Elevated Levels of the NR2C Subunit of the NMDA Receptor in the Locus Coeruleus in Depression

Beata Karolewicz[1], Craig A Stockmeier[1,3] and Gregory A Ordway*[,1,2]

[1]Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, MS, USA; [2]Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, MS, USA; [3]Department of Psychiatry, Case Western Reserve University, Cleveland, OH, USA

Low levels of the intracellular mediator of glutamate receptor activation, neuronal nitric oxide synthase (nNOS) were previously observed in locus coeruleus (LC) from subjects diagnosed with major depression. This finding implicates abnormalities in glutamate signaling in depression. Receptors responding to glutamate in the LC include ionotropic N-methyl-D-aspartate receptors (NMDARs). The functional NMDAR is a hetero-oligomeric structure composed of NR1 and NR2 (A–D) subunits. Tissue containing the LC and a nonlimbic LC projection area (cerebellum) was obtained from 13 and 9 matched pairs, respectively, of depressed subjects and control subjects lacking major psychiatric diagnoses. NMDAR subunit composition in the LC was evaluated in a psychiatrically normal subject. NR1 and NR2C subunit immunoreactivities in LC homogenates showed prominent bands at 120 and 135 kDa, respectively. In contrast to NR1 and NR2C, very weak immunoreactivity of NR2A and NR2B subunits was observed in the LC. Possible changes in concentrations of NR1 and NR2C that might occur in depression were assessed in the LC and cerebellum. The overall amount of NR1 immunoreactivity was normal in the LC and cerebellum in depressed subjects. Amounts of NR2C protein were significantly higher ($+61\%$, $p = 0.003$) in the LC and modestly, but not significantly, elevated in the cerebellum ($+35\%$) of depressives as compared to matched controls. Higher levels of NR2C subunit implicate altered glutamatergic input to the LC in depressive disorders.
Neuropsychopharmacology (2005) **30,** 1557–1567, advance online publication, 25 May 2005; doi:10.1038/sj.npp.1300781

Keywords: depression; locus coeruleus; cerebellum; glutamate; NMDA receptor

INTRODUCTION

The locus coeruleus (LC) is the largest noradrenergic nucleus in the brain and projects to several cortical and subcortical areas. Previous observations reveal that depression is associated with altered concentrations of several noradrenergic proteins in the LC. For example, elevated levels of tyrosine hydroxylase (TH) (Ordway *et al*, 1994a; Zhu *et al*, 1999), increased agonist binding to α_2-adrenergic receptors (Ordway *et al*, 1994b, 2003), and reduced levels of norepinephrine transporters (Klimek *et al*, 1997) were previously reported in the LC from major depression and in suicide victims. Interestingly, depletion of norepinephrine or repeated stress in rats can increase TH expression, increase binding to α_2-adrenergic receptors, and/or decrease binding to the norepinephrine transporter (Cubells *et al*,

1995; Lee *et al*, 1983; Melia *et al*, 1992; Torda *et al*, 1985; U'Prichard *et al*, 1979; Wang *et al*, 1998; Zafar *et al*, 1997). Together, these findings are highly suggestive of dysfunctional noradrenergic neurotransmission in depression, possibly through stress-induced activation and ultimate depletion of norepinephrine.

Stress-sensitive inputs to the LC include glutamate. Glutamate input to the LC originates predominantly in the glutamatergic paragigantocellularis nucleus (Aston-Jones *et al*, 1986, 1991), and also from the cerebral cortex (Jodo and Aston-Jones, 1997; Jodo *et al*, 1998). Glutamate receptors that modulate LC activity include the N-methyl-D-aspartate receptor (NMDAR). The NMDAR is a hetero-oligomeric structure composed of the NR1 subunit, NR2 (A–D) subunits, and a less common NR3 subunit. The NR1 subunit is expressed abundantly throughout the brain, while the NR2 subunits vary in their distribution in the central nervous system (for a review see Loftis and Janowsky, 2003). Stimulation of NMDARs results in, at least in part, the activation of nNOS. The LC region contains NMDAR (Allgaier *et al*, 2001; Shaw *et al*, 1992; Van Bockstaele and Colago, 1996), neuronal nitric oxide synthase (nNOS)-positive neurons (Cuellar *et al*, 2000; Karolewicz *et al*, 2004; Xu *et al*, 1994), and guanosine 3′,5′ cyclic monophosphate

*Correspondence: Dr GA Ordway, Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA, Tel: +1 601 984 5893, Fax: +1 601 984 5899, E-mail: gordway@psychiatry.umsmed.edu

(cGMP) (Vulliemoz *et al*, 1999; Xu *et al*, 1998), suggesting the existence of a glutamate/nitrergic transduction pathway. The exact cellular localization of these glutamate/nitrergic signaling proteins within the human LC is still unknown. However, immunohistochemical labeling of nNOS, an intracellular mediator of glutamate-NMDAR signaling, revealed localization of nNOS in neurons and glial cells in the region of the LC of a psychiatrically normal subject (Karolewicz *et al*, 2004).

Interestingly, several lines of evidence suggest a crucial involvement of glutamate signaling in the pathophysiology of depression and in the mechanism of action of antidepressant drugs. Compounds that decrease glutamatergic transmission via blockade of ionotropic NMDA or group I metabotropic glutamate receptors produce antidepressant-like effect in animal screening procedures (Layer *et al*, 1995; Moryl *et al*, 1993; Papp and Moryl, 1994; Skolnick, 1999; Tatarczynska *et al*, 2001). Moreover, recent studies have revealed that agonists of group III metabotropic glutamate receptors, known to inhibit glutamate release, exhibit antidepressant-like activity in animals (Palucha *et al*, 2004; Tatarczynska *et al*, 2002). Furthermore, ketamine, an NMDAR antagonist, exhibits antidepressant activity in humans (Berman *et al*, 2000).

Hence, converging evidence from laboratory and clinical studies provide the basis for the hypothesis that glutamatergic input to the LC may be altered in depression, leading to compensatory changes in LC proteins. Recently, a low amount of nNOS was reported in subjects diagnosed with major depression (Karolewicz *et al*, 2004). The aim of the present study was to (1) examine the composition of the NMDAR in the human LC, and (2) investigate potential abnormalities in the concentrations of NMDAR subunits in the LC that might occur in depressed subjects. For the study of depressive disorders, subjects were matched for age, sex, cigarette smoking history, and as close as possible for post-mortem interval (PMI). Brain tissue was collected from carefully screened subjects (post-mortem) who were diagnosed retrospectively with depressive disorders at the time of death, including major depression, dysthymia and adjustment disorder with depressed mood, and from controls who lacked major (Axis I) psychiatric disorders, except as indicated below for nicotine dependence.

MATERIALS AND METHODS

Human Subjects

NMDAR subunit immunoreactivity was analyzed in the LC and cerebellum from 13 and nine pairs, respectively, from subjects having depressive symptoms and individually paired control subjects. In all, 10 depressed subjects had an Axis I diagnosis of major depression, two subjects were diagnosed with dysthymia, and one subject was diagnosed with adjustment disorder with depressed mood. The two subjects diagnosed with dysthymia had a comorbid diagnosis of alcohol abuse (see Tables 1 and 2 for information on all subjects). Tissue was obtained at autopsy at the Coroner's Office of Cuyahoga County, Cleveland, OH, USA. An ethical protocol approved by the Institutional Review Board of the University Hospitals of Cleveland was used and informed written consent was obtained from the next-of-kin for all subjects. Blood and urine samples from all subjects were examined by the coroner's office for psychotropic medications and substances of abuse.

