Aggrecan and chondroitin-6-sulfate abnormalities in schizophrenia and bipolar disorder: a postmortem study on the amygdala

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INTRODUCTION

Chondroitin sulfate proteoglycans (CSPGs) are one of the main components of the brain extracellular matrix (ECM). Emerging evidence from human postmortem, animal model and genetic studies points to their involvement in the pathophysiology of schizophrenia (SZ). Postmortem studies show abnormalities of CSPG-enriched perineuronal nets (PNNs), specialized ECM aggregates enveloping distinct neuronal populations, in subjects with SZ. Specifically, PNNs labeled with a lectin CSPG marker, that is, Wisteria floribunda agglutinin (WFA), are decreased in the amygdala, entorhinal cortex and prefrontal cortex, but not visual cortex, of subjects with this disorder. In the amygdala, entorhinal cortex, WFA-positive PNN decreases were accompanied by sharp, widespread, increases of WFA-positive glial cells. Together with reduced CSPG expression in the olfactory epithelium, these findings suggest that CSPG abnormalities may be region-selective but inclusive of a wide range of neuronal structures in SZ. Rodent studies are consistent with a role of PNNs in SZ. Models of oxidative stress relevant to this disorder show disruptions of multiple PNN populations in the amygdala of SZ and, more modestly, BD. Decreases of aggrecan-IR glia and CS-6-IR glial ‘clusters’ in sharp contrast to increases of CSPG/lectin-positive glia previously observed, indicate that CSPG abnormalities may affect distinct glial cell populations and suggest a potential mechanism for PNN decreases. Together, these abnormalities may contribute to a destabilization of synaptic connectivity and regulation of neuronal functions in the amygdala of subjects with major psychoses.

PERINEURONAL NETS (PNNs)

Perineuronal nets (PNNs) are specialized extracellular matrix aggregates surrounding distinct neuronal populations and regulating synaptic functions and plasticity. Previous findings showed robust PNN decreases in amygdala, entorhinal cortex and prefrontal cortex of subjects with schizophrenia (SZ), but not bipolar disorder (BD). These studies were carried out using a chondroitin sulfate proteoglycan (CSPG) lectin marker. Here, we tested the hypothesis that the CSPG aggrecan, and 6-sulfated chondroitin sulfate (CS-6) chains highly represented in aggrecan, may contribute to these abnormalities. Antibodies against aggrecan and CS-6 (3B3 and CS56) were used in the amygdala of healthy control, SZ and BD subjects. In controls, aggrecan immunoreactivity (IR) was observed in PNNs and glial cells. Antibody 3B3, but not CS56, also labeled PNNs in the amygdala. In addition, dense clusters of CS56 and 3B3 IR encompassed CS56- and 3B3-IR glia, respectively. In SZ, numbers of aggrecan- and 3B3-IR PNNs were decreased, together with marked reductions of aggrecan-IR glial cells and CS-6 (3B3 and CS56)-IR ‘clusters’. In BD, numbers of 3B3-IR PNNs and CS56-IR clusters were reduced. Our findings show disruption of multiple PNN populations in the amygdala of SZ and, more modestly, BD. Decreases of aggrecan-IR glia and CS-6-IR glial ‘clusters’, in sharp contrast to increases of CSPG/lectin-positive glia previously observed, indicate that CSPG abnormalities may affect distinct glial cell populations and suggest a potential mechanism for PNN decreases. Together, these abnormalities may contribute to a destabilization of synaptic connectivity and regulation of neuronal functions in the amygdala of subjects with major psychoses.

Aggrecan and chondroitin-6-sulfate abnormalities in schizophrenia and bipolar disorder: a postmortem study on the amygdala

H Pantazopoulos, M Markota, F Jaquet, D Ghosh, A Wallin, A Santos, B Caterson and S Berretta

