

Brief Report

Micrometer-sized thread-like and/or spherical particles in the first fraction of cerebrospinal fluid in patients with bipolar disorder

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Objectives: Scanning electron microscopy (SEM) is a powerful tool to identify pathogenic factors for which sensitive tests are lacking. The technique has been used to recognize structures in the cerebrospinal fluid (CSF) of patients with schizophrenia. The aim of this study was to use SEM to screen for potential particles in CSF in bipolar disorder.

Methods: Fresh CSF samples from 56 euthymic bipolar patients, 31 bipolar I disorder and 25 bipolar II disorder, were compared to CSF samples from 20 controls. SEM of two portions of 200 μ L filtered CSF was performed; the first 0.6 mL of CSF and the following 12 mL. The microscopic structures were identified and the quantity and patterns were rated by two independent researchers.

Results: Quantitative SEM examinations showed that of the 56 patients, 11 were free of any SEM structures in CSF, while 45 patients displayed morphological structures in the first 0.6 mL of CSF. By contrast, only 2 patients showed structures in the second CSF fraction drawn from the following mixed 12 mL of CSF. In total, 45 of the 56 patients had either thread-like, spherical, or both structures in the CSF, compared to none of the 20 controls.

Conclusions: The identified particles in the first fraction of CSF have previously not been described in patients with bipolar disorder. Hypothetically, the amount of SEM structures in CSF, from none to many, might correlate to the degree of the alleged underlying disease processes in the central nervous system in patients with bipolar disease.

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Bipolar disorder (BP) is a severe mental illness of unknown origin. Diagnostic scanning electron microscopic (SEM) technique is a powerful tool to identify and classify pathogenic factors for

which sufficient specific and sensitive tests are still lacking. The SEM method used in the present study has previously successfully detected human immunodeficiency virus in cerebrospinal fluid (CSF) (1) and cytomegalovirus in urine (2). This method has also been employed to investigate CSF of patients with schizophrenia and identified mainly spherical structures in 20 of 22 patients, in comparison to only in 2 of 38 controls (3). Given that several similarities exist between schizophrenia and BP on a genetic susceptibility level (4–7), it was

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reasonable to study possible similarities between the two diseases regarding the presence of microscopic structures in CSF. To this end, we examined the possible occurrence of structures in CSF of patients with BP, and if present, the morphology in each individual case. In addition, we studied the microscopic content in the first and in a later fraction of CSF in BP patients and in controls, since gradients have previously been found in amounts of protein and immunoglobulin in CSF (8).

Methods and materials

Patients

A total of 56 patients with BP, 31 with bipolar I disorder (BP I), and 25 with bipolar II disorder (BP II), were included in this study. Patients were recruited during 15 months, from December 2005 to February 2007, from a long-term follow-up program at a bipolar outpatient unit at the Northern Stockholm psychiatric clinic. Consecutive new outpatients referred for treatment and continuing patients at the bipolar outpatient unit were invited to participate provided that they were at least 18 years old and met the DSM-IV (9) criteria for BP. The enrolled patients consented orally and in writing to participate in the study. The study was approved by the ethical review board of Karolinska Institutet, Stockholm, Sweden, in compliance with the Helsinki Declaration.

The clinical diagnosis of BP was established according to a standardized affective disorder evaluation (ADE), which was previously employed in the Systematic Treatment Enhancement Program for Bipolar Disorder (STEP-BD) project (10). The ADE starts with a social case history, followed by the affective module of the Structured Clinical Interview for DSM-IV (11). The number of lifetime affective episodes and their

characteristics are documented, as well as alcohol and drug abuse, violent behavior, childhood psychopathology, family history, past treatment response, reproductive history, and somatic illnesses. In addition to the ADE, the Mini-International Neuropsychiatry Interview (12) was completed at baseline in order to screen for psychiatric diagnoses other than BP. These interviews were conducted by board-certified psychiatrists working at the tertiary bipolar outpatient unit or residents in psychiatry completing their psychiatry training at this unit. The assessments were based on available information, including patient records and, if feasible, interviews with next of kin. The intake information was presented at a diagnostic case conference, at which the final diagnostic decision was made by a consensus panel of experienced board-certified psychiatrists specialized in BP. This procedure served to minimize inter-rater variability. Patients were hence diagnosed according to DSM-IV criteria and according to the International Statistical Classification of Diseases (ICD-10) (13).