Retrospective, informant-based psychiatric assessments were performed for all depressed and control subjects. The Structured Clinical Interview for DSM-IV Psychiatric Disorders (SCID-IV) was administered to next-of-kin of the 10 of depressed subjects (First *et al*, 1996). A trained interviewer administered the Schedule for Affective Disorders and Schizophrenia: lifetime version (SADS-L) to knowledgeable next-of-kin of the three remaining depressed subjects. Axis I psychopathology was assessed and consensus diagnosis was reached in conference using information from the interview and medical records. All subjects with dysthymia and adjustment disorder and seven of the 10 major depressive subjects died as a result of suicide.

Information regarding medication history came from the coroner's records (prescriptions issued to the deceased around the time of death) as well as medical records. Compliance with prescriptions at the time of death was assessed by a pill count (*versus* the date of prescription), post-mortem determination of blood or urine levels of the medication, and the interview with the next-of-kin. No antidepressant drugs were detected in the post-mortem toxicology screening of subjects in the present study (Table 1), as the presence of such drugs was a criterion of exclusion from the study. Information on smoking was also collected in the interview. In the present study, there were eight pairs of active cigarette smokers, one pair of subjects with past histories of smoking, and four pairs of nonsmokers (never smoked).

The control subjects did not meet criteria for an Axis I disorder at the time of their deaths, except as indicated above for nicotine dependence. Blocks of brain tissue were dissected, frozen in dry-ice cooled isopentane and stored at -80°C.

Dissection and Anatomical Positioning of Measurements

Frozen tissue blocks were cut along the entire length of the LC, with histological sections taken at 1 mm intervals to evaluate anatomical position along the LC axis. The LC was punched from sections and punches were stored in microfuge tubes. The exact location of the rostral and caudal end of the LC was individualized for each subject based on Nissl staining and subsequent cell counting. The LC had its rostral border defined as a point where at least 25 ± 5 neuromelanin-containing cells identified. The caudal border was defined near the caudal end at a point where 25 ± 5 or less neuromelanin-containing cells were present. The whole LC was punched into 50 μm thick sections. For each anatomical level of the LC, tissue was collected from 2 mm of sections that were each centered at points that represented 25, 50, and 75% of the total rostrocaudal length of the LC. All results for the NR1 subunit in depression were generated from these rostral, middle, and caudal portions of the LC. For the study of the NR2C subunit, pooled tissue from three anatomical levels (rostral, middle, and caudal) was used in the Western blot assays due to limitations of tissue amount. For the study of the cerebellum, several sections (total weight approximately 80 mg) of the right

Table I Demographic Characteristics of the Subjects

Subject	Age (years)	Sex	PMI (h)	Toxicology	Cause of death	Diagnosis
RR[a,b]	37	M	17	Clean	Acute hemorrhagic pancreatitis	Cont
FFI[a,b]	27	M	17	Clean	Gun shot	Cont, S
HHI[a,b]	54	M	17	Brompheniramine	Heart disease	Cont, Shx
BBI[a,b]	52	M	17	Clean	Heart disease	Cont
KS6[a,b]	43	M	22	Clean	Crushing impacts to head, trunk and extremities	Cont
KS21[a,b]	48	M	9	Clean	Heart disease	Cont, S
KS19[a]	45	F	9	Clean	Heart disease	Cont, S
KS 23[a,b]	58	M	21	Clean	Heart disease	Cont, S
KS31[a]	59	M	6	Lidocaine	Heart disease	Cont, S
KS34[a]	23	F	11	Clean	Car accident	Cont, S
SS[a,b]	38	F	11	Clean	Hypertensive cardiomyopathy	Cont, S
KS15[a]	43	M	17	CO	Accidental CO poisoning	Cont
TT[a,b]	38	F	24	Clean	Suicide by overdose unknown substance unspecified	MDD
GGI[a,b]	30	M	18	Clean	Suicide by gun shot	MDD, S
JJI[a,b]	54	M	23	CO, phenobarbital phenytoin	Suicide by CO poisoning	MDD, Shx
DDI[a,b]	52	M	18	CO	Suicide by CO poisoning	MDD
KS8[a,b]	42	M	44	Clean	Suicide by hanging	MDD
KS17[a]	45	F	27	Clean	Pulmonary embolism	MDD, S
KS12[a,b]	41	M	19	Chlorpheniramine	Heart disease	MDD, S
KS24[a,b]	64	M	26	Clean	Suicide by gun shot	MDD, S
KS32[a]	60	M	20	Clean	Suicide by gun hot	MDD, S
KS35[a]	36	F	25	Clean	Chronic asthmatic bronchitis, hypertensive cardiac disease	MD, panic disorder, S
KS-1[a,b]	33	M	33	Clean	Suicide by hanging	Dysthymia, S, alcohol
KS3[a,b]	24	M	12	Ethanol	Suicide by hanging	Dysthymia, S, alcohol
KS16[a]	31	M	5	Ethanol, CO	Suicide by CO poisoning	Adjustment disorder

PMI = post-mortem interval; MDD = major depressive disorder; M = male; F = female.
[a]Subjects used in the LC study.
[b]Subjects used in the cerebellum study; S, active smoker; Shx, history of smoking.

cerebellar hemisphere were collected into tubes and stored in −80°C until assayed. Samples of right hippocampus (located adjacent to amygdaloid complex) were dissected and used in the study of NMDAR subunit composition.

Immunobloting of NMDA Receptor Subunits

LC, cerebellum, and hippocampus tissue samples were prepared according to the method published by Nash et al (1997), with minor modifications. Briefly, 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—CompleteTM, Boehringer Mannheim GmbH, Mannheim, Germany). Total protein concentrations for all samples were determined using the bicinchoninic acid method (Pierce Biotechnology Inc., Rockford, IL). Samples were mixed with sample buffer (0.125 M 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/lane) was subjected to 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Buckinghamshire, England). After transfer, blots were blocked in 5% nonfat milk/TBS (20 mM Tris base and 0.5 M NaCl, pH 7.5) for 2 h, and then incubated (overnight at 4°C) with mouse anti-NR1 monoclonal antibody (diluted 1:1000) (Pharmingen, BD Biosciences, San Diego, CA). NR2A and NR2B subunits were labeled using rabbit polyclonal antibodies (Novus Biological Inc., Littleton, CA), and NR2C subunit was detected similarly (ABR-Affinity BioReagents, Golden, CO). Antibodies against NR2 (A–C) subunits were diluted 1:500. Membranes were washed three times for 10 min in TBS buffer and incubated with secondary anti-mouse antibody (diluted 1:2000; Amersham Biosciences, Buckinghamshire, England) for NR1 subunit and anti-rabbit secondary antibody for NR2 (A–C) (diluted 1:3000; Amersham Biosciences, Buckinghamshire, England). After incubation, blots were washed three times for 15 min in TBS buffer and developed using enhanced chemiluminescence detection (ECL; Perkin-Elmer Life Sciences Inc., Boston, MA) and immediately exposed to film (Hyperfilm-ECL; Amersham Biosciences, Buckinghamshire, England). As a control for transfer and loading, actin was detected on each blot using an anti-actin monoclonal antibody (Chemicon International Inc., Temecula, CA). Immunoreactivity of NR1 and NR2C was investigated in pairs of depressed subjects and matched controls in the LC and cerebellum. Pairs of subjects were immunoblotted on the same gel in duplicate.

