Published in final edited form as:

Bipolar Disord. 2010 February ; 12(1): 95–101. doi:10.1111/j.1399-5618.2009.00785.x.

N-methyl-D-aspartate receptor expression in parvalbumin-containing inhibitory neurons in the prefrontal cortex in bipolar disorder

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Abstract

Objectives—Inhibitory neural circuits and the glutamatergic regulation of these circuits in the cerebral cortex appear to be disturbed in bipolar disorder. In this study, we addressed the hypothesis that, in the prefrontal cortex (PFC), disturbances of glutamatergic regulation of the class of inhibitory neurons that contain the calcium buffer parvalbumin (PV) via N-methyl-D-aspartate (NMDA) receptor may contribute to the pathophysiology of bipolar disorder.

Methods—We used double *in situ* hybridization with a sulfur-35-labeled riboprobe for the NR2A subunit of the NMDA receptor and a digoxigenin-labeled riboprobe for PV in a cohort of 18 subjects with bipolar disorder and 18 demographically matched normal control subjects.

Results—We observed no differences in the relative density and laminar distribution of the PV-expressing neurons between subjects with bipolar disorder and matched normal control subjects. Furthermore, the density of the PV neurons that co-expressed NR2A messenger RNA (mRNA) or the cellular expression of NR2A mRNA in the PV neurons that exhibited a detectable level of this transcript was unaltered in subjects with bipolar disorder.

Conclusions—These findings suggest that, in the PFC, glutamatergic regulation of PV-containing inhibitory neurons via NR2A-containing NMDA receptors does not appear to be altered in bipolar disorder. However, the possibility that other subsets of γ -aminobutyric acid (GABA) neurons or other glutamate receptor subtypes are affected cannot be excluded.

Keywords

GABA; gene expression; *in situ* hybridization; NMDA; NR2A; postmortem; schizophrenia

Inhibitory neural circuits in the cerebral cortex have been established as functionally disturbed in schizophrenia, but recent studies suggest that disturbances of inhibitory neurons may also contribute to the pathophysiology of bipolar disorder (1-5). Because inhibitory

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The authors of this paper do not have any commercial associations that might pose a conflict of interest in connection with this manuscript.

neurons are functionally and connectionally diverse, with different subsets of these neurons regulating distinct aspects of information flow in the cortex (6), it is imperative to determine the specific subsets of these neurons that are affected in order to be able to truly appreciate the pathophysiologic consequences of the functional disturbances of these neurons. Toward this end, findings of postmortem studies in the past few years have led to the general agreement that inhibitory neurons that contain the calcium buffering protein parvalbumin (PV), which exhibit fast-spiking firing properties and target the perisomatic (basket cells) and axo-axonic (chandelier cells) compartments of pyramidal neurons (7,8), are functionally compromised in schizophrenia (5). For example, in the prefrontal cortex (PFC), the expression of the messenger RNA (mRNA) for PV and the γ -aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (GAD)₆₇ in the PV-containing neurons appears to be significantly decreased in subjects with schizophrenia (9). Furthermore, we have recently found that, in schizophrenia, the expression of the mRNA for the NR2A subunit of the N-methyl-D-aspartate (NMDA) receptor in these neurons also appears to be decreased (10). This latter finding supports the notion that reduced glutamatergic inputs to PV neurons via the NMDA receptor contributes to the downregulation of PV and GAD₆₇ transcripts (11,12) and hence plays a central role in the functional disturbances of PV neurons in schizophrenia (13,14).

