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ORIGINAL RESEARCH ARTICLE

Reduction of synapsin in the hippocampus of patients with bipolar disorder and schizophrenia

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Several studies suggest that decreased expression of presynaptic proteins may be characteristic of schizophrenia. We examined one such protein, synapsin, in schizophrenia and bipolar disorder. Samples of hippocampal tissue from controls ($n = 13$), patients with schizophrenia ($n = 16$), or bipolar disorder ($n = 6$), and suicide victims ($n = 7$) were used. The membrane and cytosolic fractions were analyzed by Western immunoblotting for synapsin using an antibody that detects synapsin Ia, IIa, and IIIa proteins. Synaptophysin was also measured for comparison. Total synapsin was decreased significantly in patients with schizophrenia ($P = 0.034$) and in bipolar disorder ($P = 0.00008$) as compared to controls. The synapsin/synaptophysin ratios were decreased in schizophrenia and bipolar disorder, and additionally in suicide victims ($P = 0.014$). Age, postmortem interval, percentage of protein extracted, and pH of brain were not different between groups. No changes in total synapsin or synaptophysin in the hippocampus were produced by injecting rats with either lithium or haloperidol for 30 days. Reductions in synapsin in both patients with schizophrenia (synapsin IIa and IIIa) and bipolar disorder (synapsin Ia, IIa and IIIa) imply that altered or reduced synaptic function in the hippocampus may be involved in these disorders.
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Introduction

Schizophrenia and bipolar disorder affect between 0.5% and 1.5% of the general population, respectively.[1] The pathogeneses of these diseases are largely unknown,[2,3] but some studies report the hippocampus to be structurally altered in patients with schizophrenia[4] and bipolar disorder.[5,6] Several etiological theories of schizophrenia implicate neuropathology largely in the mesial temporal lobe, including the dopaminergic,[7] glutamatergic,[8] neurodevelopmental,[9] viral,[10] autoimmunity,[11] synaptic alteration,[12] and membrane[13] hypotheses.

Decreases in several presynaptic proteins including synapsin, SNAP-25, and synaptophysin have been reported in the hippocampal formation of patients with schizophrenia.[14] In one such study, synapsin was decreased, while synaptophysin was normal.[15] Another study found a decrease in synaptosomal-associated protein-25 kDa (SNAP-25).[16] Decreases in synaptophysin mRNA have been reported in several subregions of the cerebral cortex and hippocampus in patients with schizophrenia,[17–19] while smaller or less consistent changes in synaptophysin protein have usually been reported.[16–18,20] In a prior study, we found modest decreases in synaptophysin protein as normalized to actin in patients with schizophrenia, but no change in bipolar disorder.[21] A recent microarray study of prefrontal cortex identified genes for presynaptic proteins in general, and particularly N-ethylmaleimide sensitive factor and synapsin, as showing consistent decreases in schizophrenia.[22] Thus, although synaptophysin has been studied in schizophrenia in considerably greater detail than synapsin, there are some indications that decreases in expression of synapsin may be considerably more pronounced.[15,22]

Synapsin, a presynaptic marker, is localized to the surface of synaptic vesicles that are both docked and located away from the plasma membrane.[23] Further, a high level of synapsin II relative to synapsin I may be found in excitatory synapses and a relatively high level of synapsin I may be found in inhibitory synapses.[24] It is proposed that synapsins generally cross-link or tether synaptic vesicles to one another to form a reserve pool of vesicles away from the docking site.[23]

The newly discovered synapsin III gene might be a candidate molecule in schizophrenia,[25,26] because of the role of synapsins in neurotransmitter release and synaptogenesis. Reported linkage studies in affected sib pairs showed a hot spot about 4.5 cM from the syn-

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apsin III gene on chromosome 22,[27] and the region on 22q12 is also associated with the phenotypic measures of inhibitory neurophysiological functioning, P50 auditory sensory gating, and antisaccade ocular motor performance.[28] We tested whether synapsin might also be decreased in the hippocampal formation of schizophrenia using an antibody that measures synapsin Ia, IIa, and IIIa proteins. We also determined whether there are relative differences in the expression of synapsin Ia, IIa, and IIIa in neuropsychiatric disorders. Synaptophysin was also measured for comparison.