Table 2 History of Medication of Subjects Included in the Study

Subject	Drugs
RR	Amlodipine, Ranitidine
FF1	Motrin, Tylenol
HH1	Clonazapam, Nicardipine, Digoxin, Metaprolol, Prednisone, Simvastatin, Torsemide, Glipizide, Lisinopril, Ranitidine
BB1	Unknown
KS6	None
KS21	None
KS19	None
KS23	Digoxin
KS31	None
KS34	Levonorgestril implant
SS	Cardizem, Prilosec, Albuterol, Folic acid, Aspirin
KS15	None
TT	Lorazepam, Citalopram, Paroxetine
GG1	Unknown
JJ1	Sertraline
DD1	Sumatriptan, Loracarbef
KS8	Trazodone, Xanax, Accupro, Hydrochlorothiazide
KS17	None
KS12	None
KS24	Lisinopril, Spironolactone
KS32	None
KS35	Asthma inhaler, Aspirin
KS-1	None
KS3	None
KS16	None

Relationship between the Optical Density and the Total Protein Concentration

In order to determine the relationship between optical density values and the concentrations of subunit immunoreactivities, 10, 20, and 40 μg of total LC protein was loaded into gels and immunoblotted with anti-NR1 and anti-NR2C antibodies. Optical density values of immunoreactive bands were measured and are presented as a function of total LC protein concentration (expressed in μg). Analyses of blots revealed an approximately 1:1 relationship between changes in optical density values and changes in protein concentrations. That is, a 100% increase in total protein loading resulted in an approximately 100% increase in the optical density of NR1 and NR2C immunoreactive bands (Figure 1).

Data Analysis

Band densities for NR1 and NR2C (and their control protein—actin) were analyzed using imaging software (MCID Elite 7.0; Imaging Research, St Catherines, Ontario, Canada). Relative optical density values of experimental protein were normalized to values of control protein (actin) on the same gel. For the study of depression, normalized values from each depressive subject are expressed as
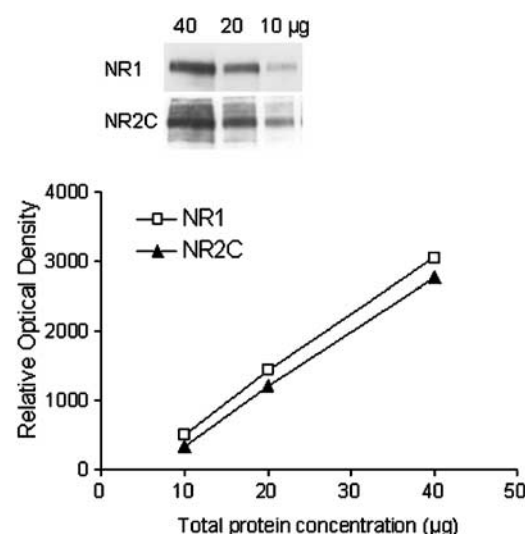

Figure 1 Relationship between the optical density values of NR1 and NR2C subunit immunoreactivity and total protein concentration in the human LC. Wells were loaded with 40, 20, and 10 μg of total human LC protein.

percentages (averaged from duplicates) of the normalized value from the paired control subject, both of which were run on the same gel. Results were analyzed statistically using the two-tailed paired Student's t-test (GraphPad Prism 3.0, GraphPad Software Incorporated, San Diego, CA). Summary statistics are reported as the mean± standard error of the mean (SEM). In order to adjust for multiple comparisons (two subunits analyzed per region), the p-value of 0.05 was adjusted to avoid a Type 1 error. Thus, a p-value < 0.025 was considered as a threshold for significance. The potential contribution of confounding factors (age, PMI, and brain pH) was evaluated in separate experiments using cerebellum tissue. For these latter experiments, all control subjects ($n = 9$) were blotted together and all depressive subjects ($n = 9$) were blotted together. Linear regression analysis (GraphPad Prism 3.0) was used to compute potential correlations between the amount of NR1 and NR2C immunoreactivity and potentially confounding factors.

RESULTS

Subunit Composition of NMDAR in the Human LC

Examination of an LC from a subject lacking a major psychiatric diagnosis revealed the presence of NR1 and NR2C subunit immunoreactivities as predominant bands at 120 and 135 kDa, respectively (Figure 2). As a comparison, an equal amount of protein from the hippocampus of the same subject was loaded for SDS-PAGE. High levels of both NR1 and NR2C subunit immunoreactivities were observed in the hippocampus. In contrast to NR1 and NR2C, very weak expression of NR2A and NR2B subunits was observed in the LC, while the hippocampus displayed prominent expression of NR2B and marked, but less abundant NR2A subunit (Figure 2). The polyclonal antibody used for identifying the NR2C subunit also detects 180 kDa proteins
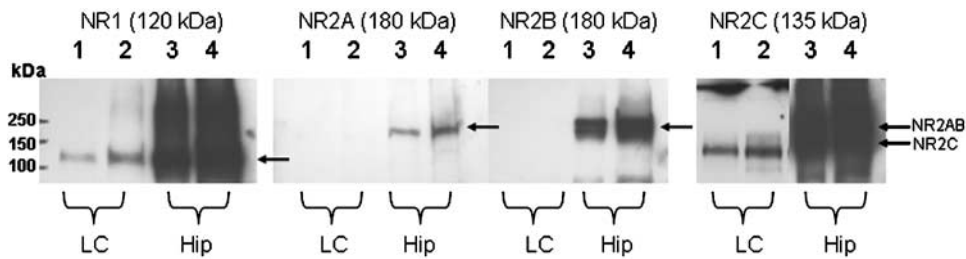
Figure 2 NMDAR subunit immunoreactivities detected in LC (lanes 1 and 2) and in hippocampus (lanes 3 and 4). In lanes 1 and 3, each well was loaded with 20 µg of total protein; for lanes 2 and 4, 40 µg of total protein was loaded into wells. The polyclonal antibody used against NR2C protein also detects a 180 kDa protein representing NR2A and NR2B subunits in the hippocampus.
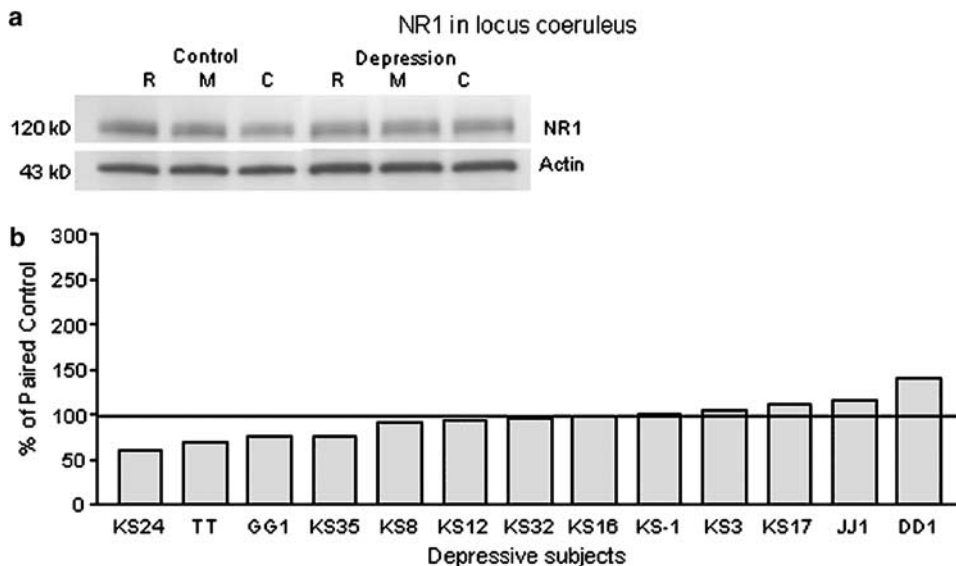

Figure 3 (a) NR1 subunit immunoreactivity in LC from a single pair used in the analysis, representing three separate anatomical levels: rostral (R), middle (M), and caudal (C). Each well was loaded with 20 µg of total protein. The bottom panel shows immunoreactive actin probed with anti-actin antibody on the same blots as a control for protein loading and transfer. (b) NR1 immunoreactivity expressed as percentages of values from paired control subjects. Each bar is an average of duplicate comparisons. The overall relative amount of NR1 in the LC was not different comparing depressive subjects to individually matched control subjects.

representing NR2A and NR2B in the hippocampus (Affinity BioReagents Cat. # OPA1-04020, Figure 2). The presence of NR2D and NR3 subunits of the NMDAR was not tested due to an unavailability of antibodies for these proteins.

