CSF SNAP-25 in Schizophrenia and Bipolar Illness
A Pilot Study

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Research efforts to identify and understand the pathophysiology of schizophrenia and bipolar illness are limited by the inability to study neuronal tissue of living patients. An alternative to sampling brain tissue from living patients is to measure neuronal proteins found in cerebral spinal fluid. One such candidate protein is synaptosomal-associated protein 25kDa. Our hypothesis is that the level of this protein in cerebral spinal fluid may be a marker of neuronal pathology. Cerebral spinal fluid from headache, schizophrenic, bipolar, and control subjects was used to measure the SNAP-25 level by quantitative dot blotting. Schizophrenic subjects had significantly elevated levels of SNAP-25 as compared to headache and control subjects. However, there was no significant difference between the bipolar group and schizophrenic or control groups. This study reports on a potentially useful clinical marker in schizophrenia, and the presence of elevated cerebral spinal fluid SNAP-25 may indicate alterations in neuronal functioning. [Neuropsychopharmacology 21:717–722, 1999] © 1999 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

In the past 20 years, scientists have conducted extensive research to identify the pathology and etiology of schizophrenia and bipolar illness. In schizophrenic subjects, neuroimaging and functional analysis has identified abnormalities in various brain structures (Nasrallah 1990). Neuropathological studies of schizophrenics have revealed brain changes ranging from loss of gray matter to disorganization of the normal neuronal architecture (Bloom 1993). More recently, changes in vesicular- and growth- associated proteins have been identified in post-mortem brains of schizophrenics (Barbeau et al. 1995; Browning et al. 1993; Eastwood et al. 1995; Perrone-Bizzozero et al. 1996). Based on the accumulated clinical and laboratory information, investigators have developed two models to explain the pathophysiology of schizophrenia.

The neurodegeneration hypothesis proposes that schizophrenia is the result of degeneration or loss of neurons, similar to Alzheimer's disease. This hypothesis dates to 1919, when schizophrenia was referred to as "dementia praecox" (Kraepelin 1919). However, post-mortem studies have not consistently identified evidence of abnormal neurodegeneration in schizophrenic brains (Arnold et al. 1991).

An alternative hypothesis states that the pathology

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of schizophrenia arises during embryonic and fetal developmental (Bloom 1993; Saugstad 1989). In this hypothesis, Weinberger (Weinberger 1987) proposes that a static brain lesion is established early in development and that neurodegeneration is not a major factor in the adult pathology. As the brain matures, it compensates for the lesion until the adaptations are overwhelmed. When the brain adaptions are insufficient to compensate, the final groups of behavioral symptoms are collectively described as schizophrenia. As in the neurodegenerative hypothesis, supporting data are circumstantial (Raedler et al. 1998).

In bipolar illness, less is known about brain pathology than with schizophrenia. Consistent findings include mood state-dependent changes in erythrocyte Na-K-ATPase activity (Looney and el-Mallakh 1997) and an increased risk for structural brain abnormalities (Altshuler et al. 1995). Both types of studies may indicate subtle neuronal damage. A question raised by both schizophrenia and bipolar studies is whether there is ongoing neuronal damage or degeneration.

The direct approach to test the hypothesis that schizophrenia and bipolar illness are not associated with ongoing neurodegeneration is to study neuronal tissue from living patients. However, moral and ethical restrictions eliminate this type of study. One way to bypass this limitation is to study a protein that has been demonstrated to be involved with the adult post-mortem pathology and can be measured in living subjects. Synaptosomal-associated protein 25kDa (SNAP-25) is a candidate protein for this type of study. We recently reported identifying this protein in human cerebral spinal fluid (CSF)(Thompson et al. 1998a), and SNAP-25 belongs to an important class of proteins involved with regulated neurotransmitter vesicle trafficking (Scheller 1995; Sollner et al. 1993). SNAP-25 is found primarily in the central nervous system and is a T-SNARE (Jacobsson et al. 1996; Roth and Burgoune 1997; Oyler et al. 1989). In human post-mortem schizophrenic cortex (Thompson et al. 1998b) the SNAP-25 levels are altered, and in an animal model of neuronal damage (Jorgensen et al. 1997), the level of SNAP-25 is decreased initially after a trauma.

In this study, we set out to determine if the level of SNAP-25 found in the CSF can differentiate pathological states. We hypothesize that SNAP-25 found primarily intracellularly will have an altered level in the extracellular space in pathological states. As a control, we used two different subject groups. The first were subjects with severe enough headaches to warrant a lumbar puncture but without other brain disorders. This group was included to demonstrate if nonspecificity exists for SNAP-25 level in nonseverely medically ill but highly stressed subjects. The second control group were normal subjects identified as free of medical or psychiatric illnesses.