Although the pathophysiology of suicide is unknown,[29] patients with mood disorders and schizophrenia are at an increased risk for suicide.[30] Therefore, as a control for diagnostic specificity and manner of death, suicide victims were included in the assays.

Materials and methods

Hippocampus samples

Postmortem brains available in the Neuropathology Section of the NIMH Intramural Research Program were obtained from the Washington, DC Medical Examiners Office. Psychiatric diagnosis was assigned after chart review by two board-certified psychiatrists using DSM III-R criteria. There were 42 samples of hippocampal tissue consisting of controls ($n = 13$), patients with schizophrenia ($n = 16$), or bipolar disorder ($n = 6$), and suicide victims ($n = 7$). There were no individuals in the suicide group for whom a definite diagnosis of unipolar or bipolar affective disorder could be established. The records indicated that four suicide victims were depressed and one individual was taking an antidepressant. Whole hippocampus samples (~ 500 mg) were previously extracted into membrane and cytosolic fractions,[31] and protein concentrations were determined by bicinchoninic acid assay (Pierce, IL, USA). The age, postmortem interval, percentage of protein extracted and pH of brain were not different between groups (Table 1), although freezer time was significantly longer in the bipolar disorder group as compared to controls ($P = 0.007$) and patients with schizophrenia ($P = 0.01$). Cause of death is summarized in Table 2.

The membrane and cytosolic fractions of hippocampus were analyzed by Western immunoblotting for synapsin, and membrane extracts were analyzed for synaptophysin.

Antibodies

Affinity purified polyclonal antibodies to synapsin (G304 directed to the C-terminus and G143 directed to the N-terminus were kindly provided by Dr HT Kao and Dr Paul Greengard). G304 has been previously characterized,[26,32] and is specific for domain E near the C-terminus of synapsin family members Ia, IIa, and IIIa, and was used at a 1: 10 000 dilution for Western immunoblots. A monoclonal antibody specific for synaptophysin (Sigma, St Louis, MO, USA, clone SVP38) was used at a 1: 1000 dilution. A polyclonal antibody to synaptotagmin[33] (Rockefeller University, RU87) was used at 1: 1000 dilution.

Western blots

The membrane and cytosolic samples were analyzed by Western immunoblotting for total synapsin, individual synapsin band immunoreactivity, and synaptophysin. The hippocampus brain samples were diluted with 2× sample loading buffer (1 volume sample: 1 volume loading buffer, SepraSol, Integrated Separation Systems, Natick, MA, USA) and 40 μg of total protein was loaded in each lane. Membrane and cytosolic samples were electrophoresed on 7.5% SDS-PAGE gels for 5.5 h at 40 mA per gel.[31] The membrane and cytosolic fractions were run on separate gels. Membrane and cytosolic fractions were prepared from a sample of control occipital cortex, and 20, 40, and 60 μg of the respective fraction was used as a reference stand and for each membrane or cytosolic gel. Gels were electroblotted to Hybond C Super supported 0.45 μm nitrocellulose membrane (Amersham) for 60 min using a semi-dry electroblotter (Bio-Rad, Hercules, CA, USA) at 800 mAMP and 25 V constant setting. The electroblotted membrane was air dried overnight at room temperature, blocked with PBS pH 7.4, containing 1% bovine serum albumin (Sigma) and 5% non-fat powdered milk for 4–8 h at 4°C. All washes were with PBS, pH 7.4, containing 0.1% Tween 20 (PBS-T). The washing sequence consisted of PBS-T applied briefly for two rinses and followed by three PBS-T washes for 20 min