NR1 in Depressed *vs* Control Subjects

NR1 protein level was analyzed by immunoblotting in the LC from 13 pairs of depressed subjects and individually paired control subjects. Western blot analyses of different subjects consistently revealed bands corresponding to the molecular mass of ~120 kDa. Figure 3a shows a representative immunoblot of NR1 from a single pair used in the analysis, representing three separate, anatomical levels of the human LC: rostral (R), middle (M), and caudal (C). Amounts of NR1 immunoreactivity, normalized by actin immunoreactivity, from depressed subjects were compared to amounts of normalized NR1 immunoreactivity of matched controls probed on the same blots. These amounts are presented individually as percentages of matched controls in Figure 3b. Averaged NR1 immunoreactivities from depressive subjects were $96 \pm 6\%$ of that from control subjects and this difference did not reach statistical significance ($t = 1.09$; $df = 12$; $p = 0.3$).

NR1 immunoreactivity was also measured in the non-limbic brain region, cerebellum, from nine of the 13 depressive/control pairs used to study the LC (cerebelli from four pairs not available). Averaged NR1 immunoreactivities of depressive subjects, expressed as a percentage of control values, were $89.8 \pm 9\%$ of that from control subjects. This difference did not reach statistical significance ($t = 1.14$; $df = 8$; $p = 0.29$; Figure 4).

NR2C in Depressed *vs* Control Subjects

Levels of NR2C protein were analyzed in the LC from 13 pairs of depressed subjects and individually paired control subjects. Western blot analyses of different subjects consistently revealed bands corresponding to the molecular mass of ~135 kDa. Figure 5a shows a representative immunoblot of NR2C from a single pair used in the analysis, representing pooled tissue from three anatomical levels of the LC (rostral, middle, and caudal). The relative amounts of NR2C immunoreactivity, normalized to actin
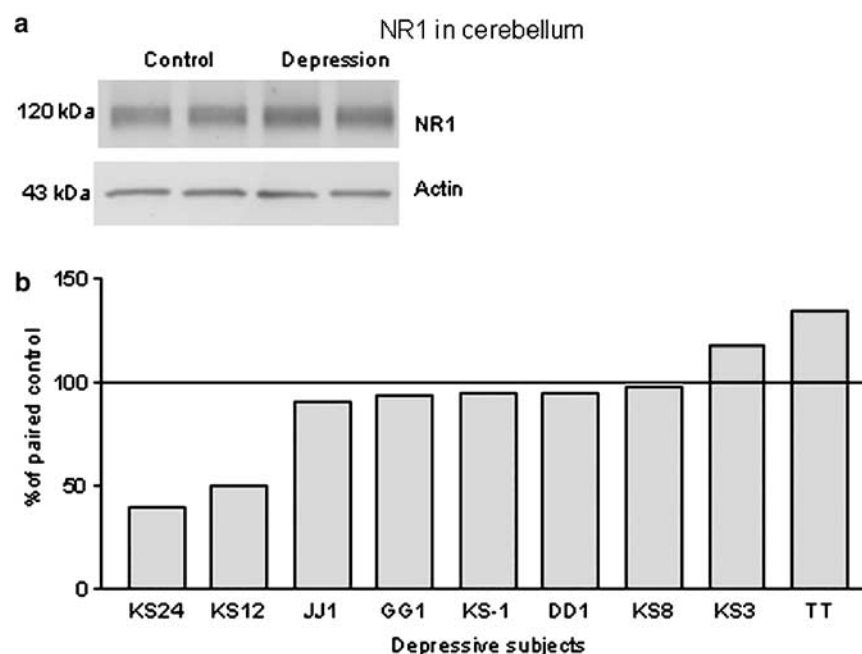
Figure 4 (a) NR1 subunit immunoreactivity in the cerebellum from a single pair used in the analysis blotted in duplicates. Each well was loaded with 20 μg of total protein. The bottom panel shows immunoreactive actin probed with anti-actin antibody on the same blots as a control for protein loading and transfer. (b) NR1 immunoreactivity expressed as percentages of values from paired control subjects. Each bar is an average of duplicate comparisons. The overall relative amount of NR1 in the cerebellum was normal among depressive subjects compared with individually matched control subjects.
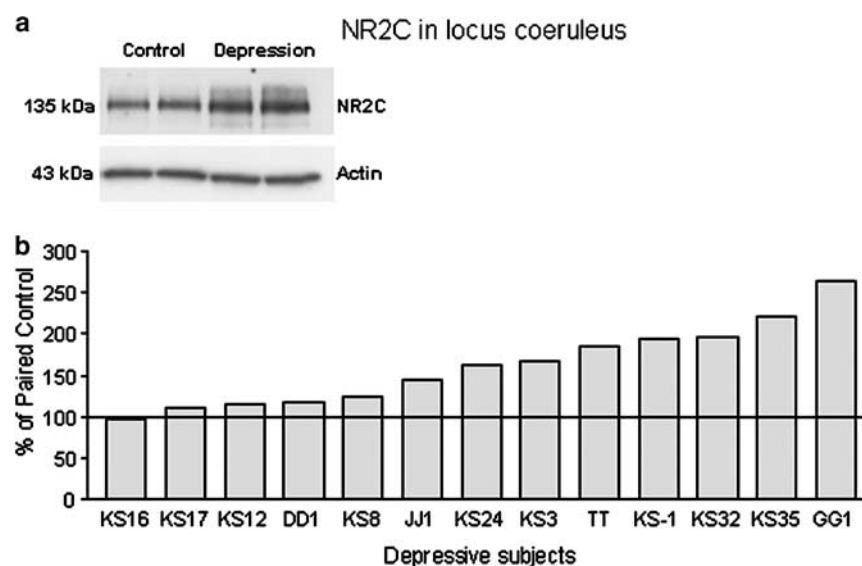

Figure 5 (a) NR2C subunit immunoreactivity in the LC from a single pair used in the analysis, representing pooled tissue from three anatomical levels (rostral, middle, and caudal). Each well was loaded with 20 μg of total protein. The bottom panel shows immunoreactive actin probed with anti-actin antibody on the same blots as a control for protein loading and transfer. (b) NR2C immunoreactivity expressed as percentages of values from paired control. Each bar is an average of duplicate comparisons. The overall relative amount of NR2C was higher in the LC (+ 61%, *p* = 0.003) among depressive subjects compared with individually matched control subjects.

immunoreactivity, from depressed subjects were compared to the amounts of normalized immunoreactivity of matched controls probed on the same blots and these are presented individually as percentages of matched controls in Figure 5b. Amounts of NR2C immunoreactivity were higher in the LC from 12 out of 13 depressed subjects relative to their matched controls. As a percentage of control values, averaged NR2C immunoreactivities from depressive sub-

jects were $161 \pm 14\%$ and this difference was statistically significant (t = 3.73; df = 12; *p* = 0.003).

