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ARTICLE

Elevated gene expression of glutamate receptors in noradrenergic neurons from the locus coeruleus in major depression

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Abstract

Glutamate receptors are promising drug targets for the treatment of urgent suicide ideation and chronic major depressive disorder (MDD) that may lead to suicide completion. Antagonists of glutamatergic NMDA receptors reduce depressive symptoms faster than traditional antidepressants, with beneficial effects occurring within hours. Glutamate is the prominent excitatory input to the noradrenergic locus coeruleus (LC). The LC is activated by stress in part through this glutamatergic input. Evidence has accrued demonstrating that the LC may be overactive in MDD, while treatment with traditional antidepressants reduces LC activity. Pathological alterations of both glutamatergic and noradrenergic systems have been observed in depressive disorders, raising the prospect that disrupted glutamate-norepinephrine interactions may be a central component to depression and suicide pathobiology. This study examined the gene expression levels of glutamate receptors in post-mortem noradrenergic LC neurons from subjects with MDD (most died by suicide) and matched psychiatrically normal controls. Gene expression levels of glutamate receptors or receptor subunits were measured in LC neurons collected by laser capture microdissection. MDD subjects exhibited significantly higher expression levels of the NMDA receptor subunit genes, *GRIN2B* and *GRIN2C*, and the metabotropic receptor genes, *GRM4* and *GRM5*, in LC neurons. Gene expression levels of these receptors in pyramidal neurons from prefrontal cortex (BA10) did not reveal abnormalities in MDD. These findings implicate disrupted glutamatergic-noradrenergic interactions at the level of the stress-sensitive LC in MDD and suicide, and provide a theoretical mechanism by which glutamate antagonists may exert rapid antidepressant effects.

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Key words: Glutamate receptors, locus coeruleus, major depression, neurons, suicide.

Introduction

Major depressive disorder (MDD) and suicide are outcomes of a complex interaction of neurochemistry, environmental, genetic, and developmental factors. Retrospective studies indicate that nearly 80% of suicide victims suffered from depressive symptoms (Beskow, 1979; Rich et al., 1986), thereby implicating depression as the leading contributor to suicide. Most depressive episodes and suicides are preceded by stressful events, such as adversity in early life (Mann and Currier, 2010). The major noradrenergic tract, the dorsal bundle, arising in the pontine nucleus locus coeruleus (LC) and innervating the dorsal brain – including the neocortex and hippocampus – is a vital component of the stress response. In rodents, stress increases LC activity (Heinsbroek et al., 1991; Melia and Duman, 1991; Kollack-Walker et al., 1997; Curtis et al., 2002) and chronic stress depletes NE in the LC and in its projection regions (Weiss et al., 1980). Norepinephrine (NE)-depletion in humans with a history of depression can re-induce depression (Berman et al., 1999; Hasler et al., 2008). Treatment with antidepressants, including selective serotonin reuptake inhibitors (SSRIs), reduces LC activity, possibly as a consequence of increased extraneuronal NE action at presynaptic autoreceptors (Melia et al., 1992; Grant and Weiss, 2001; West et al., 2009). Collectively, these studies indicate that the LC has a major role in the origin of clinical depression and possibly suicide.

A major, stress-sensitive excitatory input that intrinsically modulates LC activity is the amino acid transmitter, glutamate. Glutamatergic inputs to the LC originate from the paragigantocellularis nucleus, lateral habenula, and prefrontal cortex (Herkenham and Nauta, 1979; Aston-Jones et al., 1986; Jodo and Aston-Jones, 1997). Recent findings suggest that glutamatergic input to the LC may

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Table 1. Nomenclature for glutamate receptor genes that were studied, and their corresponding proteins

Glutamate receptor	Gene[a]	Protein[b]
N-methyl-D-aspartate (NMDA) receptor subunits	*GRIN1*	GluN1, NMDAR1, NR1
	GRIN2A	GluN2A, NMDAR2A, NR2A
	GRIN2B	GluN2B, NMDAR2B, NR2B
	GRIN2C	GluN2C, NMDAR2C, NR2C
	GRIN2D	GluN2D, NMDAR2D, NR2D
	GRIN3A	GluN3A, NMDAR3A, NR3A
	GRIN3B	GluN3B, NMDAR3B, NR3B
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunits	*GRIA1*	GluR-1, GluA1
	GRIA2	GluR-2, GluA2
	GRIA4	GluR-4, GluA4
Kainate glutamate receptor subunits	*GRIK1*	GluR-5, GluK1
	GRIK3	GluR-7, GluK3
	GRIK5	GluK5, KA2
Metabotropic glutamate receptor subunits	*GRM4*	mGluR4
	GRM5	mGluR5
	GRM8	mGluR8

[a] Derived from the HUGO gene nomenclature committee responsible for designating unique gene names (Wain et al., 2002).
[b] Official protein nomenclature showing both the recommended and alternative protein names (Uniprot, 2008).

be a key mediator of abnormal LC activity in depression. Of particular interest is the lateral habenula, where electrical stimulation inhibits habenular activity, resulting in a reduction of depressive behaviors, possibly in part due to a reduction in glutamatergic activation of the LC (Sartorius and Henn, 2007). Antagonists of the glutamate NMDA receptor decrease LC activity (Murase et al., 1992) and ketamine, an NMDA receptor antagonist, produces a rapid antidepressant effect that persists for weeks in humans (Zarate et al., 2006). In fact, a single brief infusion of ketamine reduces suicide ideation within an hour, and the effect persists up to 24 h (Price et al., 2009; Diazgranados et al., 2010). Specific abnormalities, such as increased extraneuronal glutamate levels (Sanacora et al., 2004) and altered expression of glutamate related genes (Chandley et al., 2013), have been demonstrated specifically in depressed suicide victims. These findings indicate that glutamatergic activation of the LC may be abnormal in depression.