Controls

CSF from 20 controls, who were free of psychiatric diagnosis and treatment, was sampled in connection with routine spinal anesthesia before planned surgical operation. For demographic information of the controls, see Table 1.

Blood sampling

Paired samples of serum and CSF were collected on the same day between 9 and 11 a.m. under sterile conditions after venous and lumbar punctures. The subjects were in an apparently euthymic mood state, as judged by a physician. The integrity of the blood-brain barrier was assessed by the albumin ratio, which was calculated using the following formula: $(\text{CSF albumin} \times 10^3)/\text{serum albumin}$ (14).

Table 1. Demographic data and results of scanning electron microscope (SEM) rating of structures in 200 μL of the very first 0.6 mL fraction of cerebrospinal fluid (CSF) in 56 patients^a and 20 controls

Diagnoses (DSM-IV and ICD-10) and amount of CSF structures in SEM	n (%)	Female, n	Age in years, mean \pm SD [range]
Bipolar I and II disorder	56 (100)	29	37 \pm 12 [21–67]
Category 0, no SEM structures	11 (19)	7	35 \pm 14 [22–64]
Category 1, few SEM structures	21 (39)	6	38 \pm 10 [21–58]
Category 2, several SEM structures	11 (20)	9	39 \pm 15 [22–67]
Category 3, many SEM structures	13 (22)	7	35 \pm 10 [23–51]
Controls with no psychiatric disorders displayed no SEM structures in CSF	20 (100)	10	66 \pm 8 [52–82]

For images of the structures, see Figure 1.

^a31 bipolar I disorder and 25 bipolar II disorder patients.

Collection of CSF

Most lumbar punctures were performed in a sitting position. Fine disposable needles (Becton Dickinson 22 GA 3.0 inch, 0.7×75 mm) and identical procedures were used for all individuals in the patient group. The skin in the lumbar region was thoroughly washed with sterile cotton swabs and chlorhexidine 5 mg/mL (Fresenius Kabi) before puncture. The needle was inserted in vertebral interspace L3 to L4, or L4 to L5, and the very first 12 drops of CSF, approximately 0.6 mL, were collected in a sterile test tube for microscopic examination. The following 12 mL of CSF were collected in a second test tube, which was gently inverted 10 times to secure homogeneous mixing of the components (to avoid gradient effects). An aliquot of 0.6 mL of the mixed 12 mL was transferred into a third sterile test tube for immediate filtration and gold coating before microscopic examination.

The procedure of lumbar punctures was the same in the control group as in patients with the exception that PAJUNK 24 G \times 3.5 inch (90 mm) needles were used according to the hospital routine. CSF was sampled in two fractions, with the first 12 drops (0.6 mL) in a sterile test tube and the following 12 drops in a second tube. Both CSF samples of the controls were handled in a similar way as the CSF of the patients in the preparation for SEM.

To test the possibility that the sterile lumbar needles might contain some microscopic structures, 1 mL of distilled water or 1 mL of saline solution was injected through needles to polycarbonate filters, which were vacuum dried and gold coated before SEM examination. The concentration of albumin in CSF was measured in an aliquot of the mixed second portion of 12 mL of CSF. The CSF samples were assayed for the concentrations of albumin according to the method of Blennow et al. (14).

Filtering of CSF and coating techniques

Of each 0.6 mL fresh CSF fractions, 200 μ L were pipetted and dripped onto the surface of a polycarbonate filter (Nucleopore, Inc., Pleasanton, CA, USA) with 0.6 μ m pores. The polycarbonate filters were specially prepared by GP Plastic AB (Gislaved, Sweden), supplied by Sempore[®], AB (Stockholm, Sweden). The filter was fitted to an airtight device designed with flow channels which allowed CSF to stream to the center of the filter when vacuum suction was applied from below. This design allows fluids and particles with sizes

smaller than 0.6 μ m to drip through the filter. The remaining structures in the CSF were thus concentrated in the center of the filter during the rapid drying by vacuum suction. The central area is illustrated with a red circle on the gold-coated filter inserted in the upper left corner of Figure 1A. When the filters were completely dried after about two minutes of vacuum suction at room temperature, they were subsequently coated in a JEOL JFC-1200 Fine Coater (JEOL DATUM, Tokyo, Japan) for two minutes with ionized gold to a thickness of 40 Å.