Perineuronal nets (PNNs) are specialized extracellular matrix aggregates surrounding distinct neuronal populations and regulating synaptic functions and plasticity. Previous findings showed robust PNN decreases in amygdala, entorhinal cortex and prefrontal cortex of subjects with schizophrenia (SZ), but not bipolar disorder (BD). These studies were carried out using a chondroitin sulfate proteoglycan (CSPG) lectin marker. Here, we tested the hypothesis that the CSPG aggrecan, and 6-sulfated chondroitin sulfate (CS-6) chains highly represented in aggrecan, may contribute to these abnormalities. Antibodies against aggrecan and CS-6 (3B3 and CS56) were used in the amygdala of healthy control, SZ and BD subjects. In controls, aggrecan immunoreactivity (IR) was observed in PNNs and glial cells. Antibody 3B3, but not CS56, also labeled PNNs in the amygdala. In addition, dense clusters of CS56 and 3B3 IR encompassed CS56- and 3B3-IR glia, respectively. In SZ, numbers of aggrecan- and 3B3-IR PNNs were decreased, together with marked reductions of aggrecan-IR glial cells and CS-6 (3B3 and CS56)-IR ‘clusters’. In BD, numbers of 3B3-IR PNNs and CS56-IR clusters were reduced. Our findings show disruption of multiple PNN populations in the amygdala of SZ and, more modestly, BD. Decreases of aggrecan-IR glia and CS-6-IR glial ‘clusters’, in sharp contrast to increases of CSPG/lectin-positive glia previously observed, indicate that CSPG abnormalities may affect distinct glial cell populations and suggest a potential mechanism for PNN decreases. Together, these abnormalities may contribute to a destabilization of synaptic connectivity and regulation of neuronal functions in the amygdala of subjects with major psychoses.
other molecules including growth factors and cytokines. Chondroitin-4-sulfation (CS-4) and chondroitin-6 sulfation (CS-6) are the two most common sulfation patterns in the brain, with several variations depending on the position of the sulfation on the CS chains (Figure 1). For the present investigations, we focused on the CSPG aggrecan and the CS-6 sulfation pattern. Aggrecan is a major component of the brain ECM and of at least a subgroup of PNNs, and contains numerous CS chains with a predominant CS-6 representation. In parallel to PNN development, aggrecan expression coincides with the maturation of electrophysiological properties of neurons and the formation of synapses during late stage developmental periods. Furthermore, aggrecan and CS-6 expression in glial cells are involved in the regulation of astrocyte maturation, which, in turn, has a key role in PNN formation and maintenance. With the present postmortem study, we tested the hypothesis that aggrecan and CS-6 patterns contribute to PNN and glia abnormalities in SZ. Subjects with bipolar disorder (BD) were included in these investigations to assess whether these abnormalities are specific to SZ or instead shared across major psychotic disorders.

**MATERIALS AND METHODS**

**Human subjects**

Tissue blocks containing the whole amygdala from a cohort of normal control donors \((n = 29)\), and donors with SZ \((n = 24)\) or BD \((n = 20)\) and were used for histochemical and immunocytochemical investigations (Supplementary Tables 1–3). A sub-cohort of these subjects, that is, normal control \((n = 12)\), SZ \((n = 12)\) and bipolar disorder \((n = 13)\) donors, was used for the aggrecan study. This sub-cohort overlapped to a great extent to the one used for the WFA study previously published, that is, 12 out of 12 controls, 10 out of 12 subjects with SZ and 9 out of 13 subjects with BD were included in both studies. The sub-cohorts included in the CS56 (control, \(n = 13\)); SZ, \(n = 14\); BD, \(n = 8\)) and 3B3 (control, \(n = 14\); SZ, \(n = 13\); BD, \(n = 8\)) studies largely overlap with each other, but only minimally with that used for aggrecan and WFA (Supplementary Tables 1–3). Additional tissue blocks from 14 normal controls and 14 SZ subjects were used for quantitative PCR with reverse transcription (Supplementary Materials; Supplementary Tables 2–3). All tissue blocks were obtained from...
the Harvard Brain Tissue Resource Center (HBTRC), McLean Hospital, Belmont, MA, USA. Diagnoses of SZ and BD were made by two psychiatrists on the basis of retrospective review of medical records and extensive questionnaires concerning social and medical history provided by the family members. Several regions from each brain were examined by a neuropathologist. The cohort used for this study did not include subjects with evidence of head trauma and/or macroscopic brain changes, or clinical history consistent with cerebrovascular accident or other neurological disorders. Subjects with Braak stages III or higher (modified Bielchowsky stain) were not included. None of the subjects had significant history of substance dependence within 10 or more years from death, as further corroborated by negative toxicology reports. Absence of recent substance abuse is typical for samples from the HBTRC, which receives exclusively community-based tissue donations.