In order to examine whether alterations of NR2C protein observed in the LC of depressed subjects would be observed in a nonlimbic LC projection area, for example, cerebellum, NR2C immunoreactivity was measured in cerebelli from nine of the 13 depressive/control pairs used to study the LC (Figure 6). Averaged NR2C immunoreactivities from
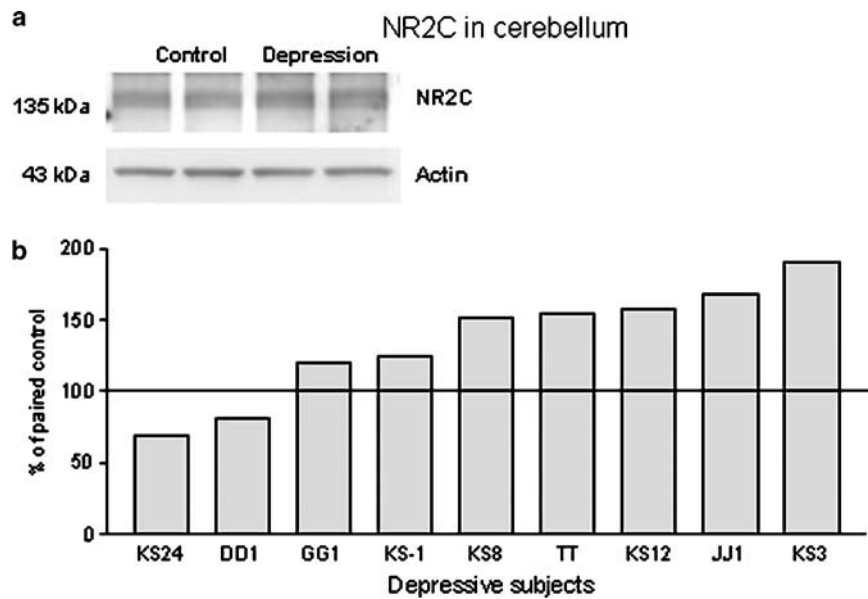
Figure 6 (a) NR2C subunit immunoreactivity in the cerebellum from a single pair used in the analysis. Each well was loaded with 20 μg of total protein. The bottom panel shows immunoreactive actin probed with anti-actin antibody on the same blots as a control for protein loading and transfer. (b) NR2C immunoreactivity expressed as percentages of values from paired control. Each bar is an average of duplicate comparisons. The overall relative amount of NR2C was elevated (+35%), but this difference did not reach statistical significance among depressives compared with individually matched control subjects.

depressive subjects were $135 \pm 13\%$ of that from control subjects. However, the difference did not reach statistical significance ($t = 1.86$; df $= 8$; $p = 0.09$). The cerebellum from four pairs of subjects investigated in the LC study was not available.

Age, PMI, Brain pH and NMDAR Subunits

The age of control subjects ranged from 27 to 59 years (43 ± 3 years) and depressed subjects ranged from 24 to 64 years (42 ± 3 years). The average ages of depressed and control subjects were not significantly different. PMIs of control and depressed subjects ranged from 6 to 22 h (14 ± 1 h) and from 5 to 44 h (21 ± 2 h), respectively. The average PMIs of depressed and control subjects were significantly different ($p < 0.05$). The average brain pH values of depressives (6.63 ± 0.07) and controls (6.67 ± 0.08) were not significantly different and ranged from 6.0 to 6.98 in controls and 6.24 to 6.91 in depressives.

The effect of potentially confounding factors on NR1 and NR2C subunits immunoreactivity was carefully examined in separate experiments where all cerebellar samples from controls and depressives subjects were run on two separate gels. This was particularly important for PMI, since there was a significant difference in PMI values comparing depressive to control subjects. There was no significant correlation between PMI and the amount of NR1 (controls, $r^2 = 0.01$; depressed $r^2 = 0.16$) and the amount of NR2C (controls, $r^2 = 0.005$; depressed, $r^2 = 0.09$; Figure 7a and b). It is noteworthy that data presented in Figure 7a and b represent two separate experiments and separate films. No attempt was made to standardize samples between experiments to generate Figure 7a and b. Hence, quantitative comparisons of gels of Figure 7a to those in Figure 7b are not valid.
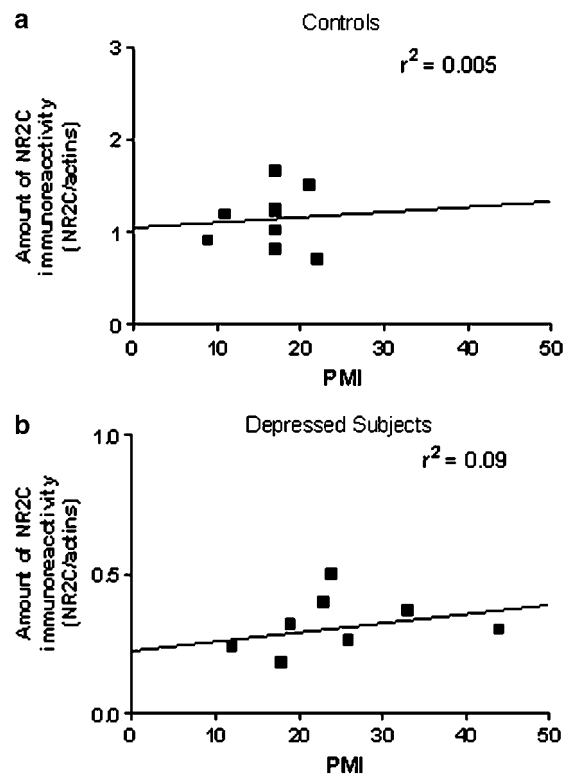

Figure 7 Relationships between the amount of NR2C immunoreactivity and PMI for control (a) and depressed subjects (b).

Using data from the experiment to examine the effect of PMI on NR1 and NR2C, the potential effect of age and brain pH on NR1 and NR2C levels was evaluated. There was no significant correlation between age and the amount of NR1

(controls, $r^2 = 0.05$; depressed $r^2 = 0.03$) and NR2C (controls, $r^2 = 0.11$; depressed $r^2 = 0.001$). Finally, there was no significant correlation between brain pH and the amount of NR1 (controls, $r^2 = 0.06$; depressed, $r^2 = 0.25$) and NR2C (controls, $r^2 = 0.12$; depressed $r^2 = 0.20$) subunit immunoreactivity in the cerebellum.

DISCUSSION

The present study provides evidence that the NMDAR in the LC is predominantly composed of a combination of NR1 and NR2C subunits. In contrast to the LC, three subunits investigated from NR2 family (NR2A, NR2B, and NR2C) were detected in the hippocampus. Hippocampal tissue was chosen as a reference region based on abundant evidence demonstrating prominent expression of NR2A and NR2B subunits. In contrast to the hippocampus, LC tissue (in equal amounts of protein as hippocampal samples) displayed a very weak, almost undetectable, expression of NR2A and NR2B subunits. Hence, it seems reasonable to conclude that NR2A and NR2B subunits are much less abundant in the LC than in the hippocampus. Moreover, the present study demonstrates that the amount of NR1 protein was normal in both the LC and cerebellum of depressed subjects. However, the amount of NR2C subunit was significantly elevated in the LC and modestly higher in the cerebellum of depressed subjects relative to the carefully matched control subjects lacking a major psychiatric diagnosis.

NMDAR Subunit Composition in the Human LC

The present data confirms that NR1 is a key abundant subunit and that it apparently coassembles with specific subunits from NR2 family depending on the brain region. These findings are consistent with *in situ* hybridization studies showing expression of the mRNA encoding NR1 subunit in human brain stem nuclei. Of the NR2 family, only NR2C was found to be expressed in these nuclei, including the LC (Rigby *et al*, 1996). Additionally, Allgaier *et al* (2001) demonstrated expression of NR2C mRNA by the majority of rat LC cells examined by single-cell RT-PCR analysis. The functional significance of this unique NR1/NR2C assembly is not understood at present and further studies are needed to elucidate its physiological relevance in the human LC. However, based on the previous observations, recombinant NR1/2C receptors have a lower current amplitude, shorter open time, smaller single conductance, and reduced sensitivity to Mg^{2+} than other subunit assemblies (Daggett *et al*, 1998; Ishii *et al*, 1993; Kutsuwada *et al*, 1992; Laurie and Seeburg, 1994; Monyer *et al*, 1994).