METHODS

Subjects

All CSF was gathered under University of New Mexico Health Sciences Center Human Research Review Committee protocol 95-338. CSF from headache subjects was collected at the University of New Mexico Health Sciences Center Hospital clinical laboratory microbiology section. This fluid was taken for clinical indications and stored at 0°C. After 7 days, the fluid was declared waste and then stored at −80°C. A retrospective chart review provided the clinical history. To have the diagnosis of headache, individuals were required to have the clinical diagnosis of migraine or headache, and CSF cultures were without bacterial growth.

For schizophrenia, bipolar, and control groups demographic, medical, and psychiatric histories were obtained after informed consent. DSM-4 Axis 1 psychiatric diagnoses were determined by the Structured Clinical Interview (Spitzer et al. 1996), followed by a physical examination and standard blood tests. Severity of psychosis in the schizophrenia group was determined by the Brief Psychotic Rating Scale (Flemenbaum and Zimmermann 1973). When the screening was complete, control, stable schizophrenic, and bipolar subjects were admitted to the Clinical Research Center, and after a 12- hour fast, an Lumbar Puncture (LP) was performed between 7–9 AM. CSF was immediately frozen and stored at −80°C. The other schizophrenic and bipolar subjects had the same procedure; however, the LP was performed at the University of New Mexico Mental Health Center inpatient ward.

SNAP-25 Analysis

1–50 μl of the first 5 ml of CSF collected was added to a dot blot apparatus and aspirated onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-PTM, Millipore) (schizophrenia, bipolar, and control groups). The last CSF from the LP or tube four (Dougherty and Roth 1986) was used for headache subjects. The membrane was blocked in 10% powdered milk, incubated in 1:12,000 SMI-81 anti-SNAP-25 primary antibody (Sternberger Monoclonals) and 1:40,000 goat antimouse secondary antibody bound to horseradish peroxidase (HRP) (ZYMED Laboratories Inc.). We visualized the dots by enhanced chemiluminescence (NEN Life Science Products). Dot intensity was then determined by scanning and comparing gray-scale values (Gel-Pro Analyzer version 3.0, Media Cybernetics) to blotted, enriched SNAP-25 standard. Enriched SNAP standard was made by immunoprecipitating SNAP-25 from postnatal day 1 rat brains using SMI-81 (Sternberger Monoclonals) bound to cyanogenbromide (CNBR) activated sepharose beads (Pharmacia Inc.). We set as an internal control level a regression coefficient $R^2 > 0.95$ for enriched SNAP-25 standard. If the data from the

standard curve fell below this level, the data were not used, and the blot was repeated. Linearity of enriched SNAP-25 has been previously published (Thompson et al. 1998b).

Statistics

All values of SNAP-25 were an average of triplicate dots repeated in three separate trials. Overall differences between group means were determined by one-way analysis of variance (ANOVA) with Fisher's least significant difference post hoc method.

RESULTS

Control subjects ($n = 6$, age $= 25.5 \pm 8$), were without medical diagnosis and without medication use, except for one subject who used medroxyprogesterone for birth control. Headache subjects ($n = 4$, age $= 38.5 \pm 11$) had the LP, which ruled out meningitis, and the subjects did not require hospitalization, Table 1. Bipolar illness ($n = 5$, age $= 37 \pm 13$) and schizophrenic ($n = 8$, age $= 41.6 \pm 9$) subjects had no medical diagnoses other than their psychiatric problems and met DSM4 criteria for their respective illnesses. All schizophrenic subjects were psychotic, and bipolar subjects were manic at the

time of LP. Table 2 lists time since last known medication, years since diagnosis, and if the subjects had a substance-or alcohol-related diagnosis. Only subject 549 had a possible diagnosis of alcohol abuse binge type. This subject reported no alcohol use for at least 2 weeks before LP.

Over-all one-way analysis of variance (ANOVA) of SNAP-25 level differences between groups was significant, $p = .03$. There are significant differences between groups for gender ($p = .001$), age ($p = .001$) and medication use ($p = .003$). To determine if these factors are involved with the main finding, medication use, gender, and race were used as covariates. The significant results were maintained, adjusting for medication use covariate ANOVA $p = .05$, adjusting for age $p = .04$, adjusting for gender $p = .01$, and adjusting for race $p = .03$. In addition to covariate analysis, we eliminated the headache group, which contained only women, and reanalyzed the data. ANOVA without the headache group remained significant, $p = .05$.