Tissue processing

Tissue blocks for immunohistochemistry were dissected from fresh brains and post-fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.1 M Na azide at 4°C for 3 weeks, then cryoprotected at 4°C for 3 weeks (30% glycerol, 30% ethylene glycol and 0.1% Na azide in 0.1 M phosphate buffer), embedded in agar and pre-sliced in 2 mm coronal slabs using an Antithetic Tissue Slicer (Stereochemical Research Lab, Aarhus, Denmark). Each slab was exhaustively sectioned using a freezing microtome (American Optical 860, Buffalo, NY, USA). Sections were stored in cryoprotectant at −20°C. Using systematic random sampling criteria, sections through the amygdala were serially distributed in 26 compartments (40 μm thick sections; 10–12 sections per compartment; 1.04-mm section separation within each compartment). All sections within one compartment per subject were selected for each marker (that is, cat-301, CS56, C8035, CS56 goat anti-mouse IgM; 1: 500; Vector Labs, Burlington, CA, USA). This step was followed by streptavidin conjugated with horse-radish peroxidase for 2 h (1:5000 μl, Zymed, San Francisco, CA, USA) and, finally, nickel-enhanced diamobenzidine/peroxidase reaction (0.022% diamobenzidine, Sigma-Aldrich, 0.088% nickel-sulfate, 0.006% hydrogen peroxide in phosphate-buffered saline) with 0.5% Triton X unless otherwise specified. All sections were mounted on gelatin-coated glass slides, coverslipped and coded for quantitative analysis blinded to diagnosis. Sections from all the brains included in the study were processed simultaneously within the same session to avoid procedural differences. Each six-well staining dish contained sections from SZ, BD and normal control subjects and was carried through each step for the same duration of time, so as to avoid sequence effects. Omission of the first (cat-301, CS56 or CS56) or secondary antibodies did not result in detectable signal.

Quantitative PCR with reverse transcription

Frozen tissue samples were processed for total RNA isolation and purification and transcript versions 1 and 2 of the human aggrecan gene (ACAN) were detected by quantitative PCR with reverse transcription using the Taqman gene expression assay Hs00153936_m1 and GAPDH, RPII and HPRT1 as reagent genes. Normalization of aggrecan gene expression to GAPDH, RPII and HPRT1 gene expression and comparison of gene expression between diagnosis groups was calculated according to the 2−ΔΔCT method by Livak and Schmittgen64,65 (for more details, see Supplementary Materials).

Data collection

A Zeiss Axioskop 2 Plus interfaced with Stereo Investigator 6.0 (Microbrightfield, Willinston, VT, USA) was used for the analysis. The borders of the lateral (LN), basal (BN), accessory basal (AB), cortical (CO), medial (ME) and central (CE) nuclei of the amygdala (Figure 2) were identified according to cytoarchitectonic criteria as described by Sims and Williams66 and Amaral et al.67 The nomenclature adopted was that used by Sorvari et al.68 The intercalated cell masses were not included within the borders of these nuclei. The paralaminar nucleus could not be distinguished reliably from the ventral basal nucleus and was thus included within its borders. A ×1.6 objective was used to trace the borders of amygdalar nuclei. Each traced region was systematically scanned through the full x, y and z axes using a × 40 objective to count each immunoreactive (IR) element within the traced borders over complete sets of serial sections (6–10 sections) representing the whole extent of the amygdala from each subject, with a section interval of 1040 μm. This method has the advantage of minimizing the risk of errors in estimating poorly represented and/or unevenly distributed elements, such as those investigated in this study.64,67

Numerical densities and total numbers estimates. Numerical densities (Nd) were calculated as Nd = Σn / ΣV, where N is the sum of cells within a region of interest, and V is the total volume of the region of interest. Total number (Tn) of IR elements (PNNs, glia, glial cell clusters) was calculated as Tn = Σn where Σn = sum of the cells counted in each subject, and i is the section interval (that is, number of serial sections between each section and the next within each compartment = 26), as described previously in detail.68

Statistical analysis

Differences between groups relative to the main outcome measures in each of the regions examined were assessed for statistical significance using an analysis of covariance stepwise linear regression process. Effect sizes were calculated according to Hedges’ g. A logarithmic transformation was uniformly applied to all original values because the data were not normally distributed. Statistical analyses were performed using JMP v5.0.1a (SAS Institute, Cary, NC, USA). BD and SZ were compared separately with normal controls. Age, gender, postmortem time interval, inflammation (classified as positive or negative for inflammatory condition at the time of death), hemisphere, cause of death, brain weight, exposure to alcohol, nicotine, electroconvulsive therapy, and lifetime as well as final 6 months, exposure to antipsychotic drugs, exposure to selective serotonin reuptake inhibitors classified as positive or negative for exposure, and lithium treatment were tested systematically for their effects on the main outcome measures, and included in the model if they significantly improved the model goodness of fit. Values relative to the r ratio and P-value for main
outcome measure differences found to be statistically significant are reported in Supplementary Tables 4–6. Any and all covariates found to affect an outcome measure significantly are also reported.