Glutamate signaling in the brainstem of both rodents (Anon-Allen brain atlas) and non-human primates (Noriega et al., 2007) is mediated by NMDA-, AMPA-, and kainate-ionotropic receptors and G protein-coupled metabotropic glutamate receptors. *In vitro* studies using brain slice preparations indicate that signaling through all glutamate receptor classes can activate LC neurons (Olpe et al., 1989; Page et al., 2005). The current study was undertaken to examine the expression of glutamate receptor genes (see Table 1 for nomenclature) in human LC neurons, and to determine if receptor gene expression levels are altered in post-mortem brain tissue of depressed subjects, the majority of which died by suicide. Given recent evidence of the rapid antidepressant properties of NMDA receptor antagonists in humans, we evaluated the expression of all human NMDA receptor subunit genes in the LC. For the remaining glutamate receptor genes, including the AMPA, kainate and metabotropic glutamate receptors, we examined only those that demonstrated measurable gene expression in the mouse LC, according to the Allen Brain Atlas (Lein et al., 2007).

Methods

Brain tissue

Tissue for the described studies were obtained from the following brain banks: Cleveland (22 subjects), Pittsburgh (13 subjects), and Quebec Suicide Brain Bank (one subject), as previously described (Chandley et al., 2013). All specimens were obtained at autopsy in accordance with the respective Institutional Review Board Protocol (IRB). Psychiatric assessment consisted of retrospective structured interviews with family members and evaluation of previous medical records by trained clinicians. A post-mortem psychiatric diagnosis obtained in this manner is considered to be comparable to the diagnosis of the living individual (Kelly and Mann, 1996; Dejong and Overholser, 2009). Control and MDD subjects were carefully matched prior to initiation of this study, primarily according to age and gender. Because of the limited number of control subjects available, perfect matching of control–MDD subject pairs for all demographic variables was not possible. The matched pairs exhibited an overall mean age difference of 4.9±0.8 yr (S.E.M.; range 0–11 yr). Cigarette smoking was matched when possible except for pair five, with smoking histories unknown for two control subjects and one MDD subject. The remaining factors, including post-mortem tissue

Table 2. Subject demographics

Pair[a]	Subject	Gender	Age	pH	RIN	PMI	Smoker	Toxicology	Tissue
Normal control donors									
1	KS57	M	17	6.71	7.40	24.0	No	Ethanol	LC
2	KS59	M	46	6.95	6.76	19.0	No	Ethanol	LC
3	KS21	M	48	6.98	7.40	9.0	Yes	NDD	LC, Ctx
4	FF1	M	27	6.88	8.40	17.0	Yes	NDD	LC, Ctx
5	KS31	M	59	6.79	7.65	6.0	No	Lidocaine	LC, Ctx
6	KS23	M	58	6.78	7.67	21.0	Yes	NDD	LC, Ctx
7	KS78	M	18	6.60	7.90	16.0	Unknown	NDD	LC
8	KS74	M	72	6.30	7.30	16.4	No	Diltiazem	LC
9[b]	KS67	M	54	6.50	6.50	26.3	No	Morphine	LC
9[b]	KS82	M	47	6.10	8.30	25.0	20 y ago	NDD	Ctx
10	KS63	M	18	6.40	6.40	31.5	Unknown	Midazolam	LC
11	KS65	M	58	5.80	6.40	27.0	No	NDD	LC
12	KS70	M	43	7.00	7.40	22.3	No	NDD	LC
13	KS72[c]	M	49	7.10	8.50	19.9	Yes	Doxylamine	LC
14	KS76[c]	M	42	6.70	7.40	23.5	No	Butalbital	LC
15	KS80[c]	M	18	7.00	8.70	14.6	No	Diazepam	LC
16	RR	M	37	6.47	7.30	17.0	No	NDD	Ctx
17	KS27	M	74	6.62	6.70	21.0	Yes	NDD	Ctx
18	VV	M	54	6.52	7.70	19.0	Yes	Lidocaine	Ctx
	KS85[c]	F	74	6.60	7.00	24.9	No	Diazepam	LC
MEAN			45.7	6.64	7.44	20.0			
S.E.M.			4.1	0.07	0.15	1.4			
Major depressed donors									
1	KS58	M	18	6.58	6.83	27.0	Unknown	CO	LC
2	KS56	M	37	6.67	6.90	31.0	No	Ethanol	LC
3	KS12	M	41	6.24	6.70	19.0	Yes	Chlorpheniramine	LC, Ctx
4	GG1	M	30	6.91	8.05	18.0	Yes	NDD	LC, Ctx
5	KS32	M	60	6.31	6.80	20.0	Yes	Ethanol	LC, Ctx
6	KS24	M	64	6.85	7.25	26.0	Yes	Ethanol	LC, Ctx
7	KS79	M	25	6.90	7.60	12.9	Yes	NDD	LC
8	KS75	M	77	N/A	6.70	19.6	No	CO, Diazepam, Temazepam	LC
9	DD	M	52	6.48	5.80	18.0	No	CO	LC, Ctx
10	KS64	M	20	6.73	6.70	20.0	No	Diphenhydramine	LC
11	KS66	M	48	6.68	6.70	17.0	No	NDD	LC
12	KS71	M	51	6.60	7.50	5.3	No	Citalopram, Venlafaxine	LC
13	KS73	M	44	6.50	7.20	11.0	Yes	NDD	LC
14	KS77	M	38	6.70	8.70	18.8	No	NDD	LC
15	KS81	M	18	7.00	8.50	10.1	No	Ethanol	LC
16	TT	M	38	6.52	7.20	24.0	No	NDD	Ctx
17	KS28	M	81	6.78	6.10	33.0	Yes	NDD	Ctx
18	WW	M	65	6.24	6.70	30.0	Yes	Codeine	Ctx
MEAN			44.8	6.63	7.11	20.0			
S.E.M.			4.5	0.05	0.18	1.8			