Recently, we found that, in bipolar disorder, the expression of the mRNA for the GluR5 subunit of the kainate class of glutamate receptor in GABA neurons was decreased in the anterior cingulate cortex (15), whereas the expression of NR2A mRNA in GABA neurons in the PFC was undisturbed (1). Therefore, it may be that, in bipolar disorder, glutamatergic regulation of GABA neurons is disturbed only in the anterior cingulate cortex, but not in the PFC. However, if NR2A expression is altered in only a subset of GABA neurons in the PFC, depending upon the magnitude of change in transcript expression and the percentage of the subset of neurons that is affected, it may not be detectable when the entire population of GABA neurons is examined. In this study, we investigated whether NR2A expression might be altered in the subset of GABA neurons that express PV, which constitute roughly 20-50 % of all GABA neurons in the primate PFC (16). Using double *in situ* hybridization to colocalize the mRNA for PV and NR2A in a cohort of subjects with bipolar disorder and demographically matched normal control subjects, we found that the expression of NR2A mRNA in PV neurons in the PFC was unchanged in bipolar disorder. Thus, although inhibitory neural transmission in the cerebral cortex is disturbed in both bipolar disorder and schizophrenia (1-5), the manner in which glutamatergic regulation of inhibitory neural circuits is impaired appears to be disease- and region-specific.

Methods

Human subjects

Postmortem human brains from 18 subjects with bipolar disorder (12 with a history of psychosis) and 18 normal control subjects, matched for age, postmortem interval (PMI), and, wherever possible, sex, hemispheric laterality, and brain pH, were obtained from the Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, MA, USA (Table 1). These same tissues have been used in a previous study (1). Two psychiatrists reviewed all medical records and an extensive family questionnaire that included medical, psychiatric, and social history and applied DSM-III-R criteria to make the diagnosis of bipolar disorder. All brains were examined by a neuropathologist to rule out any neurologic conditions. In addition, toxicologic examination confirmed that none of the subjects included in this study suffered from any active substance abuse disorders at the time of death.

Tissue processing and *in situ* hybridization

Tissue blocks containing Brodmann's area 9, each about 3 mm in thickness, were removed from fresh brain specimens at the dorsolateral level, which is bounded by the superior frontal sulcus and contained within the superior frontal gyrus (17). Specimens were fixed in 0.1% paraformaldehyde in ice-cold 0.1M phosphate buffer (pH 7.4) for 90 minutes, immersed in 30% sucrose in the same buffer overnight, and then frozen in Tissue Tek OCT (Sakura Finetek, Torrance, CA, USA). Sections of 10 μ m were made on a cryostat, mounted on slides, and stored at -70°C until use. Two sections per subject were used for *in situ* hybridization. Tissue sections were processed with a ³⁵S-radiolabeled riboprobe for NR2A (Genbank Accession No. M91561) and a digoxigenin-labeled riboprobe for PV (Genbank Accession No. NM_002854.2), as previously described (1,4,18). Control experiments with sense probes produced no specific staining (1,4,10,19).

Data collection

Sulfur-35 labeling of NR2A mRNA appeared as clusters of silver grains after processing for emulsion autoradiography, and digoxigenin labeling, in the form of a brown reaction product (Figure 1), was visualized under a bright field microscope (Laborlux, Leica Microsystems, Wetzlar, Germany) equipped with polarizing filters to enhance the optical density of the reaction product (1,4,19). Labeled neuronal profiles in a 250 μ m-wide cortical traverse extending from the pial surface to the white matter border were identified using a 100X oil immersion objective lens on the microscope, which was fitted with a solid charge coupled device (CCD) video camera connected to a Bioquant Nova Image Analysis System (R&M Biometrics, Memphis, TN, USA), as described previously (1,4,19). All cortical traverses were placed within Brodmann's area 9, which was identified based on known cytoarchitectural criteria (17). Two cortical traverses per section, and therefore four cortical traverses per case, were analyzed. Neighboring sections were stained with cresyl violet for accurate determination of laminar boundaries. For each case, cell density averaged from the four cortical traverses was used in statistical analysis. All counts were made by one investigator (BKYB) without knowledge of case number or diagnosis. Prior to the actual data collection, intra-rater reliability, as assessed by counting and recounting profiles in the same column, was established to be above 95%.

The quantification procedure applied has been described previously (1,4,18,19). Briefly, to quantify the expression level of mRNA for the NR2A subunit in individual PV cells, the area occupied by silver grain clusters was carefully outlined using a cursor displayed on the computer monitor. The cluster area was measured by highlighting the grains with a thresholding subroutine. The threshold parameters were held constant throughout the entire course of quantification. The total area covered by individual autoradiographic grains within each grain cluster was automatically computed by the Bioquant program based on the threshold value and was represented as a pixel count. This value was then divided by the cluster area to give the grain density measure for each PV+/NR2A+ neuron. Specific signal (i.e., NR2A expression level) due to hybridization was obtained by subtracting tissue background (i.e., pixel count of the area covered by autoradiographic grains per unit area in square micrometers in the white matter). The average NR2A expression level in PV neurons for each cortical layer was then computed. Intra-rater reliability in grain density measurements, which was accessed by repeating the procedures described herein on the same clusters, was determined to be consistently greater than 95% before the actual data-collection process.

Statistical analysis

The densities of PV+ and PV+/NR2A+ neurons and the grain density, which is a measure of NR2A mRNA expression level per PV+ neurons, were compared between both groups

across layers 2 through 6 using analysis of variance (ANOVA). Layer 1 was not included in the analyses as no PV+ neurons were found in this layer. We also evaluated the effect of confounding variables, such as age, PMI, brain pH, freezer storage time, and exposure to antipsychotic medications [expressed as the chlorpromazine equivalent dose (CED)] or divalproex by using analysis of covariance (ANCOVA). Because none of the conclusions derived from our findings were affected by the ANCOVA analysis, only results from repeated-measures ANOVAs are reported. In addition, Pearson's correlation was used to assess if there was any linear relationship between cell and NR2A mRNA expression levels and any of the continuous variables. Effects of hemispheric laterality and sex on our findings were evaluated by using two-tailed unpaired *t*-tests to compare the measures from the two hemispheres within individual groups. All statistical analyses were conducted using the statistical software SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) with $\alpha = 0.05$.

Results

The effect of diagnosis on the densities of PV+/NR2A+ ($F_{1,34} = 0.029$; $p = 0.866$) and PV+ ($F_{1,34} = 1.493$; $p = 0.230$) neurons was not significant (Fig. 1, Table 2). In addition, the NR2A mRNA expression level per PV+ neuron did not differ between the two subject groups (Fig. 2), indicating that in the PV cells that expressed a detectable level of NR2A mRNA, the amount of transcript expression was unaltered in subjects with bipolar disorder. Because pH for 5 of the bipolar subjects and 2 of the normal control subjects was not available, we reanalyzed the data without these subjects. Even after dropping these subjects, the effect of diagnosis on the densities of PV+/NR2A+ ($F_{1,27} = 2.091$; $p = 0.160$) and PV+ ($F_{1,27} = 3.424$; $p = 0.075$) remained nonsignificant. These conclusions were not affected when data analyses were performed separately for bipolar subjects with [PV+/NR2A+ ($F_{1,28} = 1.326$; $p = 0.259$), PV+ ($F_{1,28} = 3.238$; $p = 0.083$)] or without psychosis [PV+/NR2A+ ($F_{1,22} = 1.265$; $p = 0.273$), PV+ ($F_{1,22} = 0.39$; $p = 0.846$)]. Furthermore, no statistically significant differences were obtained for PV+ ($F_{1,16} = 0.123$; $p = 0.731$) or PV+/NR2A+ ($F_{1,16} = 2.571$; $p = 0.128$) neuronal densities when bipolar subjects not medicated with antipsychotic drugs were compared to bipolar subjects who were receiving antipsychotic treatment.

For gene expression studies, pH, age, and PMI are considered particularly important because they are indirect indicators of the quality and integrity of mRNA (20-23). In this study, there was no statistically significant difference in pH ($t = 0.369$; $p = 0.71$), age ($t = 0.324$; $p = 0.75$), or PMI ($t = 0.229$; $p = 0.82$) between the two subject groups. Correlation analyses also revealed that none of these measures or the exposure to antipsychotic medications ($n = 10$) or divalproex ($n = 8$) appear to have any effects on the dependent variables (Table 3).