Scanning electron microscopy

The filters were analyzed in an SEM microscope (Philips High Resolution SEM 515, Philips Electronic Instruments, Eindhoven, The Netherlands). The full area of the filter with a diameter of 1 cm was examined in the microscope. The peripheral area outside the center was free of structures (Figs. 1A and 1B). The total filter area was examined for particles in CSFs of the control group. As presented in the Results section and in Figure 1, we estimated the size, shape, number of particles and cells, and any other structure, since the amount and numbers present could have biological significance. To standardize the procedure, two SEM images of the central areas of the filters were enlarged to \times 500 and \times 2000. The same images were rated by two experienced researchers who estimated the SEM results independently and were blind to the diagnosis and any information about patients or controls. The diameter of the spherical particles varied from 1–2 μ m to 10–15 μ m (Fig. 1A). The sizes of structures were measured by both comparison with an inserted bar as well as the frequent presence of the 0.6 μ m-sized pores in the filter (Fig. 1B). Although the pores were 0.6 μ m, we discovered several particles with smaller diameter down to 0.1 μ m on the filter between the pores and on the larger structures. We decided that it was feasible to visually rate the quantity of morphological structures on the filters in the following four categories: 0 = none, 1 = few, 2 = several, and 3 = many (Figs. 1A–F). The most pronounced shapes and forms seen were also rated as presence of: (i) spherical particles, (ii) elongated threads, and (iii) threads with spherical particles attached (Figs. 1C–F).

Statistical analysis

The nonparametric Mann–Whitney *U*-test was used to identify differences between groups for continuous variables, and categorical variables