pH, negative $\log_{10}$ of the hydrogen ion concentration; RIN, RNA integrity number generated by the Agilent 2100e; PMI, post-mortem interval; NDD, no drugs detectable; y, years; LC, locus coeruleus; Ctx, cortex.

[a] Pair numbers here indicate the same subject pair numbers that appear in the figures.

[b] Denotes that different donor subjects were used in the two different brain areas (LC and cortex) for the same depressive subject. This was necessary because tissue for both regions were not available from the same control subject.

[c] Represents the controls that were used for the gene expression distribution study wherein RNA was extracted from trephine-punched LC; KS85 was only used for the distribution study and was not used in gene expression studies comparing control to MDD subjects.

pH (Harrison et al., 1995), RNA integrity number (RIN), post-mortem interval (PMI), and storage time in freezer were matched as closely as possible (Table 2). Tissues from matched pairs were always prepared and biochemically analyzed simultaneously. All tissues had a RIN value greater than 5.8, determined using an Agilent Bioanalyzer 2100e (Ordway et al., 2009). Toxicological evaluations were conducted with blood and

urine on all subjects to confirm medical treatment, as well as an index of drug and alcohol use or abuse at the time of death.

Brain tissues were obtained from 20 psychiatrically normal controls and 18 subjects diagnosed with MDD at time of death (Table 2). All control subjects were void of any previous major psychiatric illness. While two control and three MDD subjects had a history of alcohol use/abuse, none had active alcohol abuse at the time of death. Also, six control subjects and eight MDD subjects were smokers at the time of death. No subject in the control or MDD group had a history of other substance abuse, and there was no evidence of substance use at the time of death, as confirmed by toxicology analysis; antidepressant medication was evident in the blood of one MDD subject. Control subjects died by heart disease (8), gunshot (1), trauma (4), peritonitis (1), asthma (1), aneurysm (1), distended aorta (1), pulmonary embolism (1), subarachnoid hemorrhage (1), and acute hemorrhage (1). MDD subjects dying by suicide included those that died by self-inflicted gunshot (8), hanging (2), CO poisoning (3), unspecified suicide (1), and drowning (2). Two subjects in the MDD group died from heart disease. Comorbidities and causes of death have been excluded from Table 2 to protect subject identity.

Tissue preparation

At autopsy, blocks of tissue from the pontine LC and Brodmann Area 10 (BA10) were frozen and stored at −80 °C. Frozen tissue blocks were subsequently cut at a 10 μm thickness with a cryostat microtome (Leica CM3050 S) and mounted for histochemical analysis or laser capture microdissection (LCM). Slides were placed in a cold microslide box on ice and then transported to −80 °C for storage up to 6 months. For the distribution study, LC tissue was dissected from frozen tissue sections using a 3 mm trephine punch and then homogenized for biochemical analysis.

Staining and laser capture microdissection

Prior to sectioning, the knife holder and anti-roll plate were carefully wiped with 100% ethanol to avoid cross-contamination between subjects. Sections (10 μm) for LCM were mounted at room temperature (22 °C) on a HistoGene® LCM microslides (Molecular Devices, USA) and placed immediately in a chilled microslide box at −20 °C. Noradrenergic and cortical neurons were identified from brain sections mounted on PEN-membrane glass slides, stored at −80 °C, using the Histogene LCM Frozen Section Staining Kit (Molecular Devices, USA). This method has been described in detail (Ordway et al., 2009). LCM was performed on an Arcturus Veritas™ Microdissection Instrument; Model 704 (Molecular Devices, USA). This method has been described in detail (Ordway et al., 2009). Paired control and MDD tissues were stained and subjected to LCM sequentially within a 5 d time frame; less time was not possible because of the time required to capture a sufficient number of cells by LCM.