Role of Glutamatergic Signaling in Depression

We have previously postulated that major depression is associated with noradrenergic overdrive, as well as norepinephrine depletion, based on biochemical alterations in the LC of major depressives (Ordway *et al*, 2002). Abnormalities that have been observed in the LC from human depressives (Klimek *et al*, 1997; Ordway *et al*, 1994a, 1994b, 2003; Zhu *et al*, 1999) can be reproduced, in part, by chronic stress or pharmacological depletion of norepi-

nephrine in rats (Cubells *et al*, 1995; Lee *et al*, 1983; Melia *et al*, 1992; Torda *et al*, 1985; U'Prichard *et al*, 1979; Wang *et al*, 1998; Zafar *et al*, 1997). Given that stress activates the LC in laboratory animals (Pavcovich *et al*, 1990), that chronic stress depletes norepinephrine in the LC (Weiss and Simson, 1986), and that stress is a common precipitator of depression in humans, it seems possible that depression may be associated with elevated excitatory input to the LC.

A major excitatory input to the LC is glutamate. Interestingly, handling and immobilization stress increases glutamate measured in the rat LC by microdialysis (Singewald *et al*, 1995; Timmerman *et al*, 1999). Exposure to stress has been shown to increase mRNA levels of NR1 and NR2 subunits of NMDAR and GluR1 subunit of AMPA receptor in forebrain regions (Bartanusz *et al*, 1995; Fitzgerald *et al*, 1996; Schwendt and Jezova, 2000; Watanabe *et al*, 1995). Repeated stress also increases expression of GluR1 and NR1 subunits in the midbrain (Fitzgerald *et al*, 1996; for a review see Moghaddam, 2002). Moreover, olfactory bulbectomy (model used to study antidepressive agents) results in an increase of MK-801 binding to NMDAR measured in the medial prefrontal cortex (Webster *et al*, 2000) and in regional alterations in the glutamate metabotropic receptor 5 in rat hippocampus (Wieronska *et al*, 2001). These data demonstrate that glutamate abnormalities, precipitated by stress or generated in an animal model of depression, may contribute to functional and behavioral deficits that parallel human depression. In fact, previous post-mortem studies provide evidence that radioligand binding to NMDAR regulatory sites are altered in suicide victims and depressive disorders (Nowak *et al*, 1995; Nudmamud-Thanoi and Reynolds, 2004). Moreover, low levels of nNOS in the LC have been observed (Karolewicz *et al*, 2004) in the majority of same depressed subjects as were investigated in the present study. The activation of NMDAR leads to the activation of nNOS and stimulation of soluble guanylyl cyclase. Thus, nNOS is a part of the cascade of subcellular events linking NMDAR function with intracellular messengers systems. These findings together with the present finding of elevated amounts of the NR2C subunit of the NMDAR raise speculation that depression is associated with altered glutamatergic/nitrergic signaling in the LC.

Several indices of elevated glutamatergic transmission associated with depression have been previously demonstrated. For example, elevated levels of cerebrospinal fluid glutamine (glutamate metabolite/precursor) (Levine *et al*, 2000) and plasma glutamate were shown in depressed patients compared to controls (Altamura *et al*, 1993; Mauri *et al*, 1998). In addition, a reduction in the activity of glutamate metabolic enzyme—glutamate decarboxylase—was shown in neuropsychiatric disorders including depression (Kaiya *et al*, 1982). Recently, Sanacora *et al* (2004) demonstrated increased levels of glutamate in occipital cortex in depressed subjects as compared to healthy controls, utilizing proton magnetic resonance spectroscopy. On the other hand, Auer *et al* (2000) reported reduced glutamate level in the anterior cingulate cortex associated with depression. Consistent with the hypothesis that depression is associated with elevated glutamatergic transmission, NMDAR antagonists exhibit antidepressant-

like potential in rodent antidepressant screening procedures (Moryl *et al*, 1993; Papp and Moryl, 1994). In addition, ketamine, an NMDAR antagonist, exhibits antidepressant activity in humans (Berman *et al*, 2000). If glutamatergic input to the LC is elevated in depression, the putative antidepressant properties of NMDAR antagonists may be mediated, at least in part, by blockade of excitatory input to the LC.

Shortcomings of This Study

In the present study, the NR2C protein immunoreactivity was measured in the post-mortem tissue homogenates as opposed to measuring functional NR2C subunit protein that is expressed on the surface of the plasma membrane. Whether the increase in the total cellular NR2C protein immunoreactivity is positively correlated with an elevation of functional subunit expression cannot be answered based on the present experiments.

Since 10 out of 13 depressed subjects committed suicide, it is possible that behaviors related to suicide, but distinct from depressive symptoms associated with the depressive disorders studied might contribute to altered NR2C protein in the LC. However, it is worth noting that three subjects who died of natural causes had average amounts of NR2C higher than their respective controls, thus comparable to the levels observed in subjects dying as a result of suicide. Hence, the present findings are suggestive of altered glutamatergic signaling in the LC in depressive disorders. In the present study, three out of 13 subjects were not diagnosed with major depression but diagnosed with dysthymia, and adjustment disorder with depressed mood. The overall level of NR2C subunit in those three subjects was comparable to the levels observed in subjects diagnosed with major depression. Thus, the present data indicate that dysfunction of glutamatergic signaling may be associated with behavioral symptoms common to these depressive disorders, that is, depressed mood.

Treatment of depression may contribute to the adaptive changes seen in the human post-mortem brain. In fact, animal studies have provided evidence that antidepressant drugs produce time- and dose-dependent changes in the radioligand binding properties of the NMDAR (Nowak *et al*, 1998; Paul *et al*, 1994; Skolnick *et al*, 1996) and produce region-specific reductions of transcripts for NMDAR subunits in mouse brain (Boyer *et al*, 1998). The depressive subjects in the present study include three with a reported history of medication with antidepressants at some point during their lifetime (see Table 2, information obtained in structured clinical interviews by a trained interviewer with the next-of-kin). However, the presence of these drugs was not revealed in the post-mortem toxicology screening (Table 1). In spite of this, the influence of antidepressants was carefully considered. Two subjects examined in the present study have histories of medication with serotonin reuptake inhibitors, and one subject had a history of medication with trazodone (see Table 2). All three of these subjects had NR2C levels comparable to the average level of immunoreactivity of depressive subjects lacking the drug exposure. Hence, it is unlikely that past antidepressant drug treatment of depressed subjects contributed to the increases of NR2C.

Despite the effort to match depressive and control subjects based on several potentially confounding variables, PMIs of depressed subjects were longer than those of control subjects. Careful evaluation of this potential confound demonstrated that there was no correlation between the amount of NR2C immunoreactivity and PMI, as measured in the cerebellum (Figure 7a and b). Therefore, it is not likely that longer PMI contributes to increased levels of NR2C as observed in depressed subjects. Moreover, recent studies of other laboratories (Gonzalez-Maeso *et al*, 2002; Mato and Pazos, 2004) and our previous findings (Karolewicz *et al*, 2004) provide additional evidence that post-mortem delay does not affect receptor density and protein immunoreactivity in post-mortem tissue.

Based on the present observations, it cannot be assumed that elevated levels of NR2C subunit in depression reflect functional NMDAR subunits expressed on the surface of neurons. A potential dysfunction of glutamatergic transmission can be assessed by examination of NMDAR-associated intracellular proteins. In fact, nNOS protein level was reported to be lower (Karolewicz *et al*, 2004) in the majority of subjects who were investigated in the present study. Additional research is needed to understand how changes in glutamatergic activity alter the expression of functional NMDAR subunits, intracellular signaling molecules, and downstream cellular events.