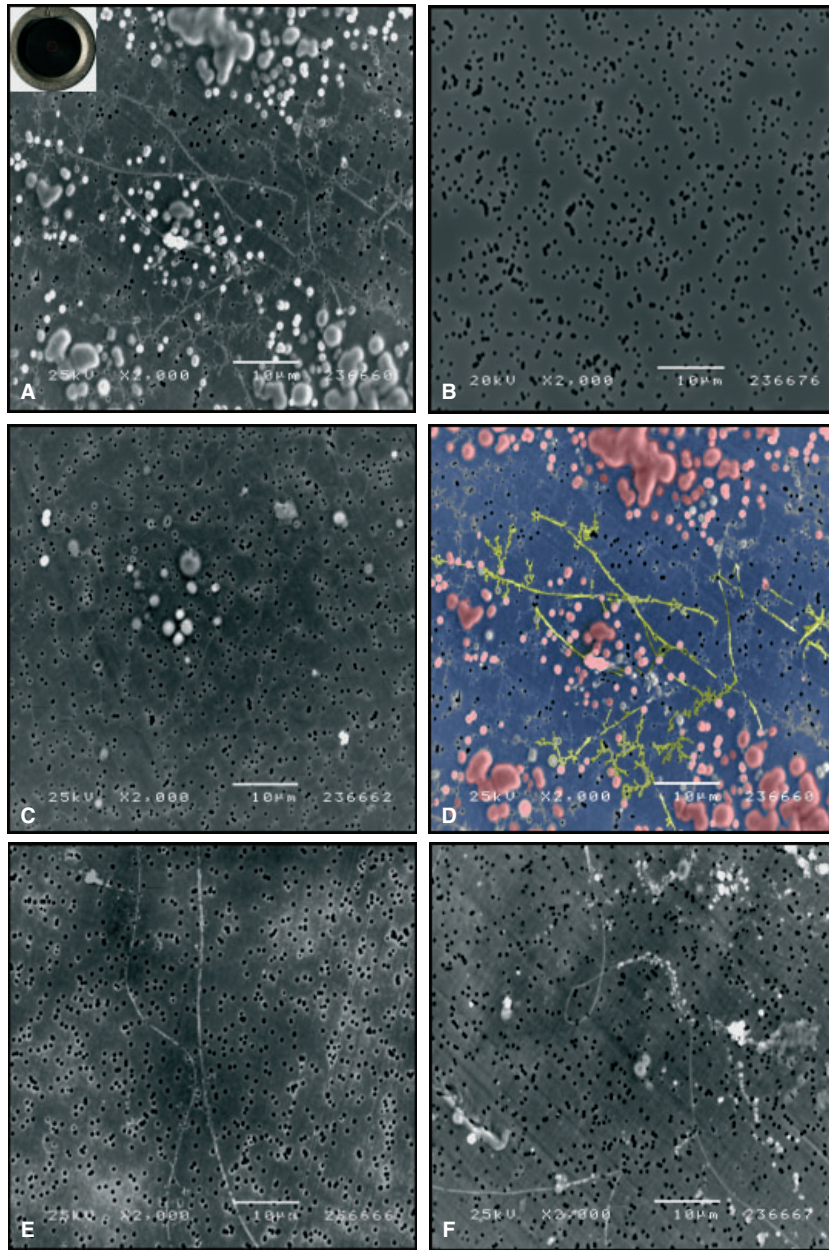


Fig. 1. Scanning electron micrographs (SEM) of 200 μL of the first 0.6 mL of cerebrospinal fluid (CSF) are shown for four patients and one control. All six SEM pictures are enlarged $\times 2000$. The scale of the white bar in each photo is 10 μm . All images, enlarged to $\times 500$ and $\times 2000$ for each subject, were rated by two experienced researchers who estimated the SEM results independently, blind to the diagnosis and other information about the patients and controls. **A.** A photo of a gold-coated polycarbonate filter with 0.6 μm pores fitted to an airtight device is inserted in the upper left corner. The red ring in the center of this filter indicates the area in which the morphological structures are concentrated during the vacuum suction of the CSF. The larger filter in black and white illustrates a CSF with many spherical particles and elongated threads, belonging to the quantitative category 3. **B.** The photo is of a filter with CSF from a control subject without any morphological structures in CSF. The 11 patients with bipolar disorder, also without microscopic structures in their CSF, were classified to the quantitative category 0. **C.** CSF of a patient with bipolar disorder containing spherical particles. **D.** The filter is from the same CSF as Figure A, with spherical particles and elongated threads, which have been colorized for illumination of the visually rated structures and patterns. **E.** Two patients showed CSF containing elongated threads. **F.** Six patients displayed threads with small spherical particles attached.

were compared by the Fisher exact test. In all statistical analyses, p -values < 0.05 were taken to indicate statistical significance. The JMP (SAS, Cary, NC, USA) and SPSS (SPSS, Inc., Chicago, IL, USA) software packages were used.

Results

Quantitative evaluation of morphological structures

The SEM results of the first 0.6 mL portion of CSF in 56 patients, 29 females and 27 males, with

BP were inspected and classified into four categories (0, 1, 2, and 3) depending upon the amount of structures observed in each individual CSF sample (Fig. 1). Structures in this fraction of CSF were found in 45 of the BP patients, 27 BP I and 18 BP II. Of these, 21 showed small amounts, 11 intermediate, and 13 large amounts in the first 0.6 mL of CSF (Table 1). Eleven patients, 4 BP I and 7 BP II, did not display any structures in this first portion of CSF. The second fraction of 12 mixed mL of CSF was free of structural particles in 54 patients, and in the 2 who also had structures in the first fraction we found few structures, rated as category 1. Thus, there is a significant difference in number of patients with structures in CSF fraction #1 (45 of 56, 80%) compared to CSF fraction #2 (2 of 56, 3.5%). No morphological structures were found in any of the 20 nonpsychiatric control patients, either in the first or in the second fraction of their CSF.

Qualitative evaluation of morphological structures

The evaluation of the image patterns of the morphological SEM structures revealed that CSF in 43 of the 56 patients showed either spherical particles alone ($n = 25$) or in combination with thread-like forms ($n = 12$), or threads with micrometer-sized spherical particles closely attached ($n = 6$) (see Fig. 1). Two patients with category 1 structures displayed threads without any spherical particles (Fig. 1E).