Cause of death was categorized as acute (for example, myocardial infarction) or chronic (for example, cancer). Data on nicotine and alcohol exposure were only available for subjects with SZ or BD; on the basis of the subjects’ record, exposure was considered as high, moderate, low and absent, as well as present or absent during the last 10 years of life. We analyzed the medical records for exposure to various classes of psychotropic and neurotropic drugs. Estimated daily mg doses of antipsychotic drugs were converted to the approximate equivalent of chlorpromazine as a standard comparator, and corrected on the basis of a qualitative assessment of treatment-adherence based on taking prescribed psychotropic medicines more or less than approximately half of the time, as indicated by the extensive antemortem clinical records. These values were reported as lifetime, as well as last 6 months’ of life, grams per patient (Supplementary Table 3). Exposure to lithium salt was estimated in the same manner (Supplementary Table 3). Exposure to lithium salt was estimated in the same manner (Supplementary Table 3). Exposure to other classes of psychotropic drugs was reported as present or absent (Supplementary Table 3). These variables, as well as subtypes of SZ (for example, paranoid, catatonic, disorganized) and measures of life quality (for example, dependent/independent), could not be tested reliably because the number of subjects in each category was too low. However, these variables were taken into account as a possible explanation when apparent clustering of subjects was observed. In addition to testing the potential effects of exposure to antipsychotics and lithium salt within our stepwise linear regression process, the effects of these variables, together with other psychotropic and neurotropic drugs, adherence to pharmacological treatment (good or poor), age of onset of the disease and duration of the illness, were tested directly in separate analyses of variance.

**RESULTS**

Aggrecan and CS-6 expression in the normal human amygdala. Aggrecan IR was observed in PNNs predominantly located within the LN. A large number of 3B3-IR PNNs were detected throughout the amygdala, outnumbering by far aggrecan-IR PNNs (Supplementary Tables 4 and 5) as well as WFA-positive PNNs, particularly within the BN, ABN, CO and CE/ME (Supplementary Tables 4 and 5). Within the LN, approximately half (54%) of the aggrecan-IR PNNs and over 80% of the 3B3-IR PNNs are also labeled with WFA (see Supplementary Materials for details). CS-6 did not label PNNs.

Glia. A small number of aggrecan-IR glia were scattered in all the amygdala nuclei examined. These cells were not labeled by WFA and did not show GFAP-IR (see also Supplementary Materials). Numerous clusters of dense CS56- and 3B3-IR product, encompassing CS56- and 3B3-IR cells, respectively, morphologically identifiable as glia and showing faint GFAP-IR, were detected in all the amygdala nuclei examined (Figures 1 and 3). WFA labeling was not detected in these clusters. We refer to these structures, that is, CS-6-IR glia surrounded by diffuse CS-6-IR, as ‘glial clusters’. Quantitative analysis for normal and comparison studies (below) focused on the number of glial clusters because under light microscopy the intensity of the diffuse immunolabeling did not allow clear identification of individual CS-6-IR glial cells (Figures 1g-i), and the large majority of these cells are contained within the clusters. We estimate that each glial cluster may contain approximately two to seven CS-6-IR glial cells. Notably, several 3B3-IR PNNs were observed in close contact with 3B3-IR glial...
clustering (Figures 1g and h), suggesting functional relationships between the two elements.

Decrease of aggrecan-IR PNNs and glia, but increased aggrecan mRNA expression, in schizophrenia

In subjects with SZ, Tn and Nd of aggrecan-IR PNNs were significantly decreased in the LN (Tn, \( P < 0.03, g = -0.95 \); Nd, \( P < 0.04, g = -0.93 \); adjusted for cause of death, \( P < 0.003 \); Figure 4, Supplementary Table 4). Aggrecan-IR glia (Tn) were significantly decreased in the LN (\( P < 0.01, g = -1.11 \)), BN (\( P < 0.01, g = -1.19 \)) and ABN (\( P < 0.005, g = -1.28 \)); significance values adjusted for the effects of PMI (\( P = 0.01 \); Figure 4, Supplementary Table 4). Aggrecan mRNA expression was significantly increased in the LN of SZ subjects (\( P < 0.03; g = 0.90 \); see Supplementary Figure 3).