RNA preparation, amplification, and polymerase chain reaction (PCR)

Initially, we were interested in a quantitative assessment of the amounts of gene expression of the different receptors. Homogenates of trephine-dissected LC tissues were used to isolate RNA for these quantitative comparisons because the number of laser captured neurons required to do this was cost-prohibitive. Messenger RNA levels for tissue homogenates ($N=4$) were assessed by quantitative polymerase chain reaction (PCR) (Biorad, USA) to determine relative receptor/receptor subunit levels in LC neurons. Each 18 μl PCR reaction for the receptor/subunit genes *GRIN1, GRIN2A, GRIN2C, GRIN2D, GRIA1, GRIA2, GRIA4, GRM5* and *GRIK5* contained 1.5 μl of cDNA, 9 μl of Platinum Supermix (Invitrogen), 250 nM sense and antisense primer (Integrated DNA Technologies, USA), 1.5 mM Mg^{2+} (Life Technologies, USA), and SYBR Green (Life Technologies, USA). Quantitative PCR reactions for *GRM4, GRM8, GRIK1*, and *GRIK3* consisted of Fast SYBR mix 10 μl (KAPA), 250 nM sense and antisense primer (Integrated DNA technologies, USA) and 1 μl cDNA. Optimal reaction temperatures and primer efficiencies were determined for each primer set using a matching synthetic standard oligonucleotide. Subsequently, oligonucleotide standards were used to determine copy numbers of all gene transcripts. Receptor/subunit gene transcripts were normalized using the geometric mean of the two reference genes (*TATA* and *GAPDH*). Genes and primers are shown in Supplementary Table 1.

Because very small amounts of RNA are available with samples collected by LCM, end-point PCR was used to quantify transcripts from laser capture neurons to determine gene expression differences between control subjects and MDD subjects as described previously (Ordway et al., 2009). Total RNA was extracted from laser captured cells using the RNA aqueous system optimized for LCM according to the manufacturer's instructions (Life Technologies, USA). Genomic DNA was removed from each sample by DNAase I treatment at 37 °C followed by a gel-based removal of enzyme (Life Technologies, USA). RNA was reverse transcribed to cDNA using the Superscript III first strand synthesis system primed with a mix of oligo$(dT)_{20}$ and random hexamers (Life Technologies, USA) and stored at −20 °C until use. Target gene expression from end-point PCR was normalized to the geometric mean of three reference genes (*GAPDH, ACTB*, and *UBC*). RNA preparation was performed simultaneously for each paired control and MDD sample. PCR reactions of paired samples were performed in triplicate at the same time in the same apparatus, and were analyzed on the same chip using the Agilent Bioanalyzer.

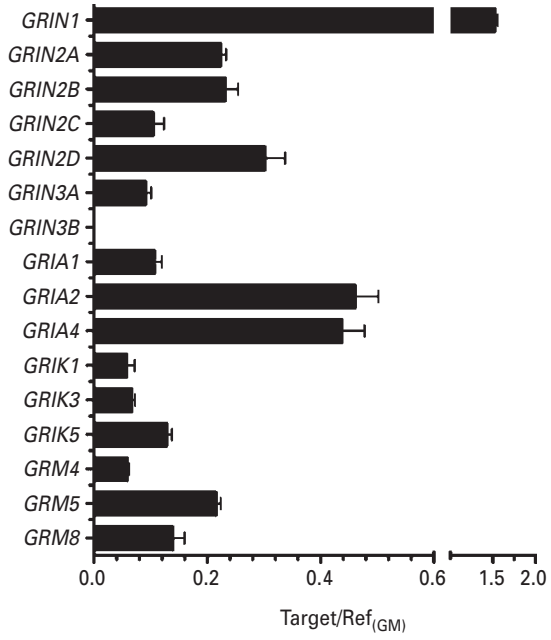

Fig. 1. Average gene expression levels of glutamate receptors/subunits in punch-dissected locus coeruleus (LC) of control subjects, as assessed by real time quantitative polymerase chain reaction (n=4). All primers are provided in Supplementary Table 1. Target gene expression was normalized to the geometric mean of two reference genes (*GAPDH* and *TATA*) determined in the same samples.

Statistical analysis

End-point PCR analyses of each receptor subtype from LC neurons captured by LCM were analyzed by paired Student's *t*-test using amplicon amounts normalized to the geometric mean of the reference genes. Donors were paired prior to laboratory experimentation (see Brain Tissue section above for more information). Percentage differences between matched control and MDD subjects were computed and reported as the mean±S.E.M. Possible relationships between post-mortem variables and levels of gene expression were evaluated using Pearson's correlation analyses. Linear correlations were performed to ensure that demographic variables did not alter statistical findings. Statistical analyses were performed using IBM SPSS Statistics (version 21) and GraphPad Prism 5.0 (GraphPad Software Incorporated, USA) using a $p<0.05$ as the significance level.

