ORIGINAL ARTICLE

Hippocampal Complexin Proteins and Cognitive Dysfunction in Schizophrenia

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Background: Converging neuroimaging and postmortem evidence indicates synaptic terminals are abnormal in schizophrenia. A putative molecular mechanism implicates abnormalities of proteins involved in the presynaptic secretory machinery, including the modulator proteins complexin I and complexin II.

Objectives: To determine the amount and distribution of complexin proteins in the hippocampus of subjects with schizophrenia, in parallel with markers for excitatory and inhibitory nerve terminals. The functional implications were also investigated.

Design: We used immunocytochemistry to study complexin I and complexin II proteins in hippocampus, as well as the vesicular transporters for γ-aminobutyric acid (GABA) and for glutamate. Immunocytochemical findings were correlated with cognitive function assessed through medical record review. To further explore the implications of the human findings, we studied rats exposed to haloperidol, amphetamine, and ketamine as well as rats trained in memory tasks.

Subjects: We studied hippocampal sections from 12 subjects with schizophrenia and 12 subjects with no known neuropsychiatric disorder.

Results: The absolute values and ratio of the hippocampal presynaptic proteins complexin II—complexin I were lower in subjects with schizophrenia. Disturbances in the complexin proteins in subjects with schizophrenia were greater than those observed for vesicular γ-aminobutyric acid or vesicular glutamate transporters. The lower complexin II–complexin I ratio in several hippocampal subfields in subjects with schizophrenia was inversely correlated with the severity of antemortem cognitive impairment. In contrast, the hippocampal complexin II–complexin I ratio was higher in rats trained in a memory task compared with untrained rats. Treatment of rats with antipsychotic drugs or with the psychotomimetic drugs amphetamine or ketamine did not alter the complexin II–complexin I ratio.

Conclusions: The pathology of hippocampal complexin proteins might play an important role in schizophrenia, especially concerning cognitive disturbances.

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SCHIZOPHRENIA IS CHARACTERized by acute periods of psychosis and chronic cognitive impairment. The functional mechanism of illness appears to involve abnormalities of information processing and neurotransmission rather than neuronal loss.[1] A putative molecular mechanism implicates abnormalities of proteins involved in the presynaptic secretory machinery.[2-6] These include the 3 soluble N-ethylmalemide-sensitive factor attachment protein (SNAP) receptor (SNARE) proteins (vesicle associated membrane protein [VAMP], syntaxin, and SNAP-25) crucial for synaptic neurotransmission[7,8] and modulator proteins such as complexin I and complexin II.[9-11]

A series of anatomical studies indicate that complexin I appears to be relatively more abundant in axosomatic presynaptic terminals (presumed inhibitory) and complexin II is enriched in axodendritic or axospinous terminals (presumed excitatory).[9,12] In a preliminary study of the medial temporal lobe, both complexin I and complexin II messenger RNA (mRNA) levels were lower in subjects with schizophrenia than in control subjects, with a greater effect on complexin II mRNA.[13] Complexin II protein levels were lower in the hippocampus of subjects with schizophrenia, but complexin I protein levels were not different from that in control subjects. A subsequent study partially replicated the mRNA findings but did not examine protein.[14] Alteration of the complexin II–complexin I (CxII/CxI) ratio in subjects with schizophrenia was suggested to indicate that excitatory neurons were more

Table. Demographic and Descriptive Data of the Sample

Sex/Age, y	Cause of Death	PMI, h	Onset, y	Antipsychotic Medication	Dose (Chlorpromazine Equivalents), mg	BFAS Total	CDR Total
			Schizophrenia: Impaired Cognition*				
M/65	Bowel obstruction	8.0	28	Haloperidol	600	18.0	16.0
M/56	Bronchopneumonia	23.0	25	Haloperidol	150	19.5	14.0
M/66	Pneumonia	18.0	28	Haloperidol	900	20.0	12.5
M/49	Unknown	7.0	17	Haloperidol	3000	20.0	12.0
M/65	Pneumonia	4.0	21	Haloperidol	2000	25.0	17.0
Mean, 60.2		12.0	23.8		1330	20.5	14.3
			Schizophrenia: Normal Cognition				
M/57	Myocardial infarction	4.0	24	Pericyazine	200	11.0	6.0
M/68	Pulmonary tuberculosis, diabetes	6.0	15	Haloperidol	150	13.5	8.0
F/36	Pulmonary tuberculosis	5.0	27	Methotrimeprazine	420	13.5	10.0
F/61	Thrombocytopenic purpura	2.0	24	Haloperidol	900	11.0	8.0
F/51	Sudden death, cause uncertain	8.7	22	Haloperidol	500	11.5	9.0
M/48	Unknown	8.7	16	Haloperidol	450	13.0	10.0
Mean, 53.5		5.7	21.3		434	12.3	8.5
			Schizophrenia: Unknown Cognition				
F/60	Pulmonary aspergillosis	9.0	49	Unknown	NA	NA	NA
			Controls				
M/33	Transected aorta, trauma	9.0					
M/29	Transected aorta, trauma	10.0					
F/70	Coronary vascular disease	11.0					
M/59	Myocardial infarction, diabetes	7.5					
M/68	Coronary vascular disease	11.5					
M/58	Coronary vascular disease	6.5					
M/57	Coronary vascular disease	15.5					
M/29	Multiple trauma	14.0					
F/68	Coronary vascular disease	17.0					
F/73	Bowel obstruction, tumor	22.0					
M/68	Acute myocardial infarction	22.0					
M/41	Acute myocardial infarction	17.5					
Mean, 54.4		13.6					

Abbreviations: BFAS, Blessed Functional Activity Scale; CDR, Clinical Dementia Rating; F, female; M, male; NA, not available; PMI, postmortem interval.
*Impaired cognition was defined by a CDR total score of 12 or more, consistent with the results of a previous study of patients with schizophrenia and definite cognitive impairment.[21]

affected than inhibitory neurons.[13] However, other results indicate that the specificity of individual complexins for excitatory or inhibitory neurons is uncertain. In glutamatergic dentate granule cells, expression of both complexins was detected.[15] In similar cells from complexin II knockout mice, complexin I was able to compensate functionally for the loss of complexin II, and the reverse was true—complexin II could compensate for the loss of complexin I—in cells from complexin I knockout mice.