In subjects with BD, aggrecan-IR PNNs (Tn, but not Nd) were decreased in the LN (\( P < 0.04, g = -0.88 \); Figure 4, Supplementary Table 4). Tn and Nd of aggrecan-IR PNNs were decreased in the ABN (Tn, \( P < 0.04, g = -0.92 \); Dn, \( P < 0.03, g = -1.37 \); corrected for lifetime exposure to lithium, \( P < 0.01 \); Figure 4, Supplementary Table 4). No changes in aggrecan-IR glia were observed in BD (Figure 4, Supplementary Table 4).

Decreased 3B3-IR PNNs and glial clusters in schizophrenia and bipolar disorder

In subjects with SZ, 3B3-IR PNNs were decreased in the LN (Tn, \( P < 0.01, g = -1.37 \); Dn, \( P < 0.03, g = -0.87 \)), BN (Tn, \( P < 0.002, g = -2.12 \); Nd, \( P < 0.006, g = -1.49 \)), ABN (Tn, \( P < 0.002, g = -1.68 \); Nd, \( P < 0.009, g = -1.44 \)), CO (Tn, \( P < 0.001, g = -1.88 \); Nd, \( P < 0.003, g = -1.65 \)), and ME (Tn, \( P < 0.04, g = -1.04 \)). In the CE, 3B3-IR PNN decreases did not reach statistical significance, although the effect sizes were very large (Tn, \( P < 0.06, g = -0.91 \); Nd, \( P = 0.07, g = -1.02 \); Figure 5, Supplementary Table 5). Significance values for each nucleus were adjusted for the effects of cause of death (\( P < 0.03 \)); PMI (\( P < 0.15 \), included a priori due to its significant positive effect on PNN Tn and Nd in SZs (\( P < 0.01 \)), and VPA (\( P < 0.03 \)). Decreases were also observed for 3B3-IR glial clusters in LN (Tn, \( P < 0.0003, g = -1.93 \); Nd, \( P < 0.0007, g = -1.74 \)), BN (Tn, \( P < 0.0006, g = -1.77 \); Nd, \( P < 0.002, g = -1.53 \)), ABN (Tn, \( P < 0.001, g = -1.63 \); Nd, \( P < 0.005, g = -1.40 \)), CO (Tn, \( P < 0.02, g = -1.12 \); Nd, \( P < 0.04, g = -0.95 \)), CE (Tn, \( P < 0.03, g = -0.98 \); Nd, \( P < 0.06, g = -0.9 \)) and ME (Tn, \( P < 0.005, g = -1.36 \); Nd, \( P < 0.03, g = -1.09 \)). Significance values for each nucleus were adjusted for effects of age (\( P < 0.001 \)) and cause of death (\( P < 0.002 \); Figure 5, Supplementary Table 5).

In subjects with BD, 3B3-IR PNNs were decreased in the LN (Tn, \( P < 0.002, g = -2.14 \); Nd, \( P < 0.002, g = -2.00 \)), BN (Tn, \( P < 0.01, g = -1.01 \); Nd, \( P < 0.05, g = -1.93 \)), AB (Tn, \( P < 0.05, g = -0.94 \); Nd, \( P < 0.004, g = -1.97 \)) and ME (Tn, \( P < 0.007, g = -2.16 \); Nd, \( P < 0.03, g = -1.32 \)). Significance values for each nucleus were adjusted for effects of lifetime lithium (\( P < 0.05 \); Figure 5, Supplementary Table 5). Decreases in 3B3-IR glial clusters were observed only in the ME (Tn, \( P < 0.03, g = -1.05 \); Figure 5, Supplementary Table 5).