Morphological structures in relation to clinical assessment and treatment

As seen in Table 2, the 45 patients with SEM structures in CSF were more often diagnosed as subtype BP I and had a history of psychosis in manic state, certified hospitalization, and less comorbidity compared to the 11 patients with no microscopic structures, either in their first or

Table 2. Demographic data, clinical assessment, course of illness, medication, and albumin ratio of 56 bipolar disorder patients (31 BP I, 25 BP II), with and without SEM structures in CSF

Variable	Category 0 (none) ($n = 11$)	Categories 1, 2, 3 ($n = 45$)	Category 3 (many) ($n = 13$)
Demographic data			
Age (years), mean \pm SD	35.4 \pm 14.0	37.2 \pm 11.0	35.1 \pm 10.0
Gender, female, %	64	49	54
Cohabitation status ^a , %	45	33	31
Higher education ^b , %	45	47	46
Clinical assessment			
Bipolar I disorder, %	36	60	62
Family history ^c , mean \pm SD	9.9 \pm 6.7	10.3 \pm 7.0	11.5 \pm 7.7
Comorbidity, %	73	40	31
Alcohol intake ^d , mean \pm SD	5.4 \pm 9.8	4.6 \pm 8.0	6.7 \pm 13.0
Smoker, %	9	22	38
Course of disease			
Age of onset (years), mean \pm SD	27.4 \pm 8.7	26.3 \pm 10.1	23.8 \pm 7.4
Duration of illness (years), mean \pm SD	12.6 \pm 14.7	15.9 \pm 10.9	14.3 \pm 9.1
Hospitalization (certified), %	27	49	46
Manic episodes for BP I, mean \pm SD	1.3 \pm 0.5	2.9 \pm 2.7	3.4 \pm 3.0
Psychosis, %	27	47	54
Depressive episodes (all), mean \pm SD	19.3 \pm 30.0	11.7 \pm 18.0	8.3 \pm 5.9
Medication, %			
Lithium	63	49	69
Antiepileptic drugs ^e	27	36	31
Antipsychotic drugs ^f	18	27	15
CSF laboratory test			
CSF albumin $\times 10^3$ /serum albumin, mean \pm SD	5.6 \pm 1.8	5.9 \pm 3.0	4.7 \pm 1.2

For category definitions, see Table 1. BP I = bipolar I disorder; BP II = bipolar II disorder; SEM = scanning electron microscope; CSF = cerebrospinal fluid.

^aLiving with partner = positive score; living alone, or with parents = negative score.

^bHigher education level defined as at least three years of university studies.

^cFamily history denotes number of points on the bipolar index scale for relatives with disease.

^dIndicated as standardized glass of ethanol used per week.

^eAntiepileptic drugs that patients used were lamotrigine, valproate, or oxcarbazepine.

^fAntipsychotic drugs that patients used were olanzapine, quetiapine, propiomazine, aripiprazole, haloperidol, or clozapine.

second fraction of CSF. There were no differences between the two groups, with and without SEM particles, with respect to age, cohabitation status, and level of education (Table 2).

Morphological structures in relation to treatment

There were no significant differences with respect to medication with lithium, antiepileptic, or antipsychotic drugs between patients with and without CSF structures (Table 2). Of the 4 patients with no medication at the time of CSF sampling, all displayed structures at SEM examination, two in category 1 and one each in categories 2 and 3. Of 10 patients who did not receive lithium, antipsychotic, or antiepileptic medication, 9 patients displayed structures in CSF. In the patient group free of particles in their CSF, 3 were on antiepileptic and 2 on antipsychotic medication.

Albumin ratio

The CSF albumin $\times 10^3$ /serum ratio was 4.7 ± 1.2 in the 13 patients in category 3, with most structures in CSF, compared to the ratio of 5.6 ± 1.8 in the 11 patients in category 0. The upper reference limit for the CSF/S albumin ratio is 6.8 for healthy individuals under 45 years of age and 10.2 for individuals over 45 years of age (14). The concentrations of albumin did not demonstrate any significant differences between the two patient groups, with and without SEM structures in the first 0.6 mL fraction of CSF.

Limitations of the study

Analyses of protein, glucose, and cell counts, which are usually included on CSF samples, were omitted in the study since we used the first 0.6 mL for microscopic and subsequent biochemical analyses of this portion of CSF. With respect to possible increased protein CSF content, however, we analyzed the integrity of the blood-brain barrier by measure of the albumin content reported in Table 2. The CSF/S albumin ratio did not differ significantly between the patients according to the amount of structures in their CSF.