Dysfunction of the hippocampus is implicated in specific forms of memory impairment in schizophrenia.[16,17] In Alzheimer disease, the loss of presynaptic terminal proteins is correlated with antemortem cognitive dysfunction.[18] However, for schizophrenia relatively few studies demonstrate associations between antemortem cognitive function and pathology.[19] We tested the hypothesis of abnormalities of complexin proteins in the hippocampus in schizophrenia and also investigated markers of inhibitory and excitatory presynaptic terminals. We also studied the relationship of complexin proteins to cognitive function in subjects with schizophrenia and in animal models.

METHODS

HUMAN TISSUE SAMPLES

Sections of the hippocampus were obtained postmortem from 12 controls (9 men, 3 women) and 12 patients with schizophrenia (8 men, 4 women). This sample was the same as that used in a previous study.[20] Specimens were reviewed by a neuropathologist and found to be free of neurological disease. A research psychiatrist made diagnoses according to *DSM-III-R* criteria following medical record review. The mean±SD age of patients with schizophrenia (56.8±9.4 years; range, 49-68 years) did not differ from the mean age of controls (54.4±16.8 years; range, 29-73 years), nor did mean postmortem interval (schizophrenia, 8.6±6.7 hours; controls, 13.6±5.3 hours). Most of the subjects with schizophrenia and all of the control subjects died of acute causes rather than chronic illness (**Table**). None died of suicide. The Scales of Cognitive Impairment Rated From Institutional Records (SCIRFIR) were also completed for each patient, using data from the year prior to death.[21] This battery provides scores for the Blessed Functional Activity scale[22] and the Clinical Dementia Rating.[23] The SCIRFIR have good reliability and validity, can demonstrate change over time, and show an association in patients with schizophrenia between cogni-

tive scores and senile plaque counts. The project was approved by the University of British Columbia Human Subjects Committee.

IMMUNOSTAINING PRESYNAPTIC PROTEINS

Complexin I, complexin II, vesicular γ-aminobutyric acid transporter (vGAT), and vesicular glutamate transporter (vGLUT1) were immunostained as described previously.[20] Following fixation in 10% buffered formalin for approximately 2 weeks, blocks of the hippocampal body at or anterior to the level of the lateral geniculate nucleus were embedded in paraffin and cut at a thickness of 3 µm for immunocytochemistry. Sections were deparaffinized by sequential immersion in xylene and decreasing concentrations of alcohol, followed by immersion in Tris-buffered saline (TBS) (10mM Tris-HCl; 140mM NaCl; pH 7.4). Following deparaffinization, sections were placed in sodium acetate (pH 4) at 95°C for 10 minutes. Endogenous peroxidase activity was blocked by incubation in TBS plus 0.2% Triton X-100 with 3% hydrogen peroxide for 30 minutes. Nonspecific antibody binding was blocked by incubation in a solution of TBS and 5% nonfat powdered milk for 1 hour at room temperature.[24] Primary antibodies were diluted in a solution of TBS and milk (anti–complexin I SP33 and anti–complexin II LP27 tissue culture supernatants $1:10^9$; anti-vGLUT1 1:500; anti-vGAT 1:500 [Synaptic Systems, Göttingen, Germany]) and incubated with sections overnight at 4°C. Following 3 washes in TBS for 10 minutes each, sections were incubated for 1 hour at room temperature with biotinylated goat antimouse IgG plus M (Jackson Immunolabs, West Grove, Pa) or biotinylated goat antirabbit (Jackson Immunolabs) diluted 1:500 in TBS. Sections were then subject to 3 washes in TBS of 10 minutes each, followed by an addition of 1:1000 peroxidase-conjugated streptavidin (Jackson Immunolabs) in TBS for 60 minutes at room temperature. After a final series of 3 TBS washes, sections were incubated in freshly prepared 0.03% diaminobenzidine (Sigma-Aldrich, St Louis, Mo), 0.015% hydrogen peroxide in 0.1 M Trizma-base, pH 7.4. The reaction was timed and terminated after 8 minutes. Slides were dried, dehydrated in increasingly concentrated alcohol solutions and finally xylene, and then coverslipped using Permount. For each antibody, sections were immunostained in a single batch, with conditioned tissue culture substituted for primary antibody as a negative control.

Colocalization studies using deparaffinized human tissue sections were not successful. We therefore used formalin-fixed sections of rat hippocampus, cut with a vibratome. Monoclonal antibodies reactive with complexins were used at 1:10 dilution; polyclonal rabbit antibodies reactive with vGAT and vGLUT1 were used at 1:250 dilution. Confocal microscopy was performed with a Zeiss Laser Scanning Microscope (Zeiss, Jena, Germany) using Alexa 488- and Alexa 555-labeled secondary antibodies (Molecular Probes, Eugene, Ore), both at 1:250 dilution. Images were analyzed using a combination of Zeiss PASCAL, NIH Image (National Institutes of Health, Bethesda, Md), and Adobe Photoshop (Adobe, San Jose, Calif) software.

QUANTITATIVE IMMUNOCYTOCHEMICAL STUDIES

Immunostaining was quantified using a previously described approach, which showed a linear relationship between immunostaining and antigen amount in a biological standard.[6,20] Additional studies using immunocytochemistry of human brain homogenate spotted onto nitrocellulose with a microarrayer (180-2.8 ng total protein per spot; 7-step 1:1 dilution) demonstrated a linear relationship between antigen amount and immunocytochemical signal ($r>0.97$) over an 8-fold range for complexin I and a 16-fold range for complexin II, vGLUT1, and vGAT. A reliability study of immunostaining using adjacent sections of human hippocampus from 5 cases indicated the following correlations between sections: complexin I, $r=0.93$; complexin II, $r=0.84$; vGAT, $r=0.72$; vGLUT1, $r=0.74$. In a series of 23 rat hippocampal sections, section-to-section correlations were complexin I, $r=0.79$, and complexin II, $r=0.87$ (vGAT and vGLUT1 were not studied in rat sections).