Decreased CS-6 (CS56) glial clusters in schizophrenia and bipolar disorder

In subjects with SZ, CS56-IR glial clusters were significantly decreased in the LN (Tn, \( P < 0.0004, g = -2.09 \); Nd, \( P < 0.002, g = -1.80 \)), BN (Tn, \( P < 0.0005, g = -2.00 \); Nd, \( P < 0.002, g = -1.74 \)), ABN (Tn, \( P < 0.01, g = -1.39 \); Nd, \( P < 0.02, g = -1.21 \)), CE (Tn, \( P < 0.007, g = -1.64 \); Nd, \( P < 0.04, g = -1.17 \)) and ME (Tn, \( P < 0.04, g = -1.08 \); Nd, \( P < 0.06, g = -0.98 \); Figure 5, Supplementary Table 6). Decreases in the CO did not reach statistical significance, although the effect size was relatively large (Tn, \( P < 0.06, g = -0.98 \); Nd, \( P < 0.06, g = -0.95 \)). Significance values were adjusted for exposure to selective serotonin reuptake inhibitors (SSRIs; \( P < 0.05 \), which showed positive correlations with CS56-IR clusters.)
In BD subjects, significant decreases of CS56-IR glial clusters were observed in the LN (Tn, \( P < 0.002, g = −2.52; \) Nd, \( P = 0.0005, g = −2.90 \)), BN (Tn, \( P < 0.0006, g = −2.19; \) Nd, \( P < 0.003, g = −2.33 \)), ABN (Tn, \( P < 0.009, g = −1.98; \) Nd, \( P < 0.004, g = −2.22 \)), and CE (Tn, \( P < 0.04, g = −1.53; \) Nd, \( P < 0.006, g = −2.11 \); Figure 5, Supplementary Table 6). Significance values were adjusted for lithium exposure (lifetime; \( P < 0.05 \)), which showed a positive correlation with CS56-IR clusters.

**DISCUSSION**

Our results point to marked abnormalities in the expression of aggrecan and CS-6 in subjects with SZ and subjects with BD (Table 1). In particular, aggrecan-IR PNNs and glia were decreased in SZ, whereas only modest aggrecan-IR PNN decreases were present in BD. Numbers of CS-6 IR glial clusters and PNNs were markedly reduced in SZ and BD. Changes in BD provide the first evidence for anomalies of PNNs and CSPG expression in this disorder, and point to partially overlapping abnormalities of these elements in SZ and BD. PNN reductions affect neuronal populations larger than previously demonstrated, encompassing several amygdala nuclei, and suggest altered neuronal maturation and firing properties and instability of neuronal synaptic connectivity. In sharp contrast to increases of WFA-positive glial cells reported previously in SZ, \( ^{3} \) aggrecan-IR glia and CS-6 (3B3 and CS56)-IR glial clusters were markedly decreased. We suggest that decreases of CSPG-positive glial cells may provide important clues on the potential mechanisms of PNN abnormalities, raising the possibility that distinct glial cell populations may fail to synthesize and secrete key CSPGs required to maintain PNN integrity.

**Technical considerations**

**Pharmacological treatment and drugs of abuse.** Effects of pharmacological treatment detected in these studies were limited to SSRIs and lithium on a small number of outcome variables (Supplementary Tables 4–6). These effects are consistent with a corrective mechanism, showing positive correlations with CSPG-IR PNNs and glia in the face of significant decreases of these elements in diagnosis groups. SSRI exposure in SZ subjects was significantly and positively correlated with Tn and Nd of CS56-IR glial cell clusters (Supplementary Table 6). Records for SSRI exposure in BD subjects were insufficient to assess these effects. Lithium exposure correlated positively with CS56-IR glia in subjects with BD. This effect was somewhat unexpected because lithium treatment was shown in rodents to facilitate enzymatic CSPG digestion. It is possible that chronic exposure to lithium treatment in BD, species differences, and perhaps the interaction of lithium with altered CSPG biochemistry in BD may account for this effect.

The subjects included in this study had no significant history of substance dependence within 10 or more years from death. Lack of recent exposure was further corroborated by negative toxicology reports provided by the HBTRC. In addition, no significant effects were observed with ethanol or nicotine exposure on any of the outcome measures tested.

**CSPG labeling.** In this study, aggrecan was detected using the antibody cat-301, widely used in investigations on PNNs.
This antibody labels a distinct glycosylated form of the aggregan core protein, whereas other glycosylated forms may be only partially recognized. Thus, our results on aggregan may be specific to the form recognized by cat-301. This consideration may, at least in part, explain the discrepancy between aggregan mRNA and protein detected in this study (see Supplementary Materials). Compensatory mechanisms leading to increased mRNA expression in response to decreased protein availability may also account for this discrepancy, as observed in other molecular systems in schizophrenia.