Table 1 Demographics of hippocampal tissue samples

	Controls	*Bipolar*	*Suicide*	*Schizophrenia*	*F (P)*
n (F/M)[a]	13 (5/8)	6 (3/3)	7 (2/5)	16 (7/9)	—
Age	51.5 ± 17.2#	45.8 ± 11.7#	51.3 ± 7.2#	51.7 ± 16.5#	0.24 (0.86)
PMI[b]	22.9 ± 11.6	29.6 ± 6.33	28.1 ± 5.33	24.0 ± 21.6	0.31 (0.82)
pH[c]	6.36 ± 0.33	6.45 ± 0.08	6.44 ± 0.21	6.48 ± 0.16	0.44 (0.72)
Protein[d]	3.59 ± 0.36	3.64 ± 0.41	3.94 ± 0.37	3.41 ± 0.56	2.09 (0.11)
Freezer time[e]	9.19 ± 2.0	13.0* ± 3.6	11.2 ± 3.7	9.48 ± 2.4	3.21 (0.03)

#Values are SD, $*P < 0.05$ for comparison to control.
[a](F/M) Female/Male. [b]Postmortem interval (h). [c]pH of postmortem brain tissue homogenate. [d]Percentage of protein extracted from brain sample (mg protein mg^{-1} weight of frozen brain tissue). [e]Storage in freezer (years). (*P*) is probability level of F-ratio for a one-way ANOVA. Each of these were entered as covariables in analyses of covariance for differences in synapsin.

Table 2 Cause of death

Cause of death	*Group*		*Suicide*	*Control*
	Bipolar	*Schizophrenia*		
Suicide	5	4	7	0
Cardiac	1	4	0	7
Multiple injuries	0	3	0	4
Respiratory	0	5	0	0
Burns	0	0	0	2
	(6)	(16)	(7)	(13)

on an orbital shaker. All antibodies were diluted in PBS with 0.1% bovine serum albumin and 2.5% nonfat powdered milk. The blocked membrane was washed, and incubated with primary antibody on a rocker for 16 h at 4°C, followed by washing. Secondary antibody was incubated for 40 min at room temperature on a rocker. After washing, the bound antibody complex was detected with an enhanced chemiluminescent (ECL) substrate (Amersham).

The resulting light emission was collected for a constant exposure time for each protein with BioMax film (Kodak, Rochester, NY, USA). The optimal exposure times varied for each protein (1–8 min) to ensure a linear range for the reference standards on each gel. Immunoreactive bands were transferred from developed film to computer by a high resolution scanner (Microtek) at 600 dots per inch resolution. The mean density of each band was measured with NIH Image software v 1.69 (Wayne Rasband, NIH Bethesda, MD, USA) by two persons blind to the diagnosis. This method for protein quantitation shows linearity between film immunodensity values and μg of protein loaded.[34,35] The synapsin and synaptophysin proteins were separated sufficiently for identification; therefore, the membranes were not stripped between antibody incubations, since stripping and additional washing decreases protein retention to the membrane.

In order to examine the effect of proteolysis on the relative integrity of synapsin and synaptophysin proteins, an experiment was performed in which a sample of brain tissue (human occipital cortex) was pulverized in liquid nitrogen and incubated at room temperature for 4 or 48 h prior to assaying. Pulverization was used to accelerate proteolytic degradation, due to exposing compartmentalized proteins to proteolytic enzymes.

In a previous study of synaptophysin, actin was measured on the same membranes and used for normalization.[21] However, the use of housekeeping genes as standards of reference for normalization, and the use of actin in particular, has recently been questioned.[36,37] Therefore, synaptophysin was employed in the present study as a reference standard and as an alternative measure of synaptic protein expression, but was not used to normalize the synapsin measurements. Actin was not measured in the present study as it was not sufficiently separated from synapsin to allow for measurement on the same blots.