For studies of human hippocampus, we used linear regression analysis to investigate the potential effects of age and postmortem interval on immunoreactivity. Hippocampal regions of interest were clustered into 4 groups (Ammon's horn [CA1 through CA3] and the dentate gyrus). Repeated-measures analysis of variance was used for each group. In the context of prior hypotheses for differences between groups for each of the 4 antibodies tested, the α level for significant effects of diagnosis was set at $P=.0125$ to control for multiple comparisons (4 regions, CA1-CA3 and the dentate gyrus). Significant interactions between diagnosis and subregion were studied with analysis of variance using age and postmortem time as covariates. The α level for significant differences was set at $P=.0125$, to control for multiple comparisons (4 subregions, the oriens, pyramidal, radiatum/lacunosum, and molecular layers). This approach to analysis is the same as that used previously.[20]

ANIMAL STUDIES OF LEARNING AND MEMORY

Adult male Sprague-Dawley rats (250-275 g; Animal Care Centre, University of British Columbia, Vancouver) were housed individually in polycarbonate cages (24×16×46 cm) in a colony room at 20±1°C on a 12-hour light-dark cycle (lights on 7 AM to 7 PM) with unlimited access to water and food (Purina Rat Chow, St Louis, Mo). Following a brief habituation to the colony, animals were placed on a restricted food schedule, whereby they were limited to 20 g of food per day (Purina Rat Chow), which maintained them at 85% of their free-feeding weight. Animals were allowed access to water ad libitum. Prior to maze habituation, animals were given 10 Noyes food pellets (Research Diet, New Brunswick, NJ) per day for 3 days in the home cage to overcome their initial feeding neophobia to the pellets.

One group of rats (n=6) was subsequently maintained on this feeding schedule but not subjected to further behavioral testing (inactive controls). The remaining animals were habituated to a wooden, 8-arm radial maze. The maze, painted white, had an octagonal center platform (40 cm diameter), which was connected to 8 equally spaced arms, each measuring 50×9 cm, with a shallow cylindrical food cup at the end of each arm. Removable pieces of opaque white plastic (9×13 cm) were used to block the arms of the maze. The maze was raised 40 cm above the floor; surrounded by numerous, salient visual cues in the testing room; and lit by overhead illumination (100 W).

During maze habituation, 12 Noyes food pellets were scattered throughout the maze and animals were placed in the maze for 10 minutes or until the pellets were consumed. By the end of the fourth day, all animals had learned to consume the food pellets. Animals were then ranked according to the latency to consume the pellets on the fourth day and subsequently divided into 3 groups in a counterbalanced manner. The first group remained as active controls (n=6), and the remaining 2 groups were assigned to either a reference memory (n=6) or a working memory (n=5) task.

Memory tasks were adapted from an earlier study that demonstrated altered levels of gene expression of SNARE proteins in a task- and region-specific manner.[25] In the reference memory task, rats were required to visit the same 4 of 8 arms on every

trial, which were baited with a food pellet. The combination of baited arms was determined in a pseudorandom order (so that no more than 2 consecutive arms were baited) and differed for each animal. Animals were required to use spatial cues within the room to learn the location of the baited arms. For the working memory task, animals had access to only 5 of the arms, which were baited and pseudorandomly chosen (no more than 2 consecutive arms). Animals were required to visit each arm once only, and the combination of arms differed for each trial. For the active control group, 5 pellets were placed in the center of the maze, and animals were allowed to wander the maze for a period equal to the average duration of the trials taken by the reference memory and working memory groups the previous day. All groups (reference memory, working memory, and active controls) were tested consecutively with 6 trials per day. Inactive control animals were given 25 pellets in the home cage.

Animals were trained to stringent standards, whereby they were required to make no more than 1 error per trial for the last 3 trials, for 2 consecutive days. Errors for the reference memory task were defined either as entry into 1 of the incorrect (nonbaited) arms or as re-entry into an arm visited previously. Errors in the working memory task were defined as entry into an arm visited previously. After each rat reached criterion, it was deeply anaesthetized with chloral hydrate, transcardially perfused with ice-cooled 200 ml of phosphate buffered saline and 200 ml of neutral buffered formalin (4%), and killed by decapitation. The brains were removed, fixed in neutral buffered formalin for 48 hours at 4°C, and then stored in a solution of TBS and azide (1% azide) at 4°C until use. All experimental procedures for these and the following studies were conducted in accordance with the guidelines provided by the Canadian Council on Animal Care and the University of British Columbia Animal Care Committee.

ANIMAL STUDIES OF ANTIPSYCHOTIC AND PSYCHOTOMIMETIC DRUGS

The rats were pair-housed as described earlier. Animals were randomly assigned to 1 of 4 groups. The first group (n=6) received daily intraperitoneal injections of haloperidol (1 mg/kg; Sigma-Aldrich, St Louis, Mo) for 21 days and were then killed as described earlier. The second group (n=9) received a series of injections of amphetamine followed by abstinence. This paradigm was shown previously to induce both behavioral and neurochemical-neuroendocrine sensitization to a stressor imposed at the end of the abstinence period.[26,27] The regimen consisted of single daily intraperitoneal injections of d-amphetamine (2 mg/kg; Smith-Kline Beecham, Oakville, Ontario) for 7 days, followed by single daily injections of a higher dose of the drug (4 mg/kg) for an additional 7 days. The third group (n=8) received a single daily intraperitoneal injection of ketamine (RBI, Natick, Mass) for 7 days at a dose (20 mg/kg) that is sufficient to induce sensorimotor gating and cognitive deficits yet is well below the anesthetic dose of the drug.[28] As part of the sensitization paradigm, both the amphetamine and ketamine groups were left undisturbed, aside from weekly weighing and cage changing, for 2 weeks following the final injection. They were then killed. The remaining group (n=12) received daily vehicle injections (isotonic sodium chloride solution, 1 ml/kg). One vehicle-treated subgroup (n=4) was treated according to the schedule for the antipsychotic group (3 weeks of injections and then killed). The other subgroup (n=8) received isotonic sodium chloride solution injections for 2 weeks and then was left undisturbed for 2 weeks prior to decapitation. All injections took place in the animals' home cages between 9 AM and 10 AM. Animals were killed as described earlier.

RESULTS

COMPLEXIN, vGAT, AND vGLUT1 DISTRIBUTION

The distribution of complexins, vGAT, and vGLUT1 was mapped in adjacent sections from human hippocampus, and confocal microscopy was used to investigate colocalization in rat brain (**Figure 1**). The distribution of complexin I and complexin II immunostaining was distinct in subregions such as the granule cell layer but overlapped in others such as the dentate gyrus molecular layer. Complexin I immunostaining overlapped with vGAT in the dentate gyrus, particularly in the granule cell layer. However, individual cells in this layer immunostained for complexin I and vGLUT1. Some complexin I and vGLUT1 overlap was also observed in the inner molecular layer. In contrast, in the subgranular layer, complexin I immunoreactivity was low, but vGLUT1 was prominent. Complexin II immunostaining overlapped with vGLUT1 in the subgranular layer. However, in the dentate inner molecular layer, complexin II immunostaining was low, but vGLUT1 immunostaining was fairly prominent. Complexin II immunostaining in the mossy fiber region largely overlapped with vGLUT1. Individual terminals positive for complexin II and negative for vGLUT1 (and vice versa) were also observed.

