Described below are two sets of studies, one describing the expression pattern of aggrecan and CS-6 in the normal human amygdala, and the other reporting abnormalities of aggrecan mRNA expression in the amygdala of subjects with SZ.

AGGRECAN AND CS-6 EXPRESSION IN THE NORMAL HUMAN AMYGDALA

As part of investigations described in this manuscript, we investigated the distribution pattern, morphology *and co-expression* of aggrecan- and CS-6-IR elements in the normal human amygdala.

Methods

Single antigen immunolabeling

Results discussed below were obtained from all healthy control subjects included in the two subcohorts used for the comparison studies. Methods for immunolabeling and data collection are included in the main text of the manuscript.

Dual antigen immunolabeling

These studies were carried out on a cohort of three control subjects (7063, 7519, and 7164; see eTable 1. *Antigen retrieval was conducted as described for single antigen immunolabeling. Sections were then co-incubated in primary antibodies (mouse anti-cat-301, 1:250 µl; mouse anti-3B3, 1:50 µl; mouse anti-CS56 1:1000 µl, rabbit anti-GFAP 1:1000 µl) and biotinylated WFA (1:500 µl; Vector Labs, Burlingame, CA) in 2% bovine serum albumin (BSA) for 72 hr at 4 °C. This step was followed by 4 hour incubation at room temperature in Alexa Fluor goat anti-mouse 594 (1:300 µl; A-11005, Invitrogen, Grand Island, NY), donkey anti-rabbit 488 (1:300 µl; A-21206, Invitrogen, Grand Island, NY), and streptavidin 488 (1:3000 µl; S-11223, Invitrogen, Grand Island, NY), followed by 10 minutes in 1 mM CuSO4 solution (pH 5.0) to block endogenous lipofucsin autofluorescence (62). Sections were mounted and coverslipped using Dako mounting media (S3023, Dako, North America, Carpinteria, CA).*

Quantitative data collection was carried out using a Zeiss AxioImager M2 system equipped

*with a Lumencor SOLA LED fluorescence lamp and motorized fluorescent filter turrets for GFP, DsRed, CY5, and DAPI, interfaced with StereoInvestigator 10.0 (Microbrightfield Inc., Willinston, VT). 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 from adjacent sections according to cytoarchitectonic criteria as described by Amaral et al, 1992 and Sims and Williams, 1990 [1,2]. The nomenclature adopted was that used by Sorvari et 1995 [3]. 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 1.6x 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 40x objective to count immunoreactive (IR) PNNs or glia for each marker, and colocalization or lack thereof with each marker, within the traced borders over four matched sections representing the whole extent of the amygdala from each subject. Sections were matched between subjects on the basis of their rostral-caudal location according to previously published anatomical criteria (see [4,5]). All PNNs were counted according to their labeling for: 1) 3B3, WFA or both, 2) cat-301, WFA or both. Cat-301-IR glia were counted according to their labeling for GFAP or WFA. Glial clusters labeled for 3B3 or CS56-IR were counted according to their labeling, or lack-there-of, for WFA and GFAP. For this latter, i.e. dual labeling of 3B3-IR or CS56-IR clusters with GFAP, a Leica TCS-SP8 confocal microscope was used to capture high resolution three-dimensional images of 3B3 and CS56-IR clusters, GFAP-IR glial cells and DAPI –positive nuclei.*

Results

PNNs

Aggrecan immunolabeling was observed primarily in PNNs, with the highest Nd in the LN (14.5/mm^3, followed by the BN (8.1/mm^3), lower Nd in ABN (5.5/mm^3) and CO (5.0/mm^3), and virtually no detectable aggrecan-IR PNNs in ME and CE (Figs. 1, 2; eTable 4). Approximately half (54%) of aggrecan-IR PNNs were also WFA-positive (across LN, BN, ABN and CO). Conversely, 30% of WFA-positive PNNs were also labeled with aggrecan (eFig. 1).

Of the two antibodies raised against CS-6, only 3B3-IR labeled PNNs, which were numerous and intensely stained (Fig. 1). No CS56-IR PNNs were detected. The highest Nd of 3B3-IR

PNNs was observed in the LN (169.1/mm^3) and CO (208.5/mm^3); given the larger size of LN and BN, these two nuclei had by far the highest Tn (30,294 and 18,860, respectively; Fig. 2; see also eTable 5). Thus, 3B3-IR PNNs were overall more numerous with respect to aggrecan-IR and WFA-positive PNNs (eTable 4 and (1), particularly in nuclei such as the ABN, CO, ME and CE, where aggrecan-IR and WFA-positive PNNs are rare or absent (Fig. 2; see also eTable 4).

Results from WFA/3B3 dual immunofluorescence also show a marked discrepancy between the distribution patterns of PNNs labeled with WFA and/or 3B3. Across all amygdala nuclei tested, the large majority of WFA-positive PNNs (78.9%) were also 3B3-IR, with similar percentages in individual nuclei (LN 82.4%, BN 80.2%, ABN 45.9%, CO 81.8%, ME 98.9%, CE 79.2%). Conversely, only 33.2% of 3B3-IR PNNs were also labeled by WFA (LN 49.6%, BN 21.6%, AB 13.3%, CO 10.3% ME 6.7%, CE 27.1%; eFigure 1). Together, these results indicate that the CS-6 pattern labeled by 3B3 is present in the majority of WFA-positive PNNs as well as in a distinct group of WFA-negative PNNs.