Our results on aggregan and CS-6, and previous findings using WFA, are consistent with regard to PNNs, but not with regard to glial cells. Although the specific binding site for WFA is known, no data are available regarding which CSPGs and sulfation patterns WFA detects. Our results show that 3B3 and aggregan only partially colocalize with WFA in PNNs, and virtually not at all in glia (Supplementary Figures 1,2). Together, these findings indicate that WFA may not detect CS-6 sulfation patterns, at least not those recognized by 3B3 and CS56, and may label only some forms of aggregan (see below). The implication is that glial cells labeled by aggregan (cat-301), 3B3 and CS56 do not express CSPGs labeled by WFA. Similarly, discrepancies in the patterns of 3B3, CS56 and aggregan labeling, that is (i) 3B3- and CS56-IR glia, but not aggregan-IR glia, express GFAP (Figure 3 and Supplementary Figure 2) and (ii) 3B3 and aggregan label both PNNs and glia whereas CS56 only labels glia, suggest that each of these antibodies preferentially recognizes distinct CSPG/CS sulfation patterns.
and BD did not correlate with duration of the illness or age at excitation, (ii) availability of key PNN components, such as psychoses. Their maturation occurs in an experience-dependent development, a time period coinciding with the typical onset of WFA. PNNs, or may re show, to our knowledge for the first time, a disruption of PNNs in the amygdala of subjects with SZ are widespread, involving the LN as a CSPG, link proteins and tenascin R, (iii) expression on excitability, receptor desensitization, long-term potentiation and synaptic plasticity characteristic of adulthood. Overall, these functions underlie the role of the adult ECM to neuronal excitability, receptor desensitization, long-term potentiation and long-term depression. A disruption of PNNs may also contribute to altered glutamatergic transmission, synaptic connectivity and plasticity in SZ and BD. In particular, in the amygdala, the process of PNN maturation is necessary to transition from a juvenile form of plasticity, during which fear conditioning can be fully erased by extinction, to an adult form of plasticity, drastically weakening the effects of extinction on fear conditioning. Our findings suggest that PNN abnormalities may impact on emotion processing and plasticity in major psychoses, potentially contributing to a disruption of fear learning reported in subjects with SZ. In the LN, these changes may directly affect fear conditioning, whereas changes in the BN may impact on hippocampal context learning, and involvement of the CE, ME and ABN suggest direct effects on the bed nucleus of the stria terminalis, speculatively impacting on sustained fear mechanisms.

**Table 1. Summary of results**

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<td>−37.1</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>248.3</td>
<td>0.2</td>
<td>−53.2</td>
<td>−32.5</td>
</tr>
<tr>
<td></td>
<td>ME</td>
<td>318.3</td>
<td>NA</td>
<td>−72.9</td>
<td>−66.5</td>
</tr>
<tr>
<td></td>
<td>CE</td>
<td>17.8</td>
<td>NA</td>
<td>−58.7</td>
<td>−39.9</td>
</tr>
</tbody>
</table>

Abbreviations: ABN, accessory basal nucleus; BD, bipolar disorder; BN, basal nucleus; CE, central nucleus; CO, cortical nucleus; Dx, diagnosis; LN, lateral nucleus; ME, medial nucleus; NA, not available; PNN, perineuronal net; SZ, schizophrenia. Percent differences for numerical densities in disease groups with respect to the controls. Bold values and arrows indicate statistically significant changes (analysis of covariance (ANCOVA) on log-transformed values; see also Supplementary Tables 4–6). Note that percent changes included in this Table were calculated on raw values (before log transformation), and do not reflect effects of covariates included in ANCOVA models (see Results and Supplementary Tables 4–6), thus explaining some discrepancies. For instance, ANCOVA analysis for CS56-IR glial cells in the CE of subjects with SZ shows a significant decrease once exposure to SSRI is included in the model (see Results and Supplementary Table 6; marked in this Table with *); however, if SSRI exposure is not taken into account, the numerical density of CS56-IR glial cells in the CE of SZ appears to be increased (that is, 63.3%).