Initial analyses of the synaptic proteins in individual layers of the human samples showed negative correlations between vGLUT1 immunoreactivity and age in 5/16 tests ($-0.516<r<-0.414$; $.01<P<.05$) and vGAT immunoreactivity and age in 6/16 tests ($-0.505<r<-0.419$; $.01<P<.05$). Negative correlations between the vGLUT1/vGAT ratio and age were seen in 1 layer ($r=-0.453$; $P=.03$) and the CxII/CxI ratio and age in 2 layers ($-0.432<r<-0.415$; $.04<P<.05$). The vGLUT1/vGAT ratio showed a positive correlation with postmortem interval in 1 layer ($r=0.485$; $P=.02$). The absence of a relationship between complexin proteins and age or postmortem interval is consistent with a previous report.[13] The values for vGLUT1, vGAT, the complexins, and the ratios were also compared between cases with schizophrenia where death was related to pulmonary disease (and possible hypoxia) and cases of schizophrenia without known pulmonary complications. There were no statistically significant differences. These findings are consistent with an earlier study that found no relationship between brain pH and complexin protein in hippocampus[13] and with our previous report of no effect of pneumonia as a cause of death on other presynaptic proteins in Alzheimer disease.[29]

The distribution of these proteins was studied in sections from cases of schizophrenia (**Figure 2**). For complexin I, CA2, CA3, and the dentate gyrus, each had less immunoreactivity in subjects with schizophrenia, and within regions, individual layers were affected more than others. For complexin II, all regions of the hippocampus had less immunostaining in the present cases of schizophrenia, and within each region, individual layers were affected more than others. As a consequence of the large differences in complexin II immunoreactivity, the CxII/CxI ratio was lower in all hippocampal regions