Glia

A small number of glial cells scattered across all amygdala nuclei were aggrecan-IR (Fig. 2). These cells present with small cell bodies and only few immunolabeled processes (Fig. 1). Virtually no aggrecan-IR glial cell were found to express GFAP or showed WFA labeling (eFigs. 1, 2).

Under light microscopy, CS-6 (3B3 and CS56) immunolabeling shows as darkly stained 'rosettes', approximately 75-100 μm in diameter (Fig. 1). High resolution confocal microscopy revealed that these rosettes are composed by CS-6 IR glial cell enveloped in round clusters of amorphous CS-6 immunolabeling, typically showing less dense immunolabeling in their center (Figs. 1, 3). We refer to these structures, CS-6-IR glia within CS-6-IR diffuse labeling, as 'glial clusters'. The highest Nd of 3B3-IR glial clusters was found in the LN (82.2/mm^3), followed by the BN (77.4/mm^3) and ABN (74.5/ mm^3), while lower Nd were found in the CO (27.4/mm^3), CE (37.8/mm^3) and ME (26.1/mm^3) (eTable 5). CS56-IR clusters showed overall lower Nd values, i.e. LN (22.3/mm^3), BN (16.1/mm^3), ABN (13.5/ mm^3), CO (17.8/mm^3), CE (15.1/mm^3) and ME (11.7/mm^3) (eTable 6).

Dual-immunofluorescence labeling in a subset of control subjects showed that many 3B3- and CS56-IR cells within the respective clusters are GFAP-IR, and thus may correspond to astrocytes (Fig. 3). A distinct group of GFAP-IR astrocytes, with no detectable CS-6-IR, surrounded CS56- and 3B3-IR clusters. The CS-6-IR glial clusters described here may correspond to structures with similar characteristics previously reported in the rodent cortex using CS56 (2, 3). Consistent with our findings, data from rodents suggest that GFAP-IR cells may express CS-6 preferentially in the most distal portion of their processes, possibly contributing to the structure of the clusters (2, 3). Finally, confocal microscopy showed that CS56- and 3B3-IR clusters are not labeled with WFA (eFig. 1), indicating that they do not correspond to WFA-positive glial cells originally shown to be increased in the amygdala of subjects with SZ (1).

Discussion

PNNs

Our results suggest that aggrecan (cat-301), 3B3 and WFA may detect distinct CSPG components, which may coexist in different combinations in heterogeneous subgroups of PNNs. 3B3 labeled the largest population of PNNs, most densely represented in the LN, BN and CO, but also robustly present in the ABN, CE and ME. In contrast, aggrecan- and WFA-positive PNNs are lower in numbers and show a more restricted distribution (1, 4). In the LN, the nucleus with the highest numbers of PNNs, the majority of WFA-positive PNNs and approximately 50% of aggrecan-IR PNNs were also labeled by 3B3, although these WFA/3B3-positive and aggrecan/3B3-positive PNN populations represented a fraction of all 3B3-IR PNNs in the same nucleus. PNN heterogeneity in the human amygdala is consistent with reports in rodents (5, 6), and suggests differential regulation of neuronal functions based on the properties of distinct CSPGs and sulfation patterns (e.g.7). Interneurons expressing parvalbumin are believed to represent one of the main neuronal populations bearing PNNs {Bruckner, 1994 #10421;Celio, 1993 #9627;Hartig, 1994 #9869;Morris, 2000 #9427;Pantazopoulos, 2006 #10161}, as approximately 40% of them were shown to be enveloped in WFA-positive PNNs in the human amygdala (4). It is possible that another subset of these interneurons is associated with aggrecan- or 3B3-IR PNNs not labeled by WFA. However, the large majority of parvalbumin-IR neurons in the human amygdala is located in the LN and, to a much lesser extent, in the BN. Thus, our results suggest that several other neuronal populations, including GABAergic projection

neurons in the ME and CE, may also be enveloped by PNNs.

Glia

Our results indicate that, in the normal human amygdala, glial cells positive for aggrecan (cat-301), CS-6 (3B3 and CS56) and WFA correspond each to distinct glial populations. In brief, i) aggrecan- and CS6- IR glial cells are not labeled by WFA; ii) aggrecan-IR glial cells are typically isolated from each other and detected in low numbers, while 3B3- and CS56-IR glia form clusters and (particularly 3B3-IR glia) are more numerous than aggrecan-IR glia (Tables 4-6; Fig. 2); iii) aggrecan-IR glial cells did not express GFAP, while virtually all WFA-positive, and the majority of CS-6-IR glial cells, were found to express GFAP (Fig. 3) (1). GFAP is typically expressed by the majority, but not all, astrocytes and a small contingent of oligodendrocyte precursor cells (unpublished results and 8, 9, 10). We suggest that specific patterns of CSPG expression in subpopulations of glial cells may underlie specialized tasks, including maintaining the integrity of PNNs expressing the corresponding CSPGs.