Results

Levels of glutamate receptor gene expression in the human LC region

Quantitative levels of the gene expression of glutamate receptors and receptor subunits in the LC from four control subjects is shown in Fig. 1. Of the 16 glutamate receptor genes observed, *GRIN1* exhibited the highest expression. Intermediate expression levels were found for *GRIN2A, GRIN2B, GRIN2D, GRIA2*, and *GRIA4*; low expression levels were found for the remaining glutamate receptor genes. A comparison of expression levels of glutamate receptor genes in mouse LC (Allen Brain Atlas (http://mouse.brain-map.org/); Lein et al., 2007) and human LC obtained in the present study (Fig. 1) is shown in Table 3. Details of statistical comparisons of gene expression levels amongst the various receptor subtypes appears in Supplementary Table 2.

Glutamate receptor/subunit gene expression in MDD

The expression level of each reference gene in captured LC neurons (normalized to the geometric mean of the other two reference genes) was not significantly different comparing control to MDD subjects (Supplementary Fig. 1), confirming the stability of these gene expressions across the two study groups. Gene expression levels for the NMDA receptor subunits *GRIN2B* and *GRIN2C* were, respectively, elevated in LC neurons by 148±11% ($p<0.001$) and 134±7% ($p<0.005$; mean±S.E.M.) in MDD subjects *vs.* matched normal control subjects (Fig. 2*a*, *b*). Although the entire study was conducted in a matched pair design (see Methods), the results of an independent *t*-test are provided for the reader in Supplementary Table 3 which shows similar statistical results for the comparison of control and MDD groups (*GRIN2B*, $p=0.003$; *GRIN2C*, $p=0.061$).

In contrast, expression levels for genes for other NMDA receptor subunits (*GRIN1, GRIN2A, GRIN2D, GRIN3A*; Supplementary Fig. 2*A–D*), for the ionotropic AMPA receptor subunits (*GRIA1, GRIA2, GRIA4*; Supplementary Fig. 3*A–C*), and kainate receptor subunits (*GRIK1, GRIK3, GRIK5*; Supplementary Fig. 3*D–F*) were not significantly different comparing MDD and control subjects. Percentage differences in expression levels of MDD *vs.* matched control subjects for these additional ionotropic glutamate receptor genes were (in percentage of controls) 109±8 (*GRIN1*), 107±9 (*GRIN2A*), 101±8 (*GRIN2D*), 122±38 (*GRIN3A*), 92±7 (*GRIA1*), 96±5 (*GRIA2*), 103±6 (*GRIA4*), 96±4 (*GRIK1*), 103±11 (*GRIK3*), and 102±7 (GRIK5).

Expression levels of genes for metabotropic receptors mGluR4 and mGluR5 (*GRM4* and *GRM5*) were significantly higher in LC neurons from MDD subjects *vs.* matched control subjects (Fig. 2*c*, *d*), although the magnitude of the difference between MDD and control subjects for *GRM4* was small. Amplimer amounts in MDD subjects as a percentage of matched controls were 116±5 (*GRM4*; $p<0.01$) and 145±9 (*GRM5*; $p<0.0005$). *GRM8* expression levels (i.e. 106±3%) were similar in MDD and matched control subjects (Supplementary Fig. 2*E*). Comparison of gene expression levels between controls and MDD groups using an independent *t*-test showed similar results for *GRM5* expression ($p=0.000$), but *GRM4* expression was not significantly different ($p=0.103$; Supplementary Table 3).

Table 3. Expression levels of glutamate receptor genes, determined using RNA from punch-dissected locus coeruleus (LC) tissue from four control subjects

Glutamate receptor gene	Glutamate receptor name	Expression level determined from Allen Brain Atlas (mouse)[a]	Expression level determined by qPCR (human)
GRIN1	Ionotropic NMDA 1	+++[b]	++++
GRIN2A	Ionotropic NMDA 2A	−−	++
GRIN2B	Ionotropic NMDA 2B	++	++
GRIN2C	Ionotropic NMDA 2C	−−	+
GRIN2D	Ionotropic NMDA 2D	−−	++
GRIN3A	Ionotropic NMDA 3A	+++	+
GRIN3B	Ionotropic NMDA 3B	−−	−−
GRIA1	Ionotropic AMPA 1	+	+
GRIA2	Ionotropic AMPA 2	+++	+++
GRIA4	Ionotropic AMPA 4	+++	+++
GRIK1	Ionotropic, kainate 1	+++	+
GRIK3	Ionotropic, kainate 3	++	+
GRIK5 (KA2)	Ionotropic, kainate 5	++	++
GRM4	Metabotropic 4	++	+
GRM5	Metabotropic 5	+++	++
GRM8	Metabotropic 8	++	++

[a] As determined using the Allen Brain Atlas, http://mouse.brain-map.org/, Lein et al. (2007).
[b] Five levels of density ranking of gene expressions were qualitatively assessed as follows: ++++, Very high levels; +++, High levels; ++, Intermediate levels; +, Low levels; −− Undetectable by method.