Statistical analysis

The ratio sample (immunodensity μg^{-1})/reference standard (immunodensity 40 μg^{-1}) was calculated for each unknown sample. The 40 μg standard was used to normalize all gels. A standard curve was used in pilot experiments to determine optimal protein loading of gels. One-way ANOVAs were used to evaluate differences between groups for total synapsin immunoreactivity, and individual synapsin bands were evaluated with a one-way ANOVA with a repeated measure factor (synapsin band) (Statistical Version 5.1, Statsoft, Tulsa, OK, USA). Total synapsin/synaptophysin ratios were also evaluated using a one-way ANOVA and planned comparisons. In addition, demographic variables of gender, age, PMI, pH, and freezer storage time were entered into separate analyses of covariance.

Drug treatment of rats

Four groups of rats (Sprague Dawley SD, Harlan Sprague Dawley, Indianapolis, IN, USA) were injected daily for 30 days with either 2 mg kg^{-1} haloperidol (McNeil Labs, PA, USA), haloperidol vehicle (1.8 mg methylparaben kg^{-1} and 0.2 mg kg^{-1} propylaraben, Sigma), lithium carbonate (2 mEq kg^{-1} day^{-1}, Sigma), or physiological saline. The animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The animals showed sedation following neuroleptic administration for the first 3 days and resumed normal activity throughout the remainder of the experiment. Two animals in the lithium group died, and the lithium dosage was decreased by one-half on day 10. On day 31, the animals were euthanized under CO_2, the hippocampus dissected and stored at −78°C for Western immunoblot assay.

Results

The synapsin antibody employed in this study reacts strongly with synapsin Ia, IIa, and IIIa. There are multiple variants associated with synapsin $IIa_{1,2,3}$, which appear on immunoblots and cannot be resolved without further treatment, and will be referred to as synapsin IIa variants or synapsin IIa(v) (Figure 1). The molecular weight (MW) values observed on immunoblots agree with previously reported MWs for synapsin Ia, $IIa_{(1,2,3)}$, and IIIa (Table 3), and confirm the specificity of the G304 antibody for synapsin. The synapsin bands

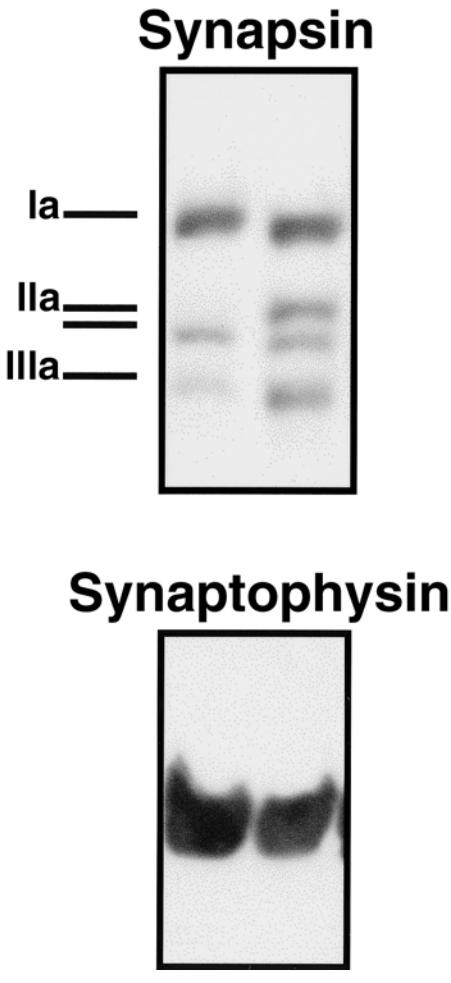

Figure 1 Examples of Western blots of hippocampal membrane extracts showing synapsin Ia, IIa, and IIIa bands (upper panel). The synapsin IIa doublet bands are both referred to as synapsin IIa(v) in Figure 2. The lower panel shows synaptophysin immunoreactivity for the same lanes.