Conclusions

The present findings indicate that the NMDAR in the human LC is composed predominantly of a combination of NR1 and NR2C subunits. Amounts of NR1 protein are normal, while amounts of NR2C are significantly elevated in the LC in depressed subjects. Increased amounts of NR2C immunoreactivity may reflect abnormal glutamatergic transmission in the LC in depressive disorders. Further study of the glutamatergic signaling pathway in depression involving non-NMDA ionotropic receptors and metabotropic glutamate receptors will lead to a better understanding of the role of glutamate in the pathology of depression.

REFERENCES

Allgaier C, Durmaz M, Muller D, Franke H, Poelchen W, Wirkner K *et al* (2001). Single-cell RT-PCR analysis of *N*-methyl-D-aspartate receptor subunit expression in rat locus coeruleus neurones. *Naunyn Schmiedebergs Arch Pharmacol* **363**: 120–123.

Altamura CA, Mauri MC, Ferrara A, Moro AR, D'Andrea G, Zamberlan F (1993). Plasma and platelet excitatory amino acids in psychiatric disorders. *Am J Psychiatry* **150**: 1731–1733.

Aston-Jones G, Ennis M, Pieribone VA, Nickell WT, Shipley MT (1986). The brain nucleus locus coeruleus: restricted afferent control of a broad efferent network. *Science* **234**: 734–737.

Aston-Jones G, Shipley MT, Chouvet G, Ennis M, Van Bockstaele E, Pieribone V *et al* (1991). Afferent regulation of locus coeruleus neurons: anatomy, physiology and pharmacology. *Prog Brain Res* **88**: 47–75.

Auer DP, Putz B, Kraft E, Lipinski B, Schill J, Holsboer F (2000). Reduced glutamate in the anterior cingulate cortex in depression: an *in vivo* proton magnetic resonance spectroscopy study. *Biol Psychiatry* **47**: 305–313.

Bartanusz V, Aubry JM, Pagliusi S, Jezova D, Baffi J, Kiss JZ (1995). Stress-induced changes in messenger RNA levels of *N*-methyl-D-aspartate and AMPA receptor subunits in selected regions of the rat hippocampus and hypothalamus. *Neuroscience* **66**: 247–252.

Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS *et al* (2000). Antidepressant effects of ketamine in depressed patients. *Biol Psychiatry* **47**: 351–354.

Boyer PA, Skolnick P, Fossom LH (1998). Chronic administration of imipramine and citalopram alters the expression of NMDA receptor subunit mRNAs in mouse brain. A quantitative *in situ* hybridization study. *J Mol Neurosci* **10**: 219–233.

Cubells JF, Kim KS, Baker H, Volpe BT, Chung T, Houpt TA *et al* (1995). Differential *in vivo* regulation of mRNA encoding the norepinephrine transporter and tyrosine hydroxylase in rat medulla and locus coeruleus. *J Neurochem* **65**: 502–509.

Cuellar B, Fernandez AP, Lizasoain I, Moro MA, Lorenzo P, Bentura ML *et al* (2000). Up-regulation of neuronal NO synthase immunoreactivity in opiate dependence and withdrawal. *Psychopharmacology (Berl)* **148**: 66–73.

Daggett LP, Johnson EC, Varney MA, Lin FF, Hess SD, Deal CR *et al* (1998). The human *N*-methyl-D-aspartate receptor 2C subunit: genomic analysis, distribution in human brain, and functional expression. *J Neurochem* **71**: 1953–1968.

First MB, Donovan S, Frances A (1996). Nosology of chronic mood disorders. *Psychiatr Clin N Am* **19**: 29–39.

Fitzgerald LW, Ortiz J, Hamedani AG, Nestler EJ (1996). Drugs of abuse and stress increase the expression of GluR1 and NMDAR1 glutamate receptor subunits in the rat ventral tegmental area: common adaptations among cross-sensitizing agents. *J Neurosci* **16**: 274–282.

Gonzalez-Maeso J, Torre I, Rodriguez-Puertas R, Garcia-Sevilla JA, Guimon J, Meana JJ (2002). Effects of age, postmortem delay and storage time on receptor-mediated activation of G-proteins in human brain. *Neuropsychopharmacology* **26**: 468–478.

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M *et al* (1993). Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. *J Biol Chem* **268**: 2836–2843.

Jodo E, Aston-Jones G (1997). Activation of locus coeruleus by prefrontal cortex is mediated by excitatory amino acid inputs. *Brain Res* **768**: 327–332.

Jodo E, Chiang C, Aston-Jones G (1998). Potent excitatory influence of prefrontal cortex activity on noradrenergic locus coeruleus neurons. *Neuroscience* **83**: 63–79.

Kaiya H, Namba M, Yoshida H, Nakamura S (1982). Plasma glutamate decarboxylase activity in neuropsychiatry. *Psychiatry Res* **6**: 335–343.

Karolewicz B, Szebeni K, Stockmeier CA, Konick L, Overholser JC, Jurjus G *et al* (2004). Low nNOS protein in the locus coeruleus in major depression. *J Neurochem* **91**: 1057–1066.

Klimek V, Stockmeier C, Overholser J, Meltzer HY, Kalka S, Dilley G *et al* (1997). Reduced levels of norepinephrine transporters in the locus coeruleus in major depression. *J Neurosci* **17**: 8451–8458.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K *et al* (1992). Molecular diversity of the NMDA receptor channel. *Nature* **358**: 36–41.

Laurie DJ, Seeburg PH (1994). Ligand affinities at recombinant *N*-methyl-D-aspartate receptors depend on subunit composition. *Eur J Pharmacol* **268**: 335–345.

Layer RT, Popik P, Olds T, Skolnick P (1995). Antidepressant-like actions of the polyamine site NMDA antagonist, eliprodil (SL-82.0715). *Pharmacol Biochem Behav* **52**: 621–627.

Lee CM, Javitch JA, Snyder SH (1983). Recognition sites for norepinephrine uptake: regulation by neurotransmitter. *Science* **220**: 626–629.

Levine J, Panchalingam K, Rapoport A, Gershon S, McClure RJ, Pettegrew JW (2000). Increased cerebrospinal fluid glutamine levels in depressed patients. *Biol Psychiatry* **47**: 586–593.

Loftis JM, Janowsky A (2003). The *N*-methyl-D-aspartate receptor subunit NR2B: localization, functional properties, regulation, and clinical implications. *Pharmacol Ther* **97**: 55–85.

Mato S, Pazos A (2004). Influence of age, postmortem delay and freezing storage period on cannabinoid receptor density and functionality in human brain. *Neuropharmacology* **46**: 716–726.

Mauri MC, Ferrara A, Boscati L, Bravin S, Zamberlan F, Alecci M *et al* (1998). Plasma and platelet amino acid concentrations in patients affected by major depression and under fluvoxamine treatment. *Neuropsychobiology* **37**: 124–129.

Melia KR, Nestler EJ, Duman RS (1992). Chronic imipramine treatment normalizes levels of tyrosine hydroxylase in the locus coeruleus of chronically stressed rats. *Psychopharmacology* **108**: 23–26.

Moghaddam B (2002). Stress activation of glutamate neurotransmission in the prefrontal cortex: implications for dopamine-associated psychiatric disorders. *Biol Psychiatry* **51**: 775–787.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**: 529–540.

Moryl E, Danysz W, Quack G (1993). Potential antidepressive properties of amantadine, memantine and bifemelane. *Pharmacol Toxicol* **72**: 394–397.

Nash NR, Heilman CJ, Rees HD, Levey AI (1997). Cloning and localization of exon 5-containing isoforms of the NMDAR1 subunit in human and rat brains. *J Neurochem* **69**: 485–493.

Nowak G, Legutko B, Skolnick P, Popik P (1998). Adaptation of cortical NMDA receptors by chronic treatment with specific serotonin reuptake inhibitors. *Eur J Pharmacol* **342**: 367–370.