Discussion

Postmortem human brain studies in the past two decades have played a pivotal role in advancing our understanding of the pathophysiology of schizophrenia (5,24-27). One of the major conclusions that can be drawn from these studies is that inhibitory neural circuits in the cerebral cortex are disturbed in schizophrenia (5,25,26,28). Specifically, the subset of inhibitory neurons that contain the calcium buffer PV appears to be preferentially affected (5). Furthermore, disturbed glutamatergic inputs to these neurons may contribute, at least in part, to their functional deficits (11,13,14). PV neurons target the perisomatic and axo-axonic compartments of pyramidal cells and exhibit fast-spiking firing properties (7,29). Together, these connectional and physiologic characteristics confer PV neurons the ability to synchronize the activities of pyramidal neural networks (30,31). Importantly, impairment in synchrony of neural activities at beta (13-30 Hz) and gamma (30-100 Hz) frequency bands is increasingly thought to be a key pathophysiologic feature of schizophrenia (32).

In contrast, there have been fewer postmortem studies that focused on bipolar disorder; a primary reason may have to do with the relative lack of available tissues (33). Nevertheless, a number of these studies suggest that inhibitory neurons in the cerebral cortex may be functionally disturbed in bipolar disorder in a fashion that is not dissimilar to what has been observed in schizophrenia (25,26,28). For example, similar to findings in schizophrenia, the expression of the mRNA for GAD₆₇ has been found to be significantly decreased in subjects with bipolar disorder (2,4,15,18,28). Interestingly, *GAD1*, the gene that encodes GAD₆₇, has been suggested to be a risk gene for bipolar disorder (34). In addition, the expression of the mRNA for various glutamate receptor subunits and postsynaptic density proteins have been shown to be altered in bipolar disorder (35-38), suggesting that glutamatergic neurotransmission is dysregulated.

Emerging evidence suggests that glutamatergic inputs to inhibitory neurons are disturbed in both bipolar disorder and schizophrenia. For example, we recently found that, in the anterior cingulate cortex, the expression of the kainate receptor GluR5 mRNA in neurons that expressed GAD₆₇, which presumably included all GABA neurons, was significantly decreased in both disorders (15). However, in the same cortical region, the expression of the mRNA for the NMDA NR2A subunit in a subset of GABA neurons that express the calcium buffer calbindin, which primarily targets the dendrites of pyramidal cells, appears to be unaltered in bipolar disorder but increased in schizophrenia (19). Furthermore, in the PFC, the expression of NR2A mRNA in GAD₆₇-expressing neurons has been shown to be unchanged in bipolar disorder but decreased in schizophrenia (1). These observations, as summarized in Table 4, together with findings of the present study, suggest that although glutamatergic regulation of inhibitory neural circuits may be disturbed in both bipolar disorder and schizophrenia, the exact nature of these disturbances appear to be brain region-, glutamate receptor subtype-, GABA neuronal class-, and disease-specific. Hence, delineating the specific elements within the inhibitory neural circuits that are disturbed may inspire the conceptualization of rational, pathophysiology-based therapeutic strategies that aim at recalibrating or normalizing the malfunctioning brain circuits in these disorders. To this end, collectively our findings (Table 3) suggest that understanding how the expression of the GluR5 glutamate receptor subunit is altered in subsets of inhibitory neurons will provide further insight into the pathophysiology of bipolar disorder.

Acknowledgments

Funding sources: Grants MH076060 and MH068541 from the National Institutes of Health.

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Fig. 1. Mean ($\pm$ SEM) density of PV+/NR2A+ neurons is unaltered in the prefrontal cortex in subjects with bipolar disorder. Inset is an example of a double-labeled neuron (arrow); scale bar = 15 μ m.

Fig. 2.