Using Fisher's post hoc least significant difference method, the level of SNAP-25 in the schizophrenic group (4.2 ± 2.9 pg/μl) is significantly greater than controls (2.0 ± 1.6 pg/μl) and headache subjects ($1.7 \pm .08$ pg/μl). Units are based on pg/ul of enriched SNAP-25 standard blotted on the same blot as the subjects. There was no significant difference between the schizo-

Table 1. Demographic Information and Level of SNAP-25 (pg/μl) for Study Subjects. A = Anglo, H = Hispanic

Research Number	Subject Group	Race	Age	Gender	Pixels Immunoreactivity/ Pg Total Protein Enriched SNAP-25 UL CSF
520	Control	A	26	Male	1.2
524		A	34	Male	0.78
553		A	18	Male	0.85
713		A	37	Male	2.7
629		H	19	Female	2.2
711		H	19	Female	4.5
503	Headache	H	24	Female	2.3
517		A	52	Female	2.0
611		A	33	Female	1.1
620		A	45	Female	1.5
519	Bipolar Illness	A	21	Male	2.2
523		A	28	Male	4.1
545		A	52	Male	3.4
547		A	51	Male	1.7
710		A	33	Male	1.8
518	Schizophrenia	A	24	Male	2.0
522		A	37	Male	3.2
538		A	39	Male	6.6
548		A	54	Male	3.1
549		A	44	Male	3.5
594		A	53	Male	3.5
715		A	37	Male	4.4
712		A	45	Female	6.9

phrenic and bipolar groups ($p > .05$), or between bipolar and control or headache groups, Figure 1. Within the schizophrenic group, the severity of psychosis (average BPRS $= 49.2 \pm 3.8$) was also not associated with SNAP-25 level ($p > .05$).

CONCLUSION

Both preclinical and clinical studies indicate that SNAP-25 may be a relevant biological marker of mental illness, especially schizophrenia. For example, preclinically SNAP-25 expression is under hormonal influence (Lustig et al. 1993). This observation may be useful when identifying markers to study the biology associated with the increased incidence of schizophrenia in adolescence (Remschmidt 1993; Kendler et al. 1999). A second preclinical observation is that developmental SNAP-25 expression changes with in utero virus exposure (Fatemi et al. 1998). Thus, measuring SNAP-25 may offer a method to study a viral etiology of schizophrenia (Wright et al. 1995). Clinically, post-mortem studies of schizophrenics show SNAP-25 has decreased immunoreactivity in the schizophrenic hippocampus (Young et al. 1998), decreased immunoreactivity in the temporal lobe, and increased in the prefrontal area 9 (Thompson et al. 1998b), decreased immunoreactivity in the cingulate with normal levels in the frontal, temporal and parietal lobe (Gabriel et al. 1997). This information demonstrates cellular pathology at the end of life, but does not indicate if the pathology is present earlier. The data presented in this report, in contrast to the above information, supports the hypothesis that neuronal pathology is present much earlier in life in schizophrenics and possibly bipolar individuals and that the process is ongoing.

What is the mechanism responsible for the elevated level of SNAP-25 found in the CSF of schizophrenics and how does it relate to the neurodevelopmental and neurodegenerative hypothesis? SNAP-25 is involved in neurotransmitter vesicle trafficking and is found in growth cones of extending neurites during the formation of synaptic connections (Jacobsson et al. 1996). Elevated SNAP-25 level may then be associated with increased synaptic activity, increased synaptogenesis, or decreased metabolism. In support of increased synaptogenesis, are data derived from brain trauma studies that show both neuronal loss and reactive synaptogenesis. Jorgensen (Jorgensen et al. 1997) measured SNAP-25 immunoreactivity in brain homogenates of brain-contused rats. His group found significantly decreased SNAP-25 levels at 3 days post-injury followed by increasing levels to 75% of baseline at day 18. Consistent with this process are observations of Geddes et al., which demonstrated increased SNAP-25 in brain regions adjacent to deafferented areas athat had reduced SNAP (Geddes et al. 1990). Possibly, our CSF SNAP-25 level, which measures the extracellular compartment, is the mirror image of Jorgensen's and Geddes's intracellular brain homogenate SNAP-25 levels. If that is the case, then CSF SNAP-25 level may indicate subtle neuronal damage, rather than the classical damage seen in major brain trauma. However, we cannot exclude the possibility that elevated levels of SNAP-25 in our psychiatrically ill subjects may represent a combination of overactive synaptic remodeling and ongoing neurodegeneration. Studying additional specific cellular markers in the CSF will clarify the process involved.