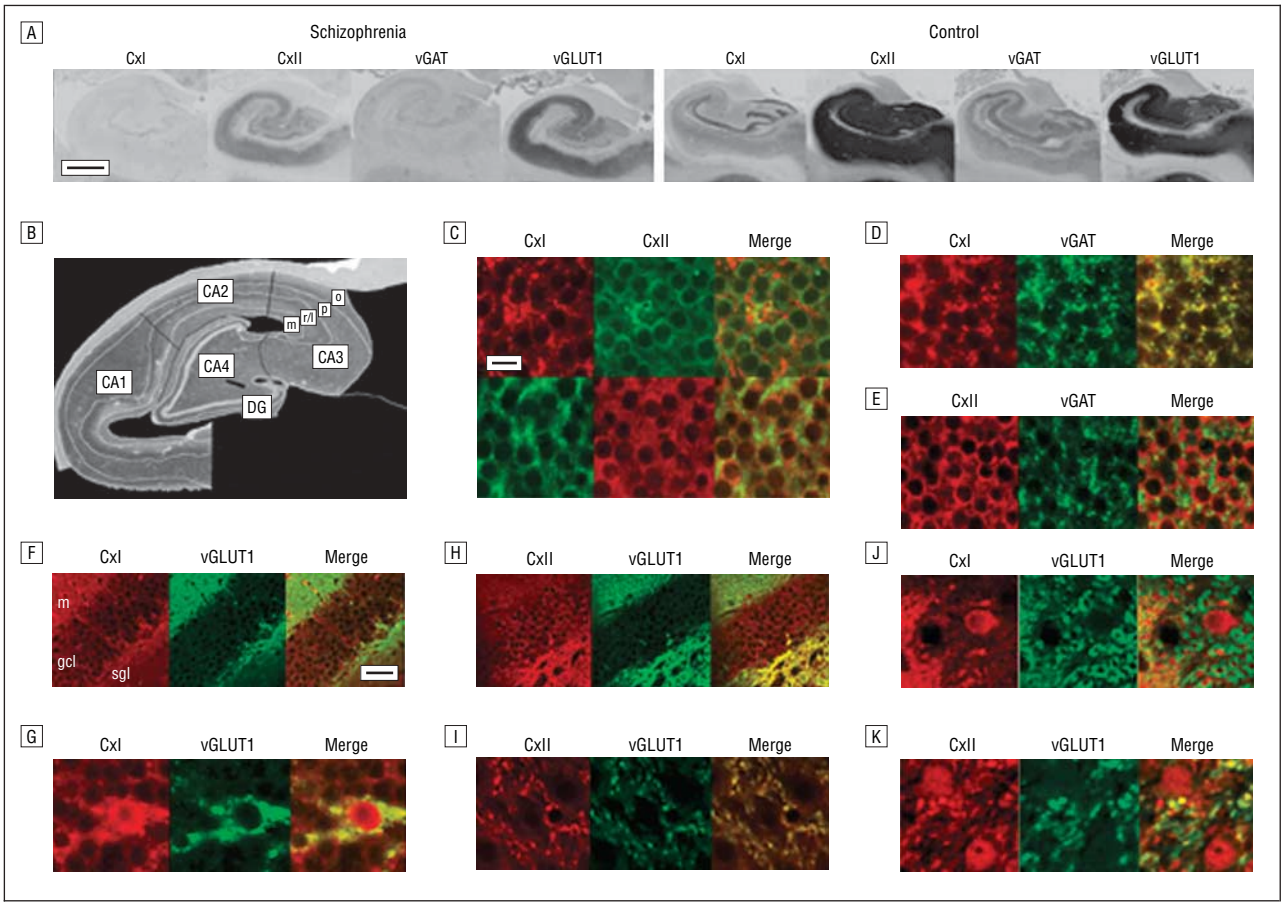

Figure 1. Complexin and vesicular transporter immunoreactivity in adjacent sections of human hippocampus from schizophrenia and control cases (A) and a reference image for layers (B). Complexin I (CxI) immunoreactivity was highest in the molecular layer of the dentate gyrus and Ammon's horn. Complexin II (CxII) immunoreactivity was more diffuse. Vesicular γ-aminobutyric acid transporter (vGAT) immunoreactivity was somewhat less intense than CxI; however, the pattern of immunostaining appeared similar. (See Figure 2 for quantification.) For the vesicular glutamate transporter (vGLUT1), immunoreactivity in the molecular layers of Ammon's horn appeared less than CxII. Scale bar represents 2 mm. Confocal microscopy was used to investigate CxI and CxII colocalization in the rat dentate gyrus granule cell layer (C). Antibodies were detected with subclass specific secondary antibodies labeled with Alexa 555 (red) or Alexa 488 (green) (Molecular Probes, Eugene, Ore). Labeling of secondary antibodies was switched in the lower row. Presynaptic terminals immunostained for CxI in the granule cell layer neuropil were negative for CxII. Scale bar represents 10 μm and applies to C-E, G, and I-K. In the granule cell layer (D, E), vGAT immunoreactivity was colocalized with CxI (D) but not CxII (E). In the dentate region (F), vGLUT1 immunoreactivity shows partial overlap with CxI in the molecular layer (m) but less in the granule cell layer (gcl) or the subgranular layer (sgl). However, isolated cells in the gcl did show CxI/vGLUT1 overlap (G). Scale bar in (F) represents 100 μm and is the same for (H). Also in the dentate (H), CxII demonstrated overlap with vGLUT1, particularly in the subgranular zone, shown at higher magnification in (I). In the mossy fiber zone (J, K), CxI was distinct in many areas from vGLUT1 (J), and CxII showed partial overlap (K). DG indicates dentate gyrus; r/l, radiatum/lacunosum layer; p, pyramidal layer; and o, oriens layer.

of subjects with schizophrenia compared with control sections. It should be noted that the results are presented as the optical density of the immunoreactivity, which appeared in our methodological studies to exhibit a linear relationship with the amount of antigen present. However, optical density and antigen amount are unlikely to have a 1:1 relationship.[6] Small percentage differences in optical density between groups might represent considerably larger percentage differences in antigen abundance.

Few statistically significant findings were observed for vGAT and were limited to effects on individual layers. Immunoreactivity of vGLUT1 was lower in several hippocampal subregions in the schizophrenia sections, although the effects were smaller compared with the differences in complexin II. The vGLUT1/vGAT ratio was altered in 1 subregion, the stratum oriens of Ammon's horn. This observation provides very limited support for the model of a reduction in the ratio of excitatory to inhibitory neurotransmission in the hippocampus of subjects with schizophrenia. The relationships between complexins, vGAT, and vGLUT1 were considered in an analysis restricted to the schizophrenia samples, with $P<.001$ as a level of significance to correct for multiple testing. Complexin I showed a correlation with vGLUT1 in the outer molecular layer of the dentate gyrus ($r=0.87$). Complexin II showed correlations with vGLUT1 in all 3 pyramidal layers; in the CA4 ($0.83\leq r\leq 0.91$), CA1, and dentate molecular layers ($0.85\leq r\leq 0.94$); and in the CA2 and CA3 radiatum/lacunosum layers ($0.89\leq r\leq 0.90$). No correlations between complexin I or complexin II and vGAT were statistically significant. Only the correlations between complexin II and vGLUT1 in the molecular layers were also statistically significant in an analysis limited to the control subjects alone. Deficits in complexin II and the glutamatergic system in the hippocampus might be linked in this series of cases of schizophrenia.