Decreases of PNN in SZ and BD In subjects with SZ, robust decreases of aggregcan- and 3B3-IR PNNs, together with marked decreases of WFA-positive PNNs previously reported, strongly indicate a widespread PNN pathology. Notably, the subject cohorts used for aggrecan (in this study) and for WFA, overlapped to a great extent. In the LN, where these three markers, at least in part, colocalize in PNNs, our findings suggest that PNNs may be lost or at least lack several key elements. Reports that aggrecan knockout results in loss of WFA-positive PNNs support this possibility. In the other amygdala nuclei tested, where the large majority of PNNs detected were labeled with 3B3, but not aggrecan or WFA, a similar conclusion can be drawn. Together, these results indicate that PNN abnormalities in the amygdala of subjects with SZ are widespread, involving the LN as well as the BN, ABN, CO and ME, and thus a range of neuronal populations broader than previously thought. In subjects with BD, marked reductions of 3B3-IR PNNs in LN, BN, ABN, CO and ME were accompanied by more modest decreases of aggrecan-IR PNNs in the LN and ABN. These findings show, to our knowledge for the first time, a disruption of PNNs in subjects with BD. PNN decreases in this disorder were not detected using WFA. Thus, CS-6 expression in PNNs in BD subjects may be decreased in the otherwise normal WFA-positive PNNs, or may reappear. In particular, in the amygdala, our findings show PNN loss/disruption in the amygdala of subjects with SZ and, perhaps to a lesser extent, BD. What may be the potential causes and consequences of the observed PNN pathology? PNNs are formed during late postnatal development, a time period coinciding with the typical onset of psychoses. Their maturation occurs in an experience-dependent manner, requiring (i) neuronal activation through glutamatergic excitation, (ii) availability of key PNN components, such as specific CSPGs, link proteins and tenascin R, (iii) expression on neurons of ECM surface receptors and (iv) transcription factors inducing neuronal maturation. Given that PNN numbers in SZ and BD did not correlate with duration of the illness or age at onset of the disorder, it is plausible that PNN decreases may already be present during the early phases of illness.
CSPGs and other PNN components. The present results help WFA-positive PNNs, because glial cells represent a main source of observed changes are interpreted as increased CSPG expression. All WFA-positive glial cells were found to express GFAP, and in turn, CSPG decreases in these cells may contribute to PNN reductions. We report, for the first time, the presence of glial/CSPG clusters in the human amygdala, and their decreases in subjects with SZ and BD. These clusters were shown in rodents to surround a small number of neurons and regulate glutamatergic transmission. This possibility is in line with growing evidence indicating that astrocytes form microdomains modulating glutamatergic synapses and extrasynaptic glutamate receptors, and that CSPGs (cell surface bound and as part of ECM) control (i) lateral diffusion of cell surface receptors, (ii) the extrasynaptic diffusion of negatively charged transmitters such as glutamate and (iii) the expression of glial transmitter transporters. Speculatively, the distinctive cellular and molecular composition of glial/CSPG clusters may underlie their role as specialized islands, perhaps representing glial/CSPG clusters in the amygdala, and affect distinct neuronal and glial cell populations in which CSPGs, including aggrecan and CS-6 sulfated CSPGs, are coexistent with abnormalities in other, distinct, glial populations in the amygdala (see Supplementary Materials). Therefore, increased expression of CSPGs, including aggrecan and CS-6 sulfated CSPGs, are instead decreased. In turn, CSPG decreases in these cells may contribute to PNN reductions. PROTEIN G Elliott 36, 37, 99 The present results help WFA-positive PNNs, because glial cells represent a main source of observed changes are interpreted as increased CSPG expression. All WFA-positive glial cells were found to express GFAP, and in turn, CSPG decreases in these cells may contribute to PNN reductions.

CONCLUSIONS Our results show aggrecan and CS-6 sulfation abnormalities in PNNs and glia within the amygdala of SZ and BD subjects. These findings indicate that CSPG abnormalities are widespread within the amygdala, and affect distinct neuronal and glial cell populations. Notably, differences between the two disorders were observed. In SZ, reductions of aggrecan and 3B3-IR PNNs in LN, together with previously reported decreases of WFA-positive PNNs in the same nucleus, suggest PNN loss. In BD, decreased aggrecan and 3B3-IR PNNs in the absence of similar changes detected by WFA labeling suggests anomalous PNN composition. Decreases of glial cells expressing CS-6 in both SZ and BD indicate that CS-6 sulfation on these cells may differentially contribute to the changes in PNNs observed in these disorders, and may impact glutamate reuptake and neurite outgrowth. In SZ and BD, CSPG abnormalities may critically contribute to a disruption of developmental and adult neuronal functions such as synaptic plasticity, glutamate signaling and firing patterns. Speculatively, a disruption of PNNs in the amygdala may lead to unstable synaptic connectivity and altered neuronal activity that may destabilize salience encoding emotion-driven learning.

CONFLICT OF INTEREST The authors declare no conflict of interest.

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