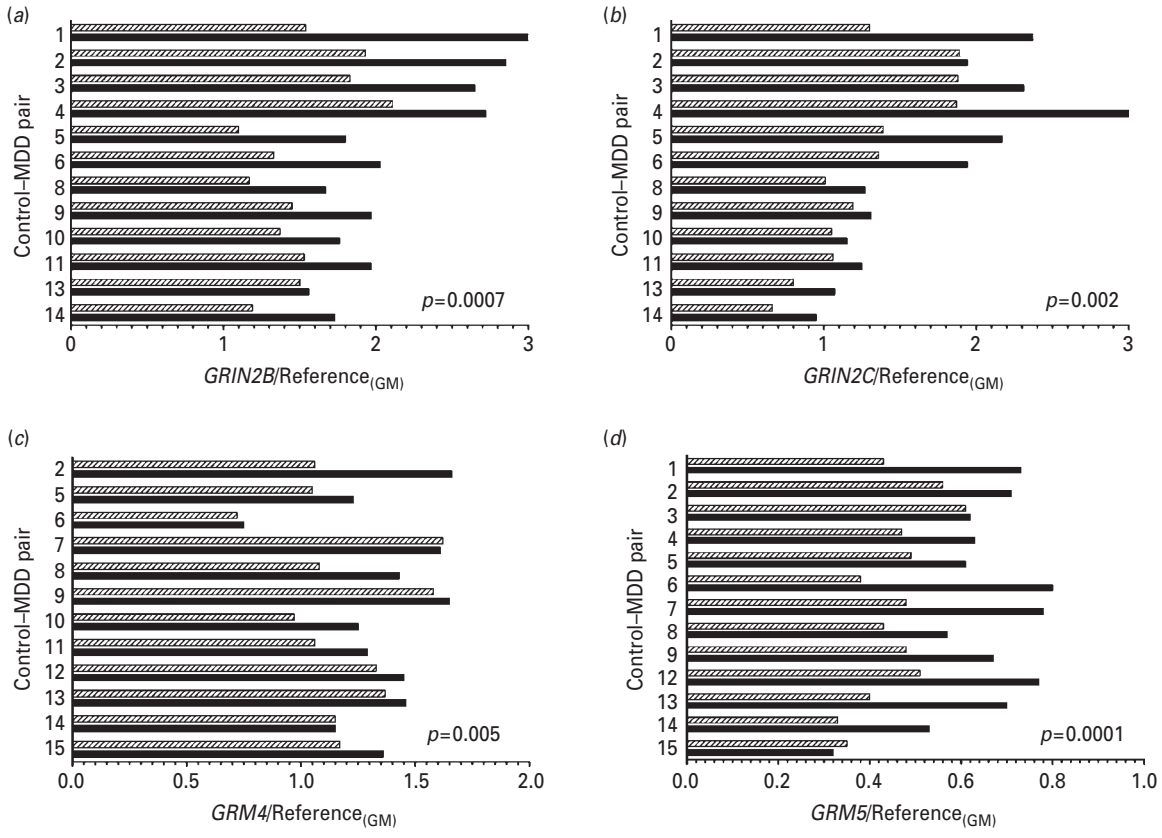

Fig. 2. Statistically significant gene expression changes of glutamate receptors or receptor subunits in LC neurons laser captured from psychiatrically normal controls (slashed bars) and subjects with major depressive disorder (MDD) (solid bars). Each control-MDD pair is identifiable by a distinct number on the *y*-axis that is consistent throughout the study. Expression of *GRIN2B* (*N*=12), *GRIN2C* (*N*=12), *GRM4* (*N*=12), and *GRM5* (*N*=13) was normalized to geometric mean (GM) of three reference genes (*GAPDH, UBC, ACTB*). Significance levels are indicated in the bottom right corner of each panel.