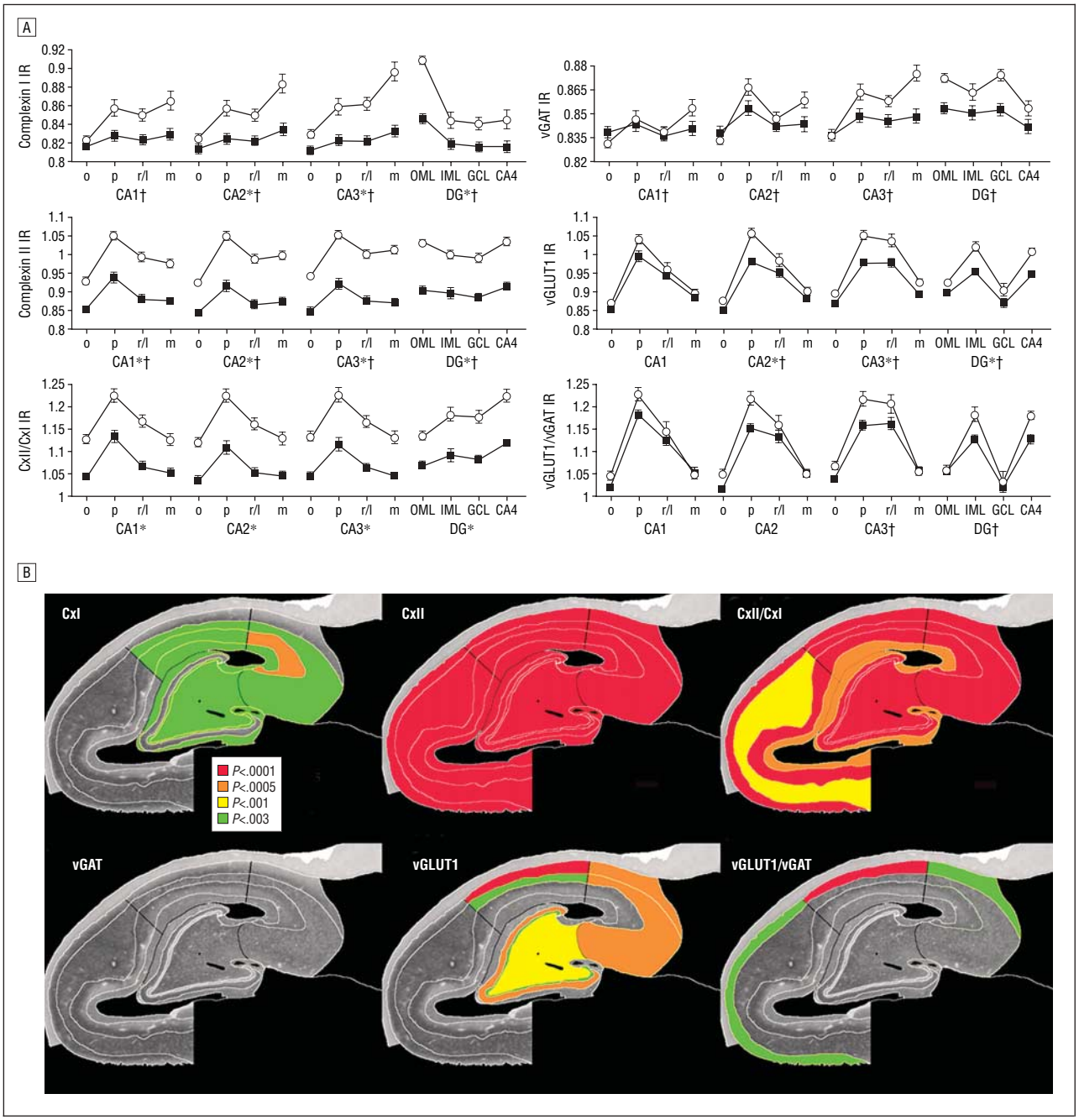

Figure 2. Quantitative studies of complexins and transporters (A). Densitometry of control (n=12, open circles) and schizophrenia (n=12, filled squares) samples indicated greater magnitude of differences between groups in complexins than in the vesicular γ-aminobutyric acid and glutamate transporters. The asterisk indicates the effect of diagnosis had $P<.01$. The dagger indicates that the diagnosis × region interaction had $P<.01$. Error bars represent SEM. (B) *P* values for regions showing statistically significant differences after correction for multiple testing. CxII/CxI indicates complexin II–complexin I ratio; DG, dentate gyrus layer; GCL, granule cell layer; IML, inner molecular layer; IR, immunoreactivity; m, molecular layer; o, stratum oriens layer; OML, outer molecular layer; p, pyramidal layer; r/l, radiatum/lacunosum layer; vGAT, vesicular γ-aminobutyric acid transporter; vGLUT1, vesicular glutamate transporter.

HIPPOCAMPAL COMPLEXINS AND MEMORY FUNCTIONS

Patient records were reviewed using rating scales that allow retrospective definition of cognitive impairment according to well-validated threshold scores.[21] Samples were categorized into 3 groups: controls, schizophrenia with cognitive impairment, and schizophrenia with no cognitive impairment (Table). Age and postmortem interval did not differ between the samples from individuals with schizophrenia with cognitive impairment and those from individuals with schizophrenia and no impairment or the control subjects. Age at illness onset did not differ between the 2 schizophrenia groups, nor did the mean of the highest dose of antipsychotic drug used in the year prior to death (in chlorpromazine equivalents). None of the individual presynaptic protein immunoreactivities or the ratio measures in any hippocampal subregion showed a statistically significant correlation with antipsychotic drug dose. We performed exploratory reanalyses with the complexin, vGAT, and

vGLUT1 data, covaried for age and postmortem interval. Complexin I did not differ between the 2 schizophrenia groups in any hippocampal region. Complexin II was lower in the sections from the group of subjects with schizophrenia with cognitive impairment compared with the sections from the group with no cognitive impairment in the CA1 oriens ($P=.02$), the CA2 pyramidal layer ($P=.03$), and the granule cell layer ($P=.02$). The CxII/CxI ratio was lower in the schizophrenia group with cognitive impairment compared with the group with no cognitive impairment in the CA1 oriens ($P=.008$), the dentate inner molecular layer ($P<.05$), and the granule cell layer ($P=.01$). Of the remaining markers, only vGAT in the CA2 oriens was lower in the cognitively impaired group ($P=.03$). Associations between global cognitive impairment (SCIRFIR score) and the CxII/CxI ratio were present for the CA1 oriens ($\rho=0.72$; $P=.03$), dentate inner molecular ($\rho=0.61$; $P=.05$) and granule cell ($\rho=0.63$; $P<.05$) layers. Cognitive functions, including memory and orientation, are particularly relevant to the hippocampus, and these showed significant correlations with the CxII/CxI ratio in the sections from the subjects with schizophrenia (**Figure 3**). Correlations were not statistically significant for complexin II or vGAT.

We investigated the possible effects of learning and memory tasks on the ratio of complexin proteins in rat hippocampus (Figure 3). Rats reached the criterion for memory performance on either the reference or working memory task within 9 days, similar to times in a previous study using the same tasks where changes in hippocampal syntaxin 1B mRNA were reported.[25] In the present study, groups of animals trained to complete reference memory or working memory tasks had higher CxII/CxI ratios in multiple hippocampal subregions compared with control animals.

HIPPOCAMPAL COMPLEXINS AND ANTIPSYCHOTIC AND PSYCHOTOMIMETIC DRUGS

Alterations in dopamine and glutamate neurotransmission might contribute to schizophrenia and to the mechanism of action of antipsychotic drugs. Antipsychotic drug treatment of the patients in the present study (all typical antipsychotics, most commonly haloperidol) could also contribute to the complexin protein findings. We studied the effects of antipsychotic and psychotomimetic drugs on complexin proteins in rat hippocampus (**Figure 4**). The effects of the antipsychotic drug haloperidol on complexin immunoreactivity were minimal, compared with animals treated with vehicle injections according to the same schedule. Treatment with the dopamine-releasing drug amphetamine or the glutamate antagonist ketamine can mimic the symptoms of schizophrenia in humans, but neither drug affected complexin proteins in rat hippocampus in a pattern similar to the observations of subjects with schizophrenia.

COMMENT

The complexin proteins are implicated in the mechanisms of schizophrenia, bipolar disorder, and Huntington disease.[3,6,13,14,30,31] Our findings support other reports of lower complexin protein and mRNA levels in the hippocampus of subjects with schizophrenia and relatively lower values of complexin II compared with complexin I.[13,14] The present results provide the first direct investigation of the hypothesis that abnormalities of the complexin proteins in the hippocampus of subjects with schizophrenia reflect an imbalance of excitatory and inhibitory neurotransmission, previously inferred through studies of the differential distribution of the complexins.[13] The results of the present parallel studies indicate relatively greater effects of schizophrenia on terminals containing vGLUT1 rather than vGAT. However, the alteration in the ratio of CxII/CxI was substantially greater than the alteration in the ratio of vGLUT1/vGAT.