Discussion

In this first report of using SEM analysis to study CSF in patients with BP, we found morphological structures in the first portion of CSF in 45 of 56 of the patients compared to none in 20 controls. We assessed the microscopic structures quantitatively (in four categories) as

well as qualitatively (in three image profiles). It is conceivable that the presence of various amounts and patterns of the SEM findings in CSF might reflect the degree of severity of the disease. This hypothesis may be tested over time when CSF of BP patients will be re-examined in manic as well as depressed states.

There was a clear gradient in the amount of structures, which was detected almost only in the first portion of CSF. There is no definite explanation for this. One possibility is that the SEM structures are adherent to endothelial cells or nerve fibers in the lumbar canal (15) and detach during the lumbar puncture procedure and thus appear only in the first fraction of CSF. Further support for a possible endothelial dysfunction in some psychiatric disorders has recently been introduced by Harris et al. (16), who found signs of a hypoinflammatory state in the cerebral microvasculature in patients with schizophrenia. The structures may thus not be specific to BP, since morphological SEM structures have previously been found in CSF of schizophrenic patients (3). In comparison with the structures in the schizophrenic patients, we found more elongated threads in the CSF of BP patients, as shown in Figures 1E and 1F. The structures in the schizophrenic patients were more similar to the spherical structures in Figure 1C, seen in 25 patients, while thread-like structures were specific to 20 BP patients.

In that study, 2 of 39 controls also showed spherical particles in CSF: one female with paraesthesia, reduced motor control, and long-lasting pain, and one male with repeated episodes of low back pain. Interestingly, Ekelund et al. (17), using a slightly different microscopic method on frozen samples, found similar structures in CSF in 3 of 9 patients with chronic pain syndrome. For ethical reasons, the CSF sampling of the controls occurred when lumbar puncture was performed as a routine medical procedure. Thus, the needle size used for lumbar puncture differed between the patient and the control group; the BP patients were punctured using a needle with a larger diameter than the controls. It is unlikely, however, that the needle size would explain the lack of SEM structures in the controls given the results of the previous study, in which larger lumbar needles (20 GA 3.5 inch, 0.9×90 mm) were used in 38 controls, of whom 36 were free of SEM particles in CSF (3). Moreover, control experiments were done by injecting distilled water or saline solution through needles to polycarbonate filters in order to exclude a possible contamination from the lumbar puncture needles. SEM examinations of the filters displayed a similar picture as seen in Figure 1B, indicating that no

microscopic structures originated from the lumbar puncture needles. Another facet that needs to be considered is that the mean age of our controls was higher than that of the BP patients. This may, however, be seen as more of an advantage since the risk for CSF disturbances increases with advanced age (18). Furthermore, the age range of the 38 controls in the earlier study was 22–62 years for 23 females, and 39–82 years for 15 males (3). It is therefore unlikely that the different results of the SEM examination of CSF between BP patients and controls in the present study are due to age difference. Results from other studies indicate that increased albumin in patients with blood-brain barrier damage might be a general risk factor in vulnerable neuropsychiatric patients, and elevated levels of protein in CSF have been reported in male individuals when studied during an episode of clinical depression (19), although no causal links have been proven with BP (20). However, there was no difference in the albumin ratio in the 13 patients with most structures (category 3) in CSF compared to the 11 patients with none (Table 2). Therefore, the explanation that increased blood-brain barrier permeability would be the cause of the increased amount of structures in the majority of patients in the cohort of BP under study may be refuted.

The presence of microscopic structures in CSF might be relevant for future studies of the possible causative role of inflammation in the pathogenesis of BP. In this context, the reports that lithium has several effects on the immune regulation in BP are of interest (21–24). Twenty-nine (52%) of the 56 patients in our study were on lithium, which may have influenced the SEM results. However, for ethical reasons, patients in this study continued to take their lithium medication during the examination period due to the risk of relapse associated with discontinuation of long-term lithium treatment (25). It is noted that 7 out of 11 patients without any SEM structures were on lithium treatment. This study might also be relevant for the study of the role of infective agents in BP, which have for a long time been thought to be involved in the origin of BP in at least some patients (26).

Further studies are needed to elucidate whether and how the CSF structures may be related to infective agents, immune or other cellular remains, autoimmune and inflammatory components, apoptotic debris, and/or other hypothetical factors. It is also important to investigate the amount and pattern of the structures in different disease states, including manic and depressive episodes.

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