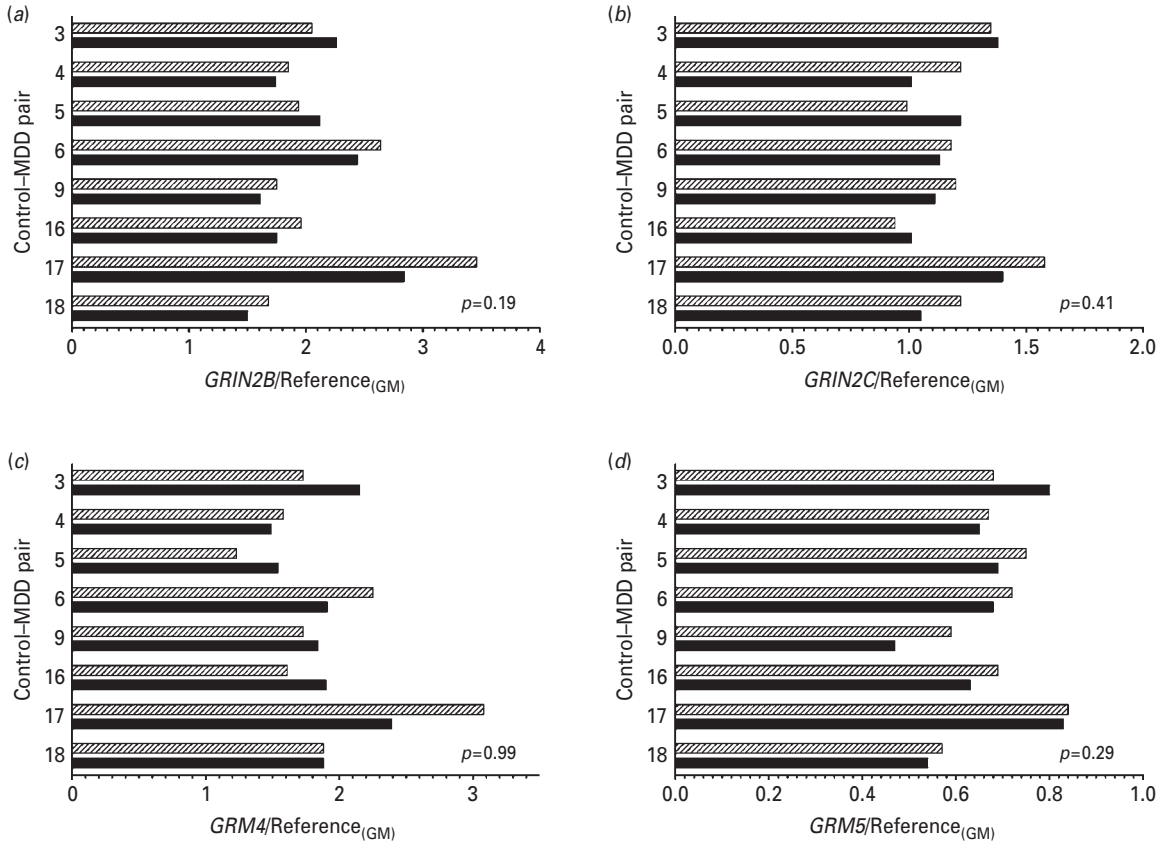

Fig. 3. Gene expression of glutamate receptors or receptor subunits in pyramidal neurons laser captured in frontal cortex, BA10, from psychiatrically normal controls (slashed bars) and subjects with major depressive disorder (solid bars). Target gene expression (*GRIN2B N*=8; *GRIN2C N*=8; *GRM4 N*=8; and *GRM5 N*=8) was normalized to the geometric mean (GM) of three reference genes (*GAPDH, UBC*, and *ACTB*). No significant changes were identified.

Glutamate receptor subunit gene expression in prefrontal cortex BA10

To determine if MDD-associated abnormalities in gene expression of glutamate receptors observed in the LC were common to brain neurons, gene expression levels were examined in pyramidal neurons captured by laser microdissection from prefrontal cortex (BA10). Reference gene comparisons between controls and MDD subjects revealed no significant differences (Supplementary Fig. 4). In contrast to LC neurons, no differences in expression levels of *GRIN2B, GRIN2C, GRM4* or *GRM5* were observed in pyramidal neurons from prefrontal cortex comparing MDD to control subjects (Fig. 3*a–d*). An independent *t*-test of cortical pyramidal neuron data yielded similar statistical findings (Supplementary Table 4).

Discussion

The present laser capture study demonstrates elevated expression levels of genes encoding specific ionotropic NMDA receptor subunits and specific metabotropic receptors in LC noradrenergic neurons of MDD subjects relative to psychiatrically normal control subjects. This study focused on single cell-type analysis of MDD pathology and extends the work of a previous study that investigated an admixture of cells wherein elevated protein levels of the NR2C subunit were observed in a multi-cell type admixture of LC tissue from MDD subjects (Karolewicz et al., 2005). In addition, we previously reported reduced expression of excitatory amino acid transporters in astrocytes surrounding the LC in MDD subjects (Chandley et al., 2013). Together, these findings provide strong support to the hypothesis that MDD is associated with disrupted communication between glutamatergic and noradrenergic LC neurons.

The present study is the first to extensively characterize the relative gene expression levels of glutamate receptors and their subunits in the human LC. In addition to the highly expressed NR1 NMDA subunit (*GRIN1*), we found moderate gene expression levels of NR2A (*GRIN2A*), NR2B (*GRIN2B*), NR2D (*GRIN2D*) subunits and low levels of NR2C (*GRIN2C*) and NR3A (*GRIN3A*) subunits. The functional NMDA receptor complex is made of a glycine binding NR1 subunit combined with at least one of the other glutamate binding NR2 or NR3 subunits. Although, permeable to both potassium and calcium, calcium is essential in activating the PI3K and CREB cell-signaling pathways that distinguish the NMDA family of receptor signaling from the other

ionotropic glutamate receptors (Lonze and Ginty, 2002; Papadia et al., 2005). This is particularly intriguing since we observed elevations in NMDA receptor subunit gene expressions in MDD, but no gene expression changes in the moderately expressed GluA1 receptor (*GRIA1*) or the highly expressed GluA2 (*GRIA2*) and GluA4 (*GRIA4*) of the AMPA ionotrophic family, nor in any of the receptor subunits (*GRIK1, GRIK3, GRIK5*) from the kainate ionotrophic class of receptors. If altered NMDA receptor subunit gene expression is secondary to elevated glutamate input to the LC, as has been presumed based on post-mortem indices of glutamatergic activity (Chandley et al., 2013), one might have expected to find alterations in other subtypes of ionotropic receptors on LC neurons. Since increases in gene expression were only identified in the NMDA family of inotropic glutamate receptors, gene expression of these subunits may be uniquely susceptible to elevated glutamatergic input. Alternatively, altered expression may be a fundamental pathology of LC neurons unrelated to any MDD-associated abnormalities in glutamatergic input. It is noteworthy that LC neurons are initially activated by glutamate via the NMDA receptor, followed by a post-activation inhibition mediated by glutamate's action at a kainate/AMPA potassium-gated channel (Zamalloa et al., 2009). It is conceivable that an elevation of NMDA receptors (or a change in their activity; see below) without any changes in kainate/AMPA receptors may result in greater or more sustained excitability of noradrenergic neurons in MDD.