Complexin proteins are important in coupling the action potential at the terminal to neurotransmitter release.[15] The distribution of complexin I and complexin II is different, and studies of mRNA expression indicate some correspondence of complexin I mRNA with inhibitory neurons and complexin II with excitatory neurons.[10,12,13,31] However, consistent with other reports,[32] the present results indicate this simple model does not apply to the distribution of complexin proteins at the presynaptic terminals where some discordance with vGAT and vGLUT1 transporters was observed. Studies of cultured neurons indicate that for several electrophysiological properties of neurons, the loss of 1 complexin isoform can be compensated for by expression of the other.[15] However, in hippocampal slice preparations, the loss of complexin II is associated with abnormalities of long-term potentiation in CA1 and in CA3.[33,34] Lower levels of the complexin proteins in subjects with schizophrenia could alter the function of individual terminals. Effects of lower complexin II might be amplified in terminals where vGLUT1 is low also.

Recent studies demonstrate that vGLUT1 is important in postnatal development and might be found in distinct sites from other glutamate transporters.[35,36] The loss of vGLUT1 results in significant impairment in neurotransmission and likely impairs different components of the release mechanism than does the loss of complexin II.[15,35] Consistent with the present results indicating lower vGLUT1 in the hippocampus of subjects with schizophrenia, a recent study indicated lower vGLUT1 mRNA expression in multiple hippocampal subfields in a different series of cases of schizophrenia.[37] However, in a third series, vGLUT1 protein values (detected with a different antibody than used here) were reported to be no different from those of control subjects in most hippocampal regions, and in a subset of cases, values were higher in the inner molecular layer of the dentate gyrus in subjects with schizophrenia.[38] In contrast to the inconsistencies with vGLUT1, the complexin results are now observed in 3 independent hippocampal series and might require a different interpretation from that of a global imbalance in the ratio of glutamate to γ-aminobutyric acid neurotransmission in the hippocampus of subjects with schizophrenia. Additional studies are required to determine whether subsets of terminals are differentially vulnerable and to further investigate the relationship between each complexin protein and neurotransmitter phenotype in human hippocampus.