Our results show, to our knowledge for the first time, the presence of high numbers of CS-6-IR glial clusters in the human brain (Figs. 1,2). Similar structures, reported in the rodent brain, have been shown to contribute to the regulation of glutamate reuptake (2, 3, 11). It is not yet clear whether CS-6 CSPGs are located uniquely in the terminal processes of astrocytes, as suggested in rodents (2, 3), and/or secreted into the extracellular space, possibly forming perisynaptic ECM specializations on neurons within the clusters. Notably, perisynaptic ECM specializations have been shown to regulate key neuronal functions such as glutamatergic transmission, synaptic sprouting and pruning (12-14).

AGGRECAN mRNA EXPRESSION ABNORMALITIES IN THE AMYGDALA OF SUBJECTS WITH SCHIZOPHRENIA

With these experiments, we tested the hypothesis that the expression of aggrecan mRNA, which encodes for aggrecan core protein, may be altered in subjects with SZ.

QRT-PCR

Frozen tissue samples from the LN were dissected, placed into microcentrifuge tubes

containing 600 µl of the miRVana RNA lysis/binding buffer then disrupted and homogenized in lysis buffer (pipetting and vortexing). Homogenates were immediately processed for total RNA isolation with silica membrane spin columns (Ambion mirVana microRNA isolation kits; Life Technologies, Carlsbad, CA). RNA purification and on-column DNase digest was performed according to kit instructions. Purified total RNA was eluted in 100µl RNase-free water. The Nanodrop ND-1000 Spectrophotometer was used to calculate RNA sample concentrations and purity (A260/280 ratios). Samples were run on a microfluidic chip for automated electrophoresis using Bio-Rad's Experion with the RNA HighSens Kit to check total RNA quality. 28S:18S ratios and RNA quality indicator (RQI) numbers (scale of 1 to 10, degraded to intact) were generated by Experion Software. For each sample, 500 ng of total RNA was primed with a mixture of oligo(dT)23 primers (3.5 µM), and dNTPs (10mM each) by incubation at 70ºC for 10 min. A cDNA synthesis master mix containing 5X First-Strand Buffer, 0.1M DTT, RNase-OUT RNase Inhibitor (40 U), and SuperScript III Reverse Transcriptase (200 U, Invitrogen, Carlsbad, CA) was added to each sample and incubated at 25ºC for 15 min, then 50ºC for 50 min. The reaction was inactivated by heating to 85ºC for 5 min. Finally RNA was removed by adding RNase H and incubating at 37ºC for 20 minutes.

Transcript variants 1 and 2 of the human aggrecan gene (ACAN) were detected using inventoried Taqman gene expression assay Hs00153936_m1 (GAPDH, RPII, and HPRT1 as reference genes). Real-time reactions were carried out using Taqman PCR Master Mix No Amperase UNG (Life Technologies, Carlsbad, CA) (thermal cycler conditions: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min). Reactions were run in triplicates (20 µl/reaction) on a 96-well plate using the Bio-Rad iQ5 instrument. Threshold settings were set above background at the beginning of the detectable log-linear phase of amplification for accurate Ct readings. Dissociation curves confirmed the specificity of amplification products and the absence of signal due to non-specific amplification.

Statistical analysis

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 $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen (15, 16).

Results

Expression of aggrecan mRNA was significantly increased in the LN of SZ subjects compared to normal controls ($p < 0.03$, 2.66 fold increase, eFig. 3).

Discussion

Consistent with previous results (1), aggrecan mRNA expression was significantly increased in the LN of SZs, perhaps representing a compensatory upregulation in response to reductions of aggrecan expression, and/or in combination with a disruption of aggrecan protein translation and/or posttranslational modifications. We cannot exclude that aggrecan forms not detected by cat-301 (17, 18) may instead be increased. Alternatively, given that gene expression analyses and cell counts were performed in two distinct subjects cohorts, it is possible that subjects with elevated aggrecan mRNA may represent a subpopulation distinct from that showing lower numbers of aggrecan-IR glia and PNNs.

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure 1. Dual immunofluorescence photomicrographs showing aggrecan, 3B3 and CS56 labeling in combination with WFA. Distinct subpopulations of aggrecan- and 3B3-IR PNNs also showed WFA labeling (A-F and G-L, respectively). In contrast, aggrecan-, 3B3- and CS56-IR glial cells were virtually void of WFA labeling (A-F, J-L, and M-O, respectively). In some instances, CS56-IR glial clusters were located in close proximity of WFA-positive PNNs (M-O). Long arrows mark PNNs; short arrows mark glial cells. Scale bars = 50 μm.

Supplementary Figure 2. Aggrecan-IR glia (A) shows astrocytic morphology but does not express GFAP (A-C). Scale bars = 50 μm.

Supplementary Figure 3. Aggrecan mRNA was significantly increased in the LN of SZ subjects compared to control subjects. Values represent gene expression normalized to GAPDH. Scatterplots show the mean (histogram) and 95% confidence intervals (black lines)

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