(Ia, IIa, and IIIa) were found in both the cytosolic and membrane extracts and analyzed together as total synapsin. When varying amounts of protein were run on immunoblots, there was a linear relationship between the concentration of cortical protein and total synapsin measurements (Pearson correlation coefficient r^2 = 0.95; data not shown).

Total synapsin was significantly different between groups ($F_{3,38}$ = 6.56; $P < 0.001$). Post-hoc comparisons showed significant decreases in total synapsin concentrations in patients with schizophrenia ($P = 0.034$) and bipolar disorder ($P = 8 \times 10^{-5}$) as compared to controls. Total synapsin was not significantly different between controls and suicide victims ($P = 0.11$).

Total synapsin did not significantly correlate with PMI, freezer storage time, age, percent of protein extracted, or brain pH and was not related to gender (not shown). Total synapsin was analyzed in separate analyses of covariance with PMI, percent of protein extracted, brain pH, gender, freezer time, and age entered as covariables. All of the analyses of covariance remained significant for synapsin when one of these covariates were entered. For the analysis of covariance for total synapsin with freezer time as a covariant (there was a difference in freezer times in the bipolar group; Table 1), the group effect remained significant ($F_{3,37}$ = 7.57, $P = 4.5 \times 10^{-4}$). The within-cells regression for freezer storage time and total synapsin was not significant ($P = 0.13$).

The densities of the individual synapsin Ia, IIa, and IIIa bands were also analyzed, confirming the changes in total synapsin described above (Figure 2). In patients with schizophrenia, there were specific reductions in synapsin isoforms IIa and IIIa ($P < 0.05$) while in bipolar disorder there were reductions in synapsin Ia, IIa, and IIIa ($P < 0.005$) as compared to controls (Figure 2). Interestingly, synapsin Ia levels did not differ between the patients with schizophrenia and controls.

Synaptophysin immunoreactivity did not differ between groups ($F_{3,38}$ = 1.34; P = 0.27). There was, however, a difference in the ratio of total synapsin/synaptophysin ($F_{3,38}$ = 8.29; $P = 2 \times 10^{-4}$), which was accounted for mainly by decreases in both bipolar disorder and schizophrenia as compared to controls ($P = 2 \times 10^{-5}$ and $P = 0.009$, respectively). Additionally, the suicide group showed a reduction in the total synapsin/synaptophysin ratio ($P = 0.014$) as compared to controls (Figure 3).

Proteolytic degradation of brain

A sample of human brain tissue was employed to study the relative susceptibility of synapsin and synaptophysin to proteolytic degradation. The tissue was pulverized, to accelerate proteolysis, and stored at room temperature for 4 or 48 h. Both synapsin and synaptophysin immunoreactivity were partially lost

Table 3 Molecular weight (MW) of synapsin observed in the present study and reported in the literature

Synapsin protein	*MW[a] observed in present study (kDa)*	*Reported MW (kDa)*	*Reference*
Ia	83	86	[15]
		85	[42]
$IIa_{(1,2,3)\ Variants}$	70, 73	74, 76, 78	[40]
		74	[15]
IIIa	66	63	[26]

[a]Mean of six determinations.

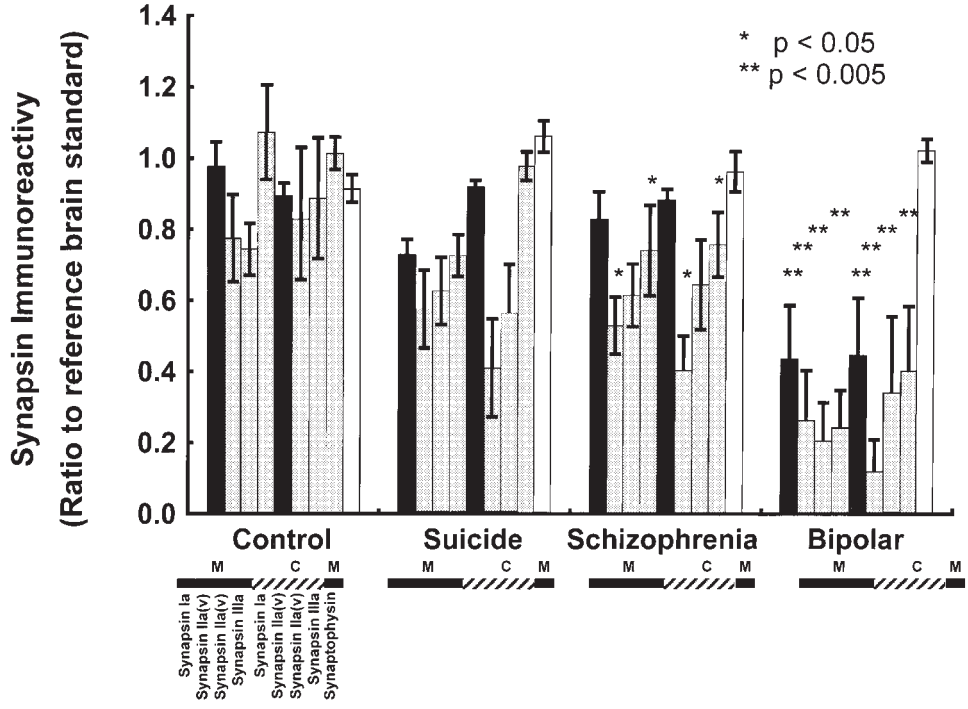

Figure 2 The ratios of synapsin isoforms to a human brain reference standard for membrane (M) and cytosolic (C) extracts. The two synapsin IIa bands shown in Figure 1 are referred to as synapsin IIa(v) in the figure. The bar on the left indicates the higher MW band, and the bar on the right indicates the band with the lower MW. There were significant reductions in synapsin isoforms Ia, IIa, and IIIa in bipolar disorder, as compared to controls. Significant reductions in synapsin IIa and IIIa were seen in schizophrenia as compared to controls (see text). Synaptophysin levels in membrane extracts (open bars) were not significantly different between groups.

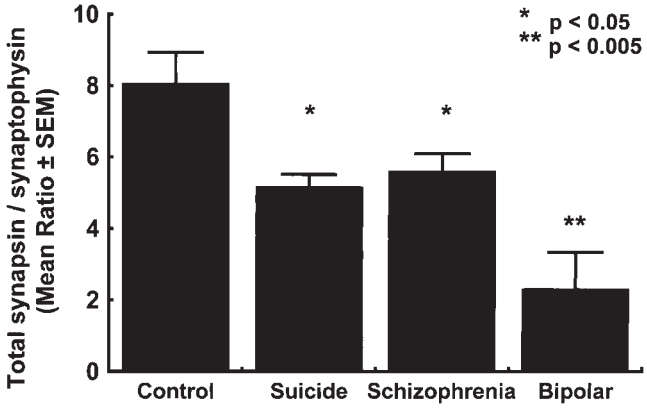

Figure 3 The total synapsin/synaptophysin ratio for the four groups. There were significant reductions in patients with schizophrenia or bipolar disorder and suicide victims as compared to controls.

after storage for 4 h, and entirely degraded after 48 h (Figure 4a).

Synaptotagmin

Bipolar (B) and control (C1, C2) hippocampus samples matched for synaptophysin immunoreactivity were assayed with an antibody to the C-terminus of synapsin Ia, IIa and IIIa (G304 antibody), the N-terminus of synapsin (G143 antibody which recognizes synapsin I strongly and synapsin II weakly, HT Kao, personal communication), and synaptotagmin (RU87). The bipolar sample showed low C-terminal synapsin immunoreactivity, low N-terminal synapsin immunoreactivity, and a high level of synaptotagmin by immunoreactivity. Ratios of synapsin G143/synaptotagmin immunoreactivity for these samples are shown in Figure 4b.

Haloperidol and lithium treatment

Synapsin immunoreactivity was analyzed in the hippocampus of lithium- and haloperidol-treated groups of rats. Synapsin did not change in either group of treated rats compared to the respective saline controls (lithium: $F_{1,10} = 2.56$; $P = 0.14$; haloperidol: $F_{1,12} = 0.039$; $P = 0.84$). Synaptophysin was also not changed by lithium: ($F_{1,11} = 1.29$; $P = 0.27$) or haloperidol: ($F_{1,12} = 0.076$; $P = 0.78$) treatments (data not shown).

Discussion

Synapsins Ia, IIa, and IIIa were significantly, and markedly, reduced in bipolar disorder as compared to controls. Patients with schizophrenia showed reductions in synapsin IIa and IIIa only. Additionally, there were reductions in the total synapsin/synaptophysin ratios in all three subject groups. Pre-agonal medication cannot entirely be ruled out in this study as an explanation for the findings; however, we did not observe changes

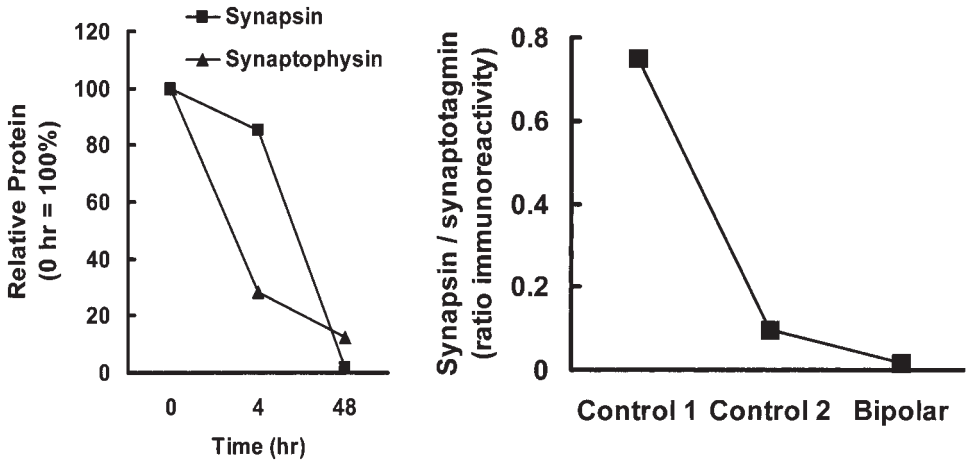

Figure 4 Human cortex proteolytic degradation (a) and synaptotagmin and synapsin (G143) immunoreactivity in hippocampal extracts (b). The proteolysis experiment (a) involved degradation of pulverized tissue at room temperature for 0, 4, and 48 h followed by immunoblotting for synapsin and synaptophysin. Concomitant decreases in synapsin and synaptophysin are seen after 4 h and 48 h. (b) A bipolar hippocampal sample and two controls matched for synaptophysin were immunoblotted for synaptotagmin and for synapsin using an antibody (G143) specific for the N-terminus. The bipolar sample showed a decrease in G143 immunoreactivity as compared to synaptotagmin.

in either synaptophysin or synapsin in the hippocampus following lithium or haloperidol treatment of rats.