Mean ($\pm$ SEM) density of silver grains over PV+ neurons in the prefrontal cortex is not different between the two subject groups, suggesting that the expression level of NR2A mRNA in parvalbumin cells is unchanged in bipolar disorder.

Table 1

Demographic information on cases used in the present study

Case	Diagnosis	Age	Sex	Side	pH	PMI	CED	Psychotropics received at time of death	Cause of death
1	CON	49	M	L	6.76	24.6		None	Myocardial infarction
2	CON	37	M	R	6.68	18.8		None	Electrocution
3	CON	54	M	L	6.53	24.2		None	Cardiopulmonary arrest
4	CON	78	F	R	6.22	14.1		None	Myocardial infarction
5	CON	53	M	R	U	20.2		None	Cardiopulmonary arrest
6	CON	65	F	R	6.40	24.3		None	Lung cancer
7	CON	89	M	R	6.39	7.42		None	Cancer
8	CON	69	M	R	6.88	15.3		None	Respiratory failure
9	CON	74	F	L	U	12.5		None	Cardiopulmonary arrest
10	CON	66	F	R	6.03	7.4		None	Cancer
11	CON	42	M	L	6.78	18.3		None	Myocardial infarction
12	CON	78	F	R	6.67	23.9		None	Breast cancer
13	CON	40	M	L	6.24	16.6		None	Myocardial infarction
14	CON	67	M	L	6.42	22.3		None	Cardiopulmonary arrest
15	CON	70	F	L	6.26	22.5		None	Liver cancer
16	CON	66	M	R	6.76	18.7		None	Myocardial infarction
17	CON	79	M	L	6.74	20.9		None	Cancer
18	CON	38	M	L	6.53	28.8		None	Myocardial infarction
Mean ± SD		61.9 ± 12.6			6.52 ± 0.21	18.9 ± 4.59			
19 ^a	BD	85	M	R	6.60	17.5	0	Divalproex (500 mg)	Cardiopulmonary failure
20	BD ^b	74	F	R	U	22.9	100	Divalproex (750 mg), lorazepam, olanzapine	Cardiopulmonary failure
21	BD	26	F	L	6.35	22.8	0	Lithium, lorazepam	Cardiopulmonary failure
22	BD ^b	52	F	R	U	17.2	200	Olanzapine	Hepatic failure
23	BD	62	F	R	U	13.4	0	Divalproex (1250 mg), bupropion, sertraline	Breast cancer
24	BD ^b	74	M	L	6.70	7.2	100	Gabapentin, olanzapine	Pneumonia
25	BD ^b	85	F	L	6.28	27.5	534	Olanzapine, divalproex (750 mg)	Cardiopulmonary failure
26	BD	51	M	L	7.02	31	0	Clonazepam, gabapentin	Suicide by overdose
27	BD	69	M	L	U	29.5	0	Lithium	Pneumonia

Case	Diagnosis	Age	Sex	Side	pH	PMI	CED	Psychotropics received at time of death	Cause of death
28	BD ^b	64	F	R	6.26	11	800	Trifluoperazine	Respiratory failure
29	BD ^b	42	F	L	6.60	15.8	960	Lithium, divalproex (1500 mg), perphenazine	Medication overdose
30	BD ^b	29	F	L	6.70	10.7	200	Divalproex (1250 mg), lithium, clonazepam, olanzapine	Cardiac arrest
31	BD ^b	36	M	L	U	9	U	Unknown	Suicide
32	BD ^b	73	F	R	6.32	20.8	50	Carbamazepine, risperidone	Sepsis
33	BD ^b	74	M	R	6.27	14.3	400	Divalproex (1500 mg)	Pneumonia
34	BD ^b	62	F	R	6.40	18.7	100	Divalproex (1250 mg), sertraline, risperidone, benzotropine, donepezil	Renal failure
35	BD	82	M	L	6.37	5.0	U	Unknown	Cardiac arrest
36	BD ^b	40	M	R	6.60	30.8	200	Gabapentin, ziprasidone, citalopram, risperidone, topiramate	Carbon monoxide poisoning
Mean ± SD		60.0 ± 15.6			6.5 ± 0.18	18.1 ± 6.55	331 ± 213		

All subjects in the cases presented in this study were of the white race. PMI = postmortem interval; CED = chlorpromazine equivalent dose; U = unknown or unavailable; CON = normal control; BD = bipolar disorder; M = male; F = female; L = left; R = right.