Expression of three metabotropic glutamate receptor genes (*GRM4, GRM5, GRM8*) has previously been demonstrated in the nonhuman primate LC (Noriega et al., 2007) and the present study confirmed their expression in the human LC. These three metabotropic receptors belong to two subclasses of G-protein coupled metabotropic glutamate receptors (mGluRs) and utilize different signaling pathways. Group I mGluRs (mGluR5, *GRM5*) are $G\alpha_q$-linked to initiate phospholipase C and the diacylglycerol signaling pathways; Group II (mGluR4 [*GRM4*] and mGluR8 [*GRM8*) are $G\alpha_{i/o}$-linked to inhibition of the cAMP pathway (as reviewed by (Conn and Pin, 1997). Our studies identified a robust increase in expression levels of *GRM5*, but not *GRM8*, in LC neurons from MDD subjects in comparison to normal control subjects. A small, but statistically significant, increase in *GRM4* expression levels was also observed. Interestingly, negative allosteric modulators of mGluR5 have antidepressant efficacy in rodent models (Hughes et al., 2013).

Limitations

There are limitations in the present study that should be considered. We were unable to examine protein levels for each receptor that exhibited elevated gene expression levels. As noted above, our laboratory has previously shown elevated NR2C subunit immunoreactivity in homogenized tissue punches of the LC in MDD subjects, most of which died by suicide (Karolewicz et al., 2005). In the same study, NR2B immunoreactivity was below levels that permitted quantification in the LC. At least for the measurable NR2C subunit, the present findings imply that elevated NR2C protein found in the previous study (which utilized LC homogenates containing multiple cell types) occurs on noradrenergic neurons in the LC. However, it remains possible that NR2C subunit protein expressed on glial cells could be similarly elevated in MDD, since the present study did not examine glutamate receptor gene expression on glia in the LC. The generally low expression levels of G-protein-coupled glutamate receptors, e.g. mGluR4, mGluR5, mGluR8, in the brain, does not permit quantification of protein levels in extremely small tissue samples typical of LC collection methods. There are not currently available antibodies for glutamate receptors that are adequate for quantitative immunolabelling of frozen human post-mortem brain sections.

The increase in *GRIN2C* expression (present study) and increase in NR2C protein levels (Karolewicz et al., 2005) in the LC of MDD subjects was not accompanied by increases in *GRIN1* expression (present study) and protein expression of the NR1 subunit of the NMDA receptor (Karolewicz et al., 2005). The NR1 subunit is a mandatory subunit of the multi-subunit NMDA receptor complex. Hence, our findings do not support the concept of more NMDA receptors in MDD, but a possible change in the subunit composition, and thereby the ionotropic properties, of the NMDA receptor in MDD.

Finally, all but two MDD subjects died by suicide. Hence, we are unable to statistically evaluate the possible association of gene expression abnormalities specific for MDD but separate from suicide. It can be noted that in every instance, the expression levels of *GRIN2B, GRIN2C, GRM4* and *GRM5* in LC neurons of the two non-suicide MDD subjects were higher than the paired normal control subjects.

Conclusion

Numerous studies in humans and laboratory animals indicate that MDD is associated with elevated glutamatergic activity. A variety of factors (psychological stress, magnesium deficiency, oxidative stress, inflammation, bioenergetic dysfunction) associated with depression are known to activate NMDA receptor signaling (Marsden, 2011). The present study contributes to growing evidence of altered glutamatergic input to the noradrenergic LC in MDD (Karolewicz et al., 2005; Miguel-Hidalgo et al., 2010; Chandley et al., 2013). Drugs that target NMDA and mGluR receptors are known to exert antidepressant effects in both humans and animals (Zarate et al., 2006; Dhir and Kulkarni, 2008; Muhonen et al., 2008; Krystal et al., 2011). It seems reasonable to suggest that at least

part of the antidepressant efficacy of drugs such as the NMDA receptor antagonist ketamine may be mediated by reducing an MDD-associated elevation of glutamatergic input to the noradrenergic LC. The importance of the effect of these drugs on LC activity seems highly relevant because traditional antidepressant drugs, including selective serotonin uptake inhibitors and norepinephrine uptake inhibitors, reduce the activity of the LC after chronic administration. Future studies designed to elucidate the specific cellular pathology associated with glutamatergic and noradrenergic dysfunction are needed to aid in the design of better drug therapies to address the core biological features of depression that may lead to suicide.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000662

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Statement of Interest

None.

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