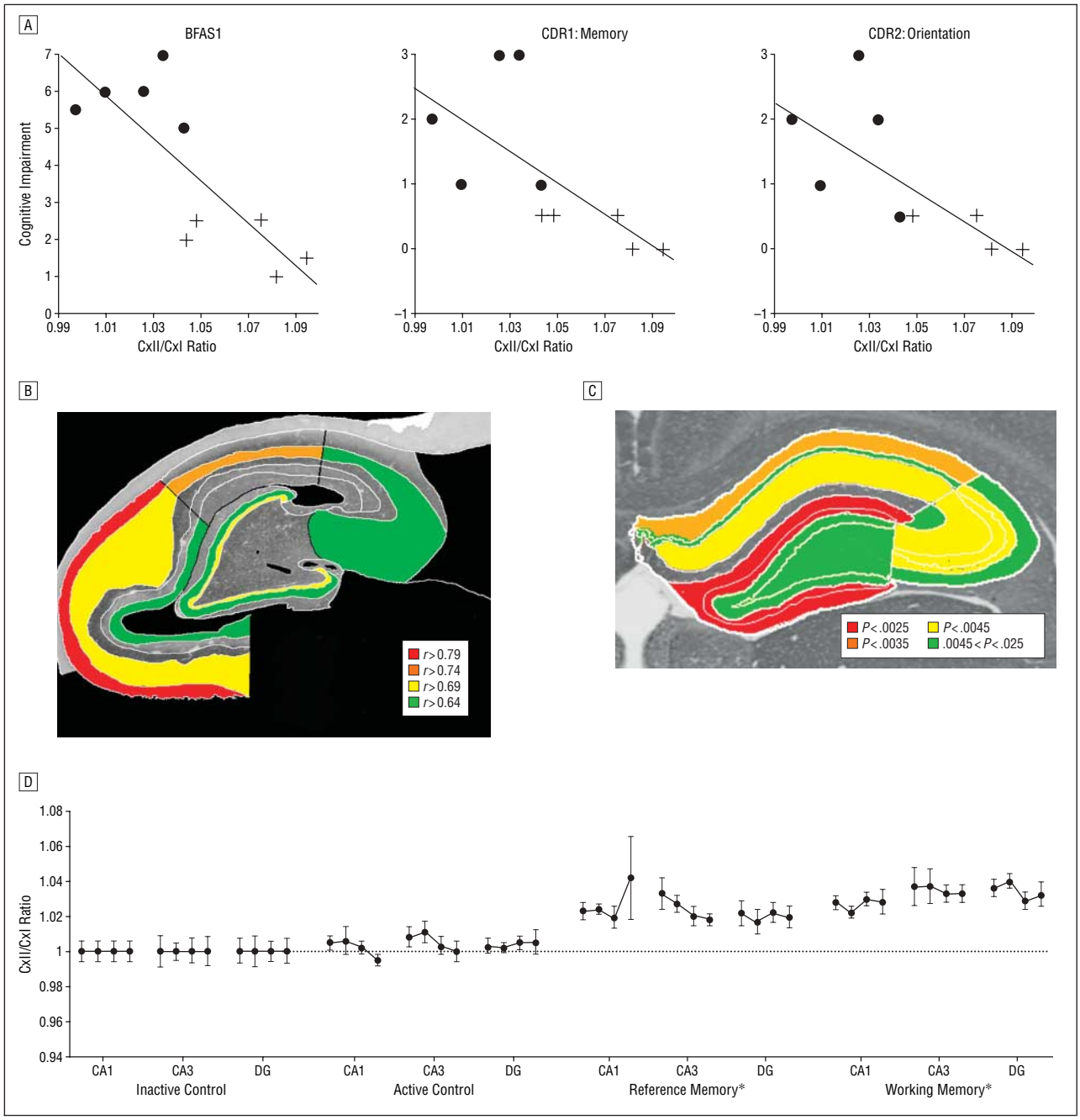

Figure 3. Complexin ratios and cognitive function in cases with schizophrenia. (A) Relationships between cognitive impairment and complexin II–complexin I (CxII/CxI) ratio in the CA1 stratum oriens. Cognitive impairment was assessed by rating medical record information using the Blessed Functional Activity Scale (BFAS) and the Clinical Dementia Rating (CDR). The first 8 items of this scale (BFAS1) refer to behavioral and cognitive impairments, with a higher score indicating more severe impairment. Circles indicate subjects with a total CDR score of 12 or higher, consistent with a clinical diagnosis of definite cognitive impairment, and crosses indicate patients with CDR total scores lower than 12. Overall scores on the BFAS and CDR correlated with the CxII/CxI ratio; individual item scores indicated the highest correlation with orientation and memory-related items. Of the 12 cases of schizophrenia, immunostaining of the stratum oriens was inadequate in 1 case, and cognitive scores could not be calculated in another. For CDR2 scores, 1 of the values of a case with no overall cognitive impairment (x=1.044, y=0.5) is obscured by the filled circle of a case with cognitive impairment and a similar CxII/CxI ratio. (B) Subregions of the hippocampus of subjects with schizophrenia where statistically significant ($P<.05$) correlations between the CxII/CxI ratio and CDR scale orientation scores were observed. (C) The rat hippocampal subregions where significant effects of learning and memory tasks on the CxII/CxI ratio were observed. (D) Ratios of rat hippocampal CxII/CxI normalized to inactive control group. Each of the 4 points in the CA regions represent in sequence the oriens, pyramidal, radiatum/lacunosum, and molecular layers. The 4 points in the dentate gyrus (DG) represent in sequence the outer molecular layer (OML), inner molecular layer (IML), granule cell layer (GCL), and CA4. Statistically significant effects on the CxII/CxI ratio were observed in relation to training in learning and memory tasks. Both tasks resulted in an increased CxII/CxI ratio. Asterisk indicates the difference between the learning and memory groups and control groups, for CA1 ($P=.0005$), CA3 ($P=.0014$), and the dentate gyrus ($P=.0014$). Error bars represent SEM.

Impaired memory function is observed in subjects with schizophrenia, with a magnitude of 1 to 2 SD from normal.[39,40] Although cognitive impairment is one of the most consistently reported abnormalities in schizophrenia, the pathological substrates remain unclear.[41] The present results indicate that the alteration in complexin proteins

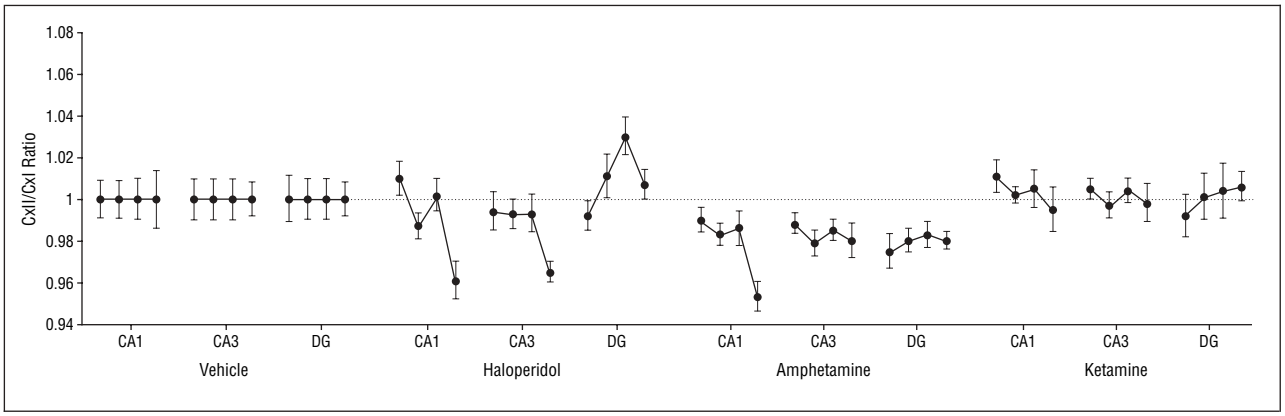

Figure 4. Ratios of rat hippocampal complexin II to complexin I (CxII/CxI) normalized to vehicle control groups. (Separate control groups were used for antipsychotic treatment and for psychotomimetic treatment.) Each of the 4 points in the CA regions represent in sequence the oriens, pyramidal, radiatum/lacunosum, and molecular layers. The 4 points in the dentate gyrus (DG) represent in sequence the outer molecular layer (OML), inner molecular layer (IML), granule cell layer (GCL), and CA4. No statistically significant effect of the antipsychotic drug haloperidol was observed. No statistically significant effects of amphetamine or ketamine administration were observed. The mean differences in the amphetamine group were in the same direction as that observed in schizophrenia. Error bars represent SEM.

in hippocampus might contribute to cognitive disturbances in the illness, particularly memory impairment and disorientation. In Alzheimer disease, lower presynaptic protein immunoreactivity in the molecular layer of the dentate gyrus was correlated with antemortem cognitive impairment, which is likely related to diffuse synaptic loss.[42] We previously reported no significant difference in synaptophysin immunoreactivity between schizophrenia and control samples in the present series,[20] suggesting that presynaptic terminals were largely intact.

On a regional basis, the strongest association between the altered CxII/CxI ratio and antemortem cognitive function was observed in the stratum oriens of Ammon's horn. Interneurons in this region might modulate activity in the perforant pathway connection of the entorhinal cortex and hippocampus, acting through projections to the stratum lacunosum-moleculare.[43] Stratum oriens interneurons also can synapse with the initial segments of pyramidal cell axons, acting to modulate output from the hippocampus.[44]

Animal models provide important contextual information to help interpret the possible origins and significance of the observed abnormalities in postmortem human samples. Schizophrenia appears to unfold on a substrate of abnormal brain development, which might begin in utero. In rats, subjecting dams to variable prenatal stress results in adult offspring with several behavioral and psychophysiological features similar to those of schizophrenia.[45] These animals also show lower complexin I mRNA levels when subject to acute stress as adults, compared with rats not subject to the developmental stressors. Studies of cognitive function in animals also help inform the present results. Complexin II knockout mice demonstrate impaired learning and memory[46] and previously noted abnormalities of long-term potentiation in hippocampus.[34] Spatial memory training in rats is associated with reduced interaction of complexin I with partner proteins in hippocampus.[47] The present results demonstrated a higher hippocampal CxII/CxI ratio in rats with training on memory tasks. If shifting the hippocampal CxII/CxI ratio to higher values is also part of learning and memory in humans, the lower baseline CxII/CxI ratio in schizophrenia could contribute to memory impairment associated with the illness.

In summary, the present findings indicate lower complexin proteins in hippocampus from cases of schizophrenia and in particular a lower CxII/CxI ratio. A disturbance in the ratio of glutamate to γ-aminobutyric acid terminals was observed also, but the 2 findings did not seem tightly associated. Normal cognitive processes in rodents were associated with dynamic modification of the CxII/CxI ratio, and impairment of complexin regulation in schizophrenia could contribute to specific cognitive dysfunction in the illness.

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