^aOnly case that was not presented in a previous study (19).

^bBipolar disorder with psychosis; parentheses after divalproex denote dosages.

Table 2

Laminar distribution of PV+/NR2A+ neurons in bipolar disorder and normal control subjects

Diagnosis	Layer	Density (neurons/mm ²)	
		Mean	± SEM
Control	2	5.80	1.06
Bipolar disorder	2	4.01	1.02
Control	3	11.53	1.12
Bipolar disorder	3	12.56	2.01
Control	4	13.33	1.87
Bipolar disorder	4	12.15	1.73
Control	5	8.90	1.03
Bipolar disorder	5	7.04	1.14
Control	6	5.08	1.21
Bipolar disorder	6	3.84	1.04

PV = parvalbumin.

Table 3

Lack of correlation between the possible confounding variables and dependent measures

	PV+		Neuronal densities		V+/NR2A+		Grain density	
	Bipolar	Control	Bipolar	Control	Bipolar	Control	Bipolar	Control
Age	$r = 0.239$; $p = 0.339$	$r = 0.091$; $p = 0.718$	$r = 0.165$; $p = 0.514$	$r = 0.271$; $p = 0.277$	$r = 0.158$; $p = 0.530$	$r = 0.207$; $p = 0.409$		
PMI	$r = 0.209$; $p = 0.406$	$r = 0.305$; $p = 0.218$	$r = 0.368$; $p = 0.133$	$r = 0.128$; $p = 0.612$	$r = 0.435$; $p = 0.071$	$r = 0.398$; $p = 0.102$		
pH	$r = -0.197$; $p = 0.640$	$r = 0.368$; $p = 0.133$	$r = 0.104$; $p = 0.681$	$r = -0.200$; $p = 0.426$	$r = -0.255$; $p = 0.306$	$r = 0.039$; $p = 0.879$		
CED	$r = 0.069$; $p = 0.786$	–	$r = 0.391$; $p = 0.109$	–	$r = 0.229$; $p = 0.361$	–		
Divalproex	$r = 0.451$; $p = 0.060$	–	$r = 0.504$; $p = 0.203$	–	$r = 0.393$; $p = 0.106$	–		

PV = parvalbumin; PMI = postmortem interval; CED = chlorpromazine equivalent dosage.

Table 4

Summary of changes in the densities of GAD₆₇-, CB-, or PV-expressing neurons that express glutamate receptor subunits (NR2A, GluR5 or GluR6) in bipolar disorder and schizophrenia^a

Transcripts	GAD ₆₇ +		NR2A+/GAD ₆₇ +		NR2A+/PV+		NR2A+/CB+		GluR5+/GAD ₆₇ +		GluR6+/GAD ₆₇ +	
Cortical region	PFC	Anterior cingulate	PFC	Anterior cingulate	PFC	Anterior cingulate	Anterior cingulate	Anterior cingulate	Anterior cingulate	Anterior cingulate	Anterior cingulate	Anterior cingulate
Bipolar disorder	Decreased (-27-36%)	Decreased (-35%)	Unchanged	Decreased (-60%)	Unchanged	Unchanged	Unchanged	Unchanged	Decreased (-40%)	Unchanged	Unchanged	Unchanged
Schizophrenia	Decreased (-27-36%)	Decreased (-28-53%)	Decreased (-49-57%)	Decreased (-52-73%)	Decreased (-48-50%)	Increased (+41-44%)	Decreased (-43%)	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged

GAD = glutamatic acid decarboxylase; CB = calbindin; PV parvalbumin; PFC = prefrontal cortex.

^aSee references 1,4,10,15,19.