Nowak G, Ordway GA, Paul IA (1995). Alterations in the *N*-methyl-D-aspartate (NMDA) receptor complex in the frontal cortex of suicide victims. *Brain Res* **675**: 157–164.

Nudmamud-Thanoi S, Reynolds GP (2004). The NR1 subunit of the glutamate/NMDA receptor in the superior temporal cortex in schizophrenia and affective disorders. *Neurosci Lett* **372**: 173–177.

Ordway GA, Klimek V, Mann JJ (2002). Neurocircuitry of mood disorders. In: Davis KL, Charney D, Coyle JT, Nemeroff C (eds). *Neuropsychopharmacology: The Fifth Generation of Progress*. Lippincott Williams & Wilkins: Philadelphia. pp 1051–1064.

Ordway GA, Schenk J, Stockmeier CA, May W, Klimek V (2003). Elevated agonist binding to alpha(2)-adrenoceptors in the locus coeruleus in major depression. *Biol Psychiatry* **53**: 315–323.

Ordway GA, Smith KS, Haycock JW (1994a). Elevated tyrosine hydroxylase in the locus coeruleus of suicide victims. *J Neurochem* **62**: 680–685.

Ordway GA, Widdowson PS, Smith KS, Halaris A (1994b). Agonist binding to alpha 2-adrenoceptors is elevated in the locus coeruleus from victims of suicide. *J Neurochem* **63**: 617–624.

Papp M, Moryl E (1994). Antidepressant activity of non-competitive and competitive NMDA receptor antagonists

in a chronic mild stress model of depression. *Eur J Pharmacol* **263**: 1–7.

Palucha A, Tatarczynska E, Branski P, Szewczyk B, Wieronska JM, Klak K et al (2004). Group III mGlu receptor agonists produce anxiolytic- and antidepressant-like effects after central administration in rats. *Neuropharmacology* **46**: 151–159.

Paul IA, Nowak G, Layer RT, Popik P, Skolnick P (1994). Adaptation of the *N*-methyl-D-aspartate receptor complex following chronic antidepressant treatments. *J Pharmacol Exp Ther* **269**: 95–102.

Pavcovich LA, Cancela LM, Volosin M, Molina VA, Ramirez OA (1990). Chronic stress-induced changes in locus coeruleus neuronal activity. *Brain Res Bull* **24**: 293–296.

Rigby M, Le Bourdelles B, Heavens RP, Kelly S, Smith D, Butler A et al (1996). The messenger RNAs for the *N*-methyl-D-aspartate receptor subunits show region-specific expression of different subunit composition in the human brain. *Neuroscience* **73**: 429–447.

Sanacora G, Gueorguieva R, Epperson CN, Wu YT, Appel M, Rothman DL et al (2004). Subtype-specific alterations of gamma-aminobutyric acid and glutamate in patients with major depression. *Arch Gen Psychiatry* **61**: 705–713.

Schwendt M, Jezova D (2000). Gene expression of two glutamate receptor subunits in response to repeated stress exposure in rat hippocampus. *Cell Mol Neurobiol* **20**: 319–329.

Shaw PJ, Ince PG, Johnson M, Perry EK, Candy JM (1992). The quantitative autoradiographic distribution of [^{3}H]MK-801 binding sites in the normal human brainstem in relation to motor neuron disease. *Brain Res* **572**: 276–280.

Singewald N, Zhou GY, Schneider C (1995). Release of excitatory and inhibitory amino acids from the locus coeruleus of conscious rats by cardiovascular stimuli and various forms of acute stress. *Brain Res* **704**: 42–50.

Skolnick P (1999). Antidepressants for the new millennium. *Eur J Pharmacol* **375**: 31–40.

Skolnick P, Layer RT, Popik P, Nowak G, Paul IA, Trullas R (1996). Adaptation of *N*-methyl-D-aspartate (NMDA) receptors following antidepressant treatment: implications for the pharmacotherapy of depression. *Pharmacopsychiatry* **29**: 23–26.

Tatarczynska E, Klodzinska A, Chojnacka-Wojcik E, Palucha A, Gasparini F, Kuhn R et al (2001). Potential anxiolytic- and antidepressant-like effects of MPEP, a potent, selective and systemically active mGlu5 receptor antagonist. *Br J Pharmacol* **132**: 1423–1430.

Tatarczynska E, Palucha A, Szewczyk B, Chojnacka-Wojcik E, Wieronska J, Pilc A (2002). Anxiolytic- and antidepressant-like effects of group III metabotropic glutamate agonist (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) in rats. *Pol J Pharmacol* **54**: 707–710.

Timmerman W, Cisci G, Nap A, de Vries JB, Westerink BH (1999). Effects of handling on extracellular levels of glutamate and other amino acids in various areas of the brain measured by microdialysis. *Brain Res* **833**: 150–160.

Torda T, Kvetnansky R, Petrikova M (1985). Effect of repeated immobilization stress on central and peripheral adrenoceptors in rats. *Endocrinol Exp* **19**: 157–163.

U'Prichard DC, Bechtel WD, Rouot BM, Snyder SH (1979). Multiple apparent alpha-noradrenergic receptor binding sites in rat brain: effect of 6-hydroxydopamine. *Mol Pharmacol* **16**: 47–60.

Van Bockstaele EJ, Colago EE (1996). Selective distribution of the NMDA-R1 glutamate receptor in astrocytes and presynaptic axon terminals in the nucleus locus coeruleus of the rat brain: an immunoelectron microscopic study. *J Comp Neurol* **369**: 483–496.

Vulliemoz Y, Whittington RA, Virag L (1999). The nitric oxide-cGMP system of the locus coeruleus and the hypnotic action of alpha-2 adrenergic agonists. *Brain Res* **849**: 169–174.

Wang P, Kitayama I, Nomura J (1998). Tyrosine hydroxylase gene expression in the locus coeruleus of depression-model rats and rats exposed to short- and long-term forced walking stress. *Life Sci* **62**: 2083–2092.

Watanabe Y, Weiland NG, McEwen BS (1995). Effects of adrenal steroid manipulations and repeated restraint stress on dynorphin mRNA levels and excitatory amino acid receptor binding in hippocampus. *Brain Res* **680**: 217–225.

Webster HH, Flores G, Marcotte ER, Cecyre D, Quirion R, Srivastava LK (2000). Olfactory bulbectomy alters NMDA receptor levels in the rat prefrontal cortex. *Synapse* **37**: 159–162.

Weiss JM, Simson PG (1986). Depression in an animal model: focus on the locus ceruleus. *Ciba Found Symp* **123**: 191–215.

Wieronska JM, Branski P, Szewczyk B, Palucha A, Papp M, Gruca P et al (2001). Changes in the expression of metabotropic glutamate receptor 5 (mGluR5) in the rat hippocampus in an animal model of depression. *Pol J Pharmacol* **53**: 659–662.

Xu ZQ, de Vente J, Steinbusch H, Grillner S, Hokfelt T (1998). The NO-cGMP pathway in the rat locus coeruleus: electrophysiological, immunohistochemical and *in situ* hybridization studies. *Eur J Neurosci* **10**: 3508–3516.

Xu ZQ, Pieribone VA, Zhang X, Grillner S, Hokfelt T (1994). A functional role for nitric oxide in locus coeruleus: immunohistochemical and electrophysiological studies. *Exp Brain Res* **98**: 75–83.

Zafar HM, Pare WP, Tejani-Butt SM (1997). Effect of acute or repeated stress on behavior and brain norepinephrine system in Wistar–Kyoto (WKY) rats. *Brain Res Bull* **44**: 289–295.

Zhu MY, Klimek V, Dilley GE, Haycock JW, Stockmeier C, Overholser JC et al (1999). Elevated levels of tyrosine hydroxylase in the locus coeruleus in major depression. *Biol Psychiatry* **46**: 1275–1286.