Table 2. Medication Use, Alcohol/Substance Abuse Diagnosis and Time Since First Diagnosis for Schizophrenic and Bipolar Subjects

| Research Number	Years after First Diagnosis	Time without Medication	Alcohol/Drug Use
518	5	Current fluphenazine, benztropine for 7 days after no neuroleptic for > 1 month	No diagnosis
522	0	> 1 month	No diagnosis
538	6	> 1 month	No diagnosis
548	22	> 1 month	No diagnosis
549	28	3 weeks	Binge drinker last use > 2 weeks prior
594	20	> 1 month	No diagnosis
712	17	Current olanzapine, lorazepam	
715	0	> 1 month	No diagnosis
519	5	> 1 month	No diagnosis
523	6	Current thiothixene, valproate	No diagnosis
545	20	Current carbamazepine	No diagnosis
547	7	Current perphenazine, benztropine for 6 days after no medication	No diagnosis
710	0	> 1 month	No diagnosis
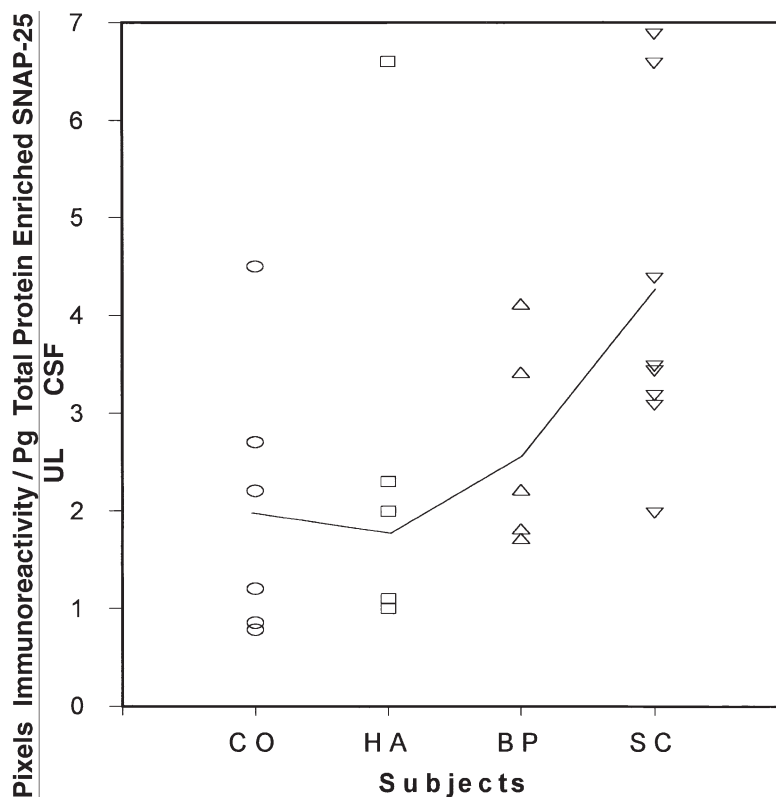
Figure 1. Graph of SNAP-25 level (pg/μl). Each symbol represents a separate subject. CO: control; HA: headache; BP: bipolar; SC: schizophrenic. Solid lines indicate mean value.

Although the present study demonstrates elevated levels of CSF SNAP-25 in schizophrenia, several confounding factors affect our interpretation. The first is that CSF from headache subjects was not collected in a controlled research protocol; rather, it was taken from medically ill patients at different times of the day and stored for a week at 0°C. To determine if storage conditions altered SNAP-25 level, we examined repeated measurements of control SNAP-25 maintained at −80°C. After repeated freeze/thaw cycles, we observed progressively decreasing levels of immunoreactive SNAP-25 (data not shown). By measuring the SNAP-25 level between the different fractions collected in the normal controls, we also determined that there is no concentration gradient for SNAP-25 in the CSF (data not shown). These observations suggest that the SNAP-25 level difference between the headache group and controls is less than reported. Other possible confounding issues are racial and gender differences and the use of medication between groups. To address the gender differences, the headache group, which contained the majority of women, was removed from the analysis, and the over-all findings remained significant. We also used race, gender, and medication use as covariants, and, again, the results remained significant. In addition, medication use was studied preclinically, and perphenazine treatment does not effect SNAP-25 immunoreactivity in rats (Fog et al. 1976; Jorgensen 1995).

As a pilot study, only a limited number of subjects were available for this report. This small sample is likely responsible for the lack of significance between the bipolar and the other groups. Expanding the present sample should provide better resolution and increased significance between normals and affected groups.

Identifying the pathology and etiology of mental illnesses are priorities in mental health research. However, current research techniques have many limitations. For example, post-mortem studies are restricted by the availability of brain donations and cannot address what is occurring earlier in life. Noninvasive study techniques such as magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI), and positron emission tomography (PET) scanning are expensive and lack ligands to many relevant targets. The method reported here circumvents the need for destructive in vivo brain biopsies and adds valuable information that other types of studies cannot.

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