanged in the amygdala in depression, despite a significant elevation of NR2A subunit immunoreactivity. Unchanged levels of NR1 subunit in amygdala in depression is consistent with our previous observations in the locus coeruleus and cerebellum of depressed subjects (Karolewicz et al., 2005b) and no changes in NR1 immunoreactivity in subregions of the hippocampal dentate gyrus and in the orbitofrontal cortex (Toro and Deakin, 2005).

Only two previous investigations have measured PSD-95 in brain tissues from depressed subjects. Normal levels in depressed subjects were reported for PSD-95 mRNA in the striatum (Kristiansen and Meador-Woodruff, 2005) and for PSD-95 protein in the hippocampus and orbitofrontal cortex (Toro and Deakin, 2005). In the lateral amygdala, we found markedly elevated levels of PSD-95 in depressives as compared to matched control subjects. These findings suggest that PSD-95 involvement in depression is neuroanatomically limited. PSD-95 belongs to the large family of membrane-associated guanylate kinases (MAGUKs) and is exclusively localized to dendritic spines at glutamatergic synapses (Hunt et al., 1996). PSD-95 binds to the NMDA receptor subunits NR2A and NR2B (Kornau et al., 1995) and also couples synaptic NMDA receptors to downstream signalling

molecules such as nNOS (Brenman et al., 1996; Sattler et al., 1999) which serves as an intracellular mediator of NMDA receptor activation. MAGUKs appear to play a fundamental role in the transport, anchoring, and signalling of glutamate NMDA receptors. In the present study, the increase in PSD-95 immunoreactivity in depressives was highly correlated with an increase in NR2A subunit immunoreactivity. Hence, the depression-associated elevation in levels of PSD-95 imply increased intracellular trafficking, synaptic delivery, and coupling of the NMDA receptors to intracellular signalling cascades in the amygdala. The lateral amygdala receives its major glutamatergic afferents from the cortical areas and from the thalamus (Mahanty and Sah, 1999). These inputs activate synapses containing both NMDA and non-NMDA receptors. Depression-associated perturbations in markers of glutamate transmission in cortical regions, as revealed by imaging and post-mortem cell counting studies (Hasler et al., 2007; Rajkowska et al., 1999, 2005; Sanacora et al., 2004; Si et al., 2004) could result in abnormalities of glutamatergic signalling in cortical projection areas, including the lateral amygdala. Recent imaging studies in depressed humans point to reduced prefrontal cortical function that is linked to increased amygdala activity (Drevets, 1999; Siegle et al., 2007). Elevated levels of NR2A/PSD-95 may reflect an adaptive response to altered cortical input to the amygdala and may contribute to altered amygdala activity in depression.

A shortcoming of the present study is that NMDA receptor subunits were measured as a total pool of proteins that included intracellular pools of un-assembled proteins and assembled NMDA receptors expressed at the cell membrane, complicating the prediction of functional significance. However, it has been proposed that the NR1 subunit, which is synthesized in great excess (~10-fold), accounts for most of the intracellular pool (Huh and Wenthold, 1999). The excess production of NR1 increases the chance that NR2 subunits will find NR1 partners. In contrast to NR1, only a small percentage of NR2 subunit protein is intracellular (Huh and Wenthold, 1999); almost all NR2 subunits are expressed on the cell surface. Hence, the total number of functional NMDA receptors appears to be controlled by expression of the NR2 subunits (Prybylowski et al., 2002). Based on this, the elevation of NR2A immunoreactivity in homogenates of lateral amygdala from depressives is likely to reflect an increase of functional surface NMDA receptors. Of course, a depression-associated disruption in the normal cellular trafficking of NMDA receptor subunits cannot be ruled out with the present data.

Treatment of depression may contribute to the adaptive changes seen in the human post-mortem brain. This study includes only two subjects with a reported history of medication with antidepressants at some point during their lifetime. In both cases, the selective serotonin reuptake inhibitor, sertraline, was prescribed. However, the presence of this drug was not revealed in the post-mortem toxicology screening and both subjects died as a result of suicide (Table 1). One of these two subjects had PSD-95 and NR2A levels unchanged compared to their matched control, while the other subject had levels higher than that of the matched control subject and comparable to the average level of immunoreactivity of depressive subjects lacking the drug exposure. It can be inferred from these observations that the past antidepressant drug treatment is unlikely to contribute to the elevation of PSD-95 or NR2A levels observed in the depressed subjects.

In summary, a marked elevation of NR2A subunit and PSD-95 immunoreactivities in the lateral amygdala of depressed subjects compared to control subjects were observed. A strong correlation between levels of NR2A subunit and PSD-95 immunoreactivities were observed in depressed subjects. Changes in NR2A and its anchoring protein in depression may reflect an adaptive response to a decreased excitatory input from cortical regions. Further studies of the glutamatergic signalling markers in depression involving non-NMDA ionotropic receptors, metabotropic receptors, and glutamate transporters will lead to a better understanding of the role of glutamate in the pathology of depression.

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#### Statement of Interest

None.



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