To our knowledge, this study is the first to measure synapsin proteins in both schizophrenia and bipolar disorder. Other presynaptic proteins including SNAP-25 and complexin I and II have been found to be decreased in the hippocampal formation in schizophrenia.[14] The presynaptic protein complexin I is a marker of axosomatic (inhibitory) synapses, whereas complexin II is a marker of axodendritic (mainly excitatory) synapses.[38] The amounts of both complexin mRNAs were lower in patients with schizophrenia, while the complexin II/complexin I ratio (for both mRNA and protein) was lower in schizophrenia.[38] This finding suggests a relatively greater loss of excitatory (glutamatergic) synapses. At present, a decrease in synapsin IIa was found in both schizophrenia and bipolar disorders, which is also consistent with a decrease in excitatory synapses.[24]

Two recent postmortem studies suggest that decreased expression of synapsin may be related to cognitive disorders in human subjects. Mirnics and coworkers[22] found decreased expression of synapsin II mRNA and several other synaptic markers in the prefrontal cortex in schizophrenia using microarrays and *in situ* hybridization. The present study confirms at the protein level that synapsin is decreased in schizophrenia. Another recent study[39] showed that early stage Alzheimer's patients had marked decreases in expression of synapsin protein and mRNA in the entorhinal, but not the visual cortex. The present study suggests that synapsin may also be altered in bipolar disorder. Taken together, these studies suggest that synapsin may be a labile gene, which is sensitive to alteration in disorders of mood and cognition.

In a separate study (MP Vawter *et al*, unpublished data), a difference in synapsin expression in specific subfields in the hippocampal formation was found by immunohistochemistry, although the degree of change was much less than that found with Western immunoblots. By immunoblotting, the synapsin Ia isoform is quantitatively predominant (Figure 1). Since changes were seen primarily for the synapsin IIa and IIIa isoforms, differences might not be seen by immunohistochemistry because they would be overwhelmed by synapsin Ia immunostaining. *In situ* hybridization might help to resolve differences in isoform specificity and localization in the hippocampus.

Previous studies have found that postmortem interval and freezer storage time do not correlate with synapsin II concentrations.[40,41] In the present study, there were no relationships between the decreases in synapsin and PMI, pH of the samples, or freezer storage time. The experiment in which degradation was accelerated by pulverization of the tissue indicates that synapsin and synaptophysin are degraded concomitantly. Therefore, decreases in synapsin in bipolar disorder and schizophrenia are unlikely to be related to differential degradation of the samples, since synaptophysin was not different between groups.

In a previous study, synaptophysin was found to be decreased in schizophrenia when expressed as a ratio to actin.[21] In bipolar patients, the synaptophysin/actin ratio was also smaller by approximately 40%, but this difference was not statistically significant. In the present study, no change in synaptophysin was found. We suspect that this difference is related to the fact that synaptophysin was normalized to actin concentrations in the prior study. Nonetheless, it is apparent that there is a much greater relative decrease in synapsin in both schizophrenia and bipolar disorder than any change in synaptophysin protein. Synaptophysin may be viewed as a measure of synapse number, and/or general integrity of synaptic protein expression. There-

fore, the loss of synapsin seems to greatly exceed any general loss of synaptic proteins in both bipolar disorder and schizophrenia.

The bipolar samples showed apparently normal synaptophysin levels, but a 66% reduction in synapsin proteins. If the reduction in synapsin had been caused by proteolytic degradation, it would be expected that synaptophysin would have been similarly degraded. Since synaptophysin was similar in all of the diagnostic groups, it appears that the change in synapsin represents decreased expression of the protein. Other prior investigators have also ruled out differential degradation as the possible cause of synapsin reduction in the hippocampus.[15]

Conclusion

Our findings suggest that in the hippocampus of patients with bipolar disorder and schizophrenia, abnormalities in synaptic proteins, specifically synapsin IIa and IIIa, may be related to a common pathophysiology of these disorders. The role of each synapsin gene has not been fully elucidated; however, it is currently thought that synapsins are involved in tethering synaptic vesicles to one another and in linkage of synaptic vesicles to the cytoskeleton[23] and could allow for the regulation of a reserve pool of vesicles. Our data therefore suggest a disturbance in regulation of synaptic function in both bipolar disorder and schizophrenia that is consistent with common susceptibility loci.

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