Cerebrospinal fluid proteomics targeted for central nervous system processes in bipolar disorder

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ABSTRACT

The etiopathology of bipolar disorder is largely unknown. We collected cerebrospinal fluid (CSF) samples from two independent case-control cohorts (total n=351) to identify proteins associated with bipolar disorder. A panel of 92 proteins targeted towards central nervous system processes identified two proteins that replicated across the cohorts: the CSF concentrations of testican-1 were lower, and the CSF concentrations of C-type lectin domain family 1 member B were higher (CLEC1B), in cases than controls. In a restricted subgroup analysis, we compared only bipolar type 1 with controls and identified two additional proteins that replicated in both cohorts: draxin and tumor necrosis factor receptor superfamily member 21 (TNFRSF21), both lower in cases than controls. This analysis additionally revealed several proteins significantly associated with bipolar type 1 in one cohort, falling just short of replicated statistical significance in the other (tenascin-R, disintegrin and metalloproteinase domain-containing protein 23, cell adhesion molecule 3, RGM domain family member B, plexin-B1, and brorin). Next, we conducted genome-wide association analyses of the case-control-associated proteins. In these analyses, we found associations with the voltage-gated calcium channel subunit *CACNG4*, and the lipid droplet-associated gene *PLIN5* with CSF concentrations of TNFRSF21 and CLEC1B, respectively. The reported proteins are involved in neuronal cell-cell and cell-matrix interactions, particularly in the developing brain, and in pathways of importance for lithium's mechanism of action. In summary, we report four novel CSF protein associations with bipolar disorder, replicated in two independent case-control cohorts, shedding new light on the central nervous system processes implicated in bipolar disorder.

BACKGROUND

Bipolar disorder is a severe and often chronic psychiatric disorder featuring recurrent episodes of depression and (hypo-)mania interspersed with, at best, inter-episodic remission. Psychotic symptoms are common during an acute manic episode (1). Bipolar type 1 denotes patients with at least one manic episode, whereas patients with bipolar type 2 have had at least one hypomanic and one depressive episode, but no manic episode. With early onset and a global prevalence of 2.4% (2), the disorder not only accounts for more than 1% of years lived with disability (3) but also incurs substantial societal costs (4).

The bipolar disorder diagnosis rests solely on observed behavior and reported symptoms with no bio-signature test (*e.g.*, genetic, protein, or imaging biomarker) to aid diagnostic decisions or help personalize treatment. Given that family history is the greatest risk factor for bipolar disorder—with heritability estimates ranging from 60 to 90% (5, 6)—much research has focused on identifying genetic variants that confer increased risk. Recent large-scale genome-wide association studies (GWAS) have successfully identified 64 common genetic variants associated with the disorder, including genes that encode ion channels, neurotransmitter transporters, and synaptic elements (7-11). Still, the explained variance is low and the biological mechanisms by which the genetic risk loci confer risk for bipolar disorder remains largely unknown.

Human neuronal tissue is not accessible for *in vivo* studies but investigating cerebrospinal fluid (CSF) provides an option to study brain chemistry. CSF reflects central nervous system (CNS) processes better than peripheral blood (12, 13). A 2018 review of bipolar disorder CSF studies identified 34 studies investigating a total of 117 unique biomarkers (14). Among 40 markers reported to differ significantly between cases and controls, the only replicated

findings were higher CSF concentrations of the monoamine metabolites homovanillic acid (15-17) and 5-hydroxyindoleactic acid (16, 18) in bipolar disorder compared with healthy controls. This review not only illustrates the limitations of the hypothesis-driven approach but also the dearth of studies targeting a broader set of CSF proteins. In fact, only one prior small study has explored the global CSF proteome in bipolar disorder (11 cases and 27 controls), identifying 197 altered proteins where none survived correction for multiple testing (19).

The aim of this study was to identify novel CSF protein associations with bipolar disorder using a 92-plex immunoassay panel targeting neurobiological processes. We analyzed CSF from two independent case-control cohorts with a total sample size of 351 individuals.

METHODS

Study participants and ethics

The St. Göran bipolar project (SBP) is a naturalistic study of patients with bipolar syndromes and healthy controls. Study persons are enrolled at two bipolar outpatient units in Stockholm (SBP-S) and Gothenburg (SBP-G) in Sweden. The cohort collections have been described previously (20-22). Briefly, a Swedish version of the structured interview instrument Affective Disorder Evaluation (ADE) (23) was used to capture clinical information. The diagnosis of bipolar disorder was established according to the DSM-IV criteria using the Structured Clinical Interview for DSM-IV (SCID) as included in the ADE. The Mini International Neuropsychiatric Interview (M.I.N.I.) was used to screen for comorbid psychiatric conditions. A final best estimate diagnostic decision was made by board-certified psychiatrists specialized in bipolar disorder. The inclusion criteria for this report were age ≥18 years, and a DSM-IV bipolar spectrum diagnosis (type 1, type 2, not otherwise specified, cyclothymia, or schizoaffective syndrome bipolar type). The severity of bipolar disorder was rated at the interview using the Clinical Global Impression (CGI) rating scale, and the Montgomery-Åsberg Depression Rating Scale (MADRS) and the Young Ziegler Mania Rating Scale (YMRS) were used to assess depressive and manic symptoms at the time of lumbar puncture.

Statistics Sweden (www.scb.se) randomly selected controls from the general population living within the same catchment area as enrolled patients. Those who responded to an invitation letter were first interviewed over phone by research nurses who screened for the exclusion criteria: any current psychiatric disorder or any current use of psychotropic drugs, bipolar disorder or schizophrenia in first degree relatives, neurological diseases (excluding

mild migraine), substance abuse, untreated endocrine disorders, or pregnancy. Controls were not excluded due to past minor psychiatric morbidity such as mild depressive episodes, mild eating disorders, isolated episodes of panic disorder that had remitted spontaneously or with brief counselling. Eligible persons were scheduled for a personal examination where M.I.N.I. and selected parts of the ADE were used to collect information.

The SBP-study has been approved by the Stockholm Regional Ethics Committee and is conducted in accordance with the Declaration of Helsinki. All study participants provided oral and written consent. Controls but not patients were reimbursed for participating in the study.

CSF sampling

CSF sampling occurred between 9–10 AM after a night of fasting, and during a period (>8 weeks) when patients were in a stable mood as judged by a physician, *i.e.*, not during an acute episode of depression, hypomania, or mania. For ethical reasons, patients remained on their prescribed medication. Lumbar puncture was performed in a sitting position with a fine disposable spinal needle inserted into the L3/L4 or L4/L5 interspace. A total volume of 12 ml CSF was collected, and the tube was inverted to avoid concentration gradients. For a subset of the study population (n=235), blood cells were counted in CSF. The samples were aliquoted and stored at -80°C pending analysis. All samples in SBP-G, but only 6 in SBP-S, were centrifuged prior to freezing.

Laboratory analyses and data preparation

A total of 351 CSF samples were analyzed across 5 plates using the commercially available Proseek Neurology I panel (v.8001) designed for protein biomarker discovery (Olink® Proteomics, Uppsala, Sweden). Olink panels are based on the proximity extension assay

technique using paired oligonucleotide-labeled antibodies that bind the target protein and activate a quantitative polymerase chain reaction readout (24). The Neurology I panel contains 92 proteins related to neurobiological processes (*e.g.*, neural development, axon guidance, and synaptic function), and more general processes such as cellular regulation, immunology, development, and metabolism.

Samples from cases and controls were randomly distributed across plates, with the two study cohorts on separate plates. Laboratory technicians were blinded to clinical information. Initial preprocessing and quality control were performed by Olink® using NPX manager, exporting normalized protein expression (NPX) values on a log2-scale (24). Three samples were flagged in quality control (https://www.olink.com/resources-support/white-papers-from-olink/) but were included as no data anomaly was identified. Brain-derived neurotrophic factor (BDNF) was excluded by Olink® due to technical issues. The NPX-values were median centered per protein and plate to adjust for batch effects. No protein was significantly correlated with CSF sampling date in both cases and controls in any of the cohorts. Proteins with >20% of values below the limit of detection in both cases and controls were excluded from further analyses (9 in SBP-S and 11 in SBP-G) leaving 82 proteins for study in the SBP-S cohort and 80 in the SBP-G cohort. Values below the limit of detection were kept for these remaining assays. Supplementary Table 1 lists all studied proteins. In total, five samples were excluded from the final analysis due to clinical diagnosis other than bipolar spectrum (n=3), or samples from SBP-S that were incorrectly aligned on SBP-G plates (n=2).

To assess blood-CSF barrier function, we calculated the CSF/serum albumin ratio by dividing albumin concentration in CSF with albumin concentration in serum (25). Albumin concentrations were analyzed by immunonephelometry on a Beckman Immage

Immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA) at the Clinical Neurochemistry Laboratory in Mölndal, Sweden. The method was accredited by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC).

Genotyping and imputation

A subset of the study participants (n=262) was genotyped as previously described (26). Genotyping was conducted in three waves using different genotyping arrays: Affymetrix 6.0 (Affymetrix, Santa Clara, CA, USA), Illumina OmniExpress chips (Illumina, San Diego, CA, USA), and Infinium PsychArray-24 v1.2 BeadChip (Illumina, San Diego, CA, USA). The Ricopilli pipeline was used for quality control (27), and genotypes were imputed to the HRC 1.1 reference panel on the Sanger imputation server (28).

Statistics

Case-control analyses were conducted separately for each cohort. In all other analyses the two cohorts were harmonized and combined. To explore covariance across the protein panel, we used protein-protein rank correlations (aka. Spearman's correlation) and principal component analysis (PCA) of all included proteins. A PCA can reduce data dimensionality by creating orthogonal principal components that explain a significant portion of variance in a dataset if variables are intercorrelated. We tested biological correlates to the primary three principal components by rank correlations. Case-control comparisons were carried out using both unadjusted and covariate-adjusted logistic regression models, where age, sex, and albumin ratio were included as covariates. To rectify false positives, we required a replication of significant findings across the cohorts rather than correcting for multiple testing: proteins that differed significantly ($P<0.05$) and in the same direction in both cohorts were considered replicated. Results are presented as standardized odds ratios, where the odds represent one

standard deviation increase in CSF protein concentration. We also present results from the combined cohorts, where *P*-values were adjusted for false discovery rate (FDR) (29). Replicated case-control associated proteins were subsequently tested for association with disease severity indices by linear regression, adding bipolar subtype as an additional covariate (cases only). To estimate the influence of psychiatric drugs, we included four drug categories (see definition in Supplementary material) in a linear regression as above (cases only), reporting standardized β-coefficients.

Genome-wide association analyses were conducted in plink v.2.0 (30, 31) using additive regression models where NPX values were rank-based and inversely normal-transformed. To account for population stratification, we included the primary six genetic multidimensional scaling components as covariates, as well as age, sex, CSF/serum albumin ratio, and cohort. The association analyses were conducted in each wave separately and meta-analyzed using the inverse-variance-weighted approach in METAL (release: 2011-03-25) (32), where results from genetic variants passing filtering thresholds (INFO score≥0.6, minor allele frequency (MAF)≥5%, Hardy-Weinberg equilibrium *P*>1e-10, detected in ≥2 waves) are reported. In a hypothesis-driven approach, we also specifically looked at the results for the 64 risk loci for bipolar disorder recently published (11). Gene-based tests were conducted in MAGMA v.1.0.9 (33) using the 'snp-wise=mean' model, where genes were annotated in a 10kb upstream and 2.5kb downstream window (GRCh37/hg19). Phase 3 of 1000 genomes (European sample) were used for computations of linkage disequilibrium. *P*-values were adjusted for the number of tested genes (*P*<0.05/18020).

Protein data were analyzed in R (v.3.6.3) using the external packages tidyverse (v.1.3.0), arsenal (v.3.5.0), Hmisc (v.4.4-1), ropls (v.1.16.0), and qqman (v.0.1.8). Code is available at github.com/andreasgoteson.

RESULTS

Demographics and clinical characteristics

We analyzed CSF from 221 individuals in SBP-S (133 cases and 88 controls) and 125 individuals in SBP-G (70 cases and 55 controls). Demographic data are presented in Table 1. There were no case-control differences in age or sex in either cohort, but BMI and CSF/serum albumin ratio were higher in cases than controls in the SBP-S cohort but not in the SBP-G cohort. Nicotine use was more frequent in bipolar disorder in both cohorts.

Patients in both cohorts had a similar duration of illness (median 16–18 years) and lifetime symptom severity (median CGI=4), and were in a stable mood at CSF sampling as indicated by low scores in MADRS and YMRS. Bipolar type 1 was the most common subtype in SBP-S, whereas type 2 was the more common subtype in SBP-G. In the SBP-G cohort, patients had more recorded depressive, (hypo-)manic, and mixed episodes, while a lifetime history of psychosis was more common among SBP-S patients. Lithium was the most used drug in both cohorts.

Insert Table 1 here

Protein panel overview

We first conducted PCA analyses to capture covariance across the studied panel (Supplementary figure 1). The first principal component (PC1) explained ≥60% of the total variance (R2X=0.61), which was also reflected in overall high protein-protein correlations (rho median (IQR) = 0.60 (0.41-0.74)). Whereas most proteins had a similar loading in PC1, PC2 (R2X=5%) loaded strongly on neuronal cell adhesion proteins (*e.g.*, Nr-CAM, Thy-1, neurocan), and PC3 (R2X=5%) was highly influenced by mainly blood-derived proteins (*e.g.*,

C-type lectin domain family 1 member B (CLEC1B), macrophage scavenger receptor type I/II, Ezrin).

Next, we tested biological correlates to the principal components. PC1 was correlated with CSF/serum albumin ratio, age, and sex. Including CSF/serum albumin ratio, age, and sex in a multiple linear regression explained ~30% of the variance in PC1 (multiple R^2=0.28). PC2 was not correlated with any of the tested variables, whereas PC3 was correlated with CSF/serum albumin ratio, age, sex, BMI, nicotine use, and use of antipsychotics.

Case-control analyses

We tested case-control differences adjusted for age, sex, and CSF/serum albumin ratio (Supplementary table 2). These analyses revealed 9 proteins that differed (P<0.05) between cases and controls in the in SBP-S cohort, and 4 in the SBP-G cohort. Two proteins replicated across cohorts: the CSF concentrations of testican-1 were lower, and CLEC1B were higher, in cases than controls. Five additional proteins (all lower in cases than controls) were significantly associated with bipolar disorder in one cohort, while falling just short of replicated statistical significance in the other: draxin, tenascin-R, disintegrin and metalloproteinase domain-containing protein 23 (ADAM 23), tumor necrosis factor receptor superfamily member 21 (TNFRSF21), and cell adhesion molecule 3 (CADM3). Kynureninase was highly significant in SBP-S, higher in cases than controls. Significant findings are listed in Table 3 and Figure 2. Results from unadjusted case-control comparisons, as well as analyses of the combined cohorts, are available in Supplementary Tables 3-4.

Insert Table 2 here

Secondary subgroup analyses

We performed three subgroup comparisons (complete results are available in the Supplementary Tables 5-7). First, we compared bipolar type 1 with controls and found a total of 14 CSF proteins (9 in SBP-S, 9 in SBP-G) that differed significantly between groups. Most proteins that showed a tendency towards a replicated case-control association in our primary analysis were strengthened in this restricted comparison. Testican-1 and CLEC1B again replicated (P<0.05) across cohorts. Additionally, we found significantly lower CSF concentrations of draxin and TNFRSF21 in bipolar type 1 compared with controls in both cohorts. As in the primary case-control comparison, several proteins were significantly lower in bipolar type 1 in one cohort while falling just short of replicated significance in the other (tenascin-R, ADAM 23, RGM domain family member B (RGMB), CADM3, Plexin-B1, and Brorin). Second, we compared bipolar type 1 with type 2 and found 13 CSF proteins (13 in SBP-S, 1 in SBP-G) that differed between the subtypes. The CSF concentrations of CMRF35-like molecule 1 (CLM-1) were higher in type 1 than type 2 in both cohorts. Third, we compared bipolar disorder with and without a history of psychosis. This comparison revealed one statistically significant finding in SBP-S that did not replicate in SBP-G.

Insert Figure 1 here

Blood contamination sensitivity analysis

Five samples in SBP-S were suspected of blood contamination at lumbar puncture, as they had either high red blood cell count (>500 per microliter, n=4) or otherwise similar protein profile (n=1, cell count data missing). In a sensitivity analysis, we excluded these 5 samples together with the 6 samples that were centrifuged prior to storage and repeated preprocessing and analyses in SBP-S (n=210). All replicated associations remained when blood

contaminated and centrifuged samples in SBP-S were excluded except the association of TNFRSF21 with bipolar type 1, which was similar in effect size but now fell short of statistical significance in the SBP-S cohort (OR (95% CI)=0.71 (0.50-1.0), *P*=0.06).

Associations with disease severity and psychotropic drugs

None of the four case-control associated proteins (testican-1, CLEC1B, draxin, and TNFRSF21) were associated with disease severity indices (lifetime CGI, illness duration, or total lifetime mood episodes). As controls were not exposed to psychoactive drugs, we were not able to adjust our case-control associations for this potential confounder. Instead, we explored the effect of psychiatric drugs in cases only and found that the CSF concentration of three proteins were significantly positively associated with lithium use after correction for multiple testing (Supplementary Table 8): N-acylethanolamine-hydrolyzing acid amidase (β (95% CI)=0.61 (0.32-0.89), *P*=3.5e-5, FDR=0.006), dipeptidyl peptidase 1 (β (95% CI)=0.58 (0.30-0.85), *P*=6.4e-5, FDR=0.007), and neutral ceramidase (β (95% CI)=0.60 (0.33-0.86), *P*=1.6e-5, FDR=0.005). With respect to the proteins that differed between cases and controls, testican-1 (β (SE)=-0.42 (0.15), *P*=0.005, FDR=0.17) was nominally associated with antipsychotic use. However, the concentration of testican-1 remained significantly lower in bipolar disorder cases than controls when we excluded cases using antipsychotic drugs (OR (95% CI)=0.36 (0.17-0.78), *P*=0.01, both cohorts combined).

Genetic association analyses

Finally, we conducted genome-wide analyses to identify regulatory genes and protein quantitative trait loci (pQTL) for our case-control associated proteins (complete results in Supplementary material). No genome-wide significant (*P*<5e-8) loci were identified for any of the tested proteins. However, in genome-wide gene-based analyses, averaging the effects

of independent risk loci within a gene, *CACNG4* on chromosome 17 was significantly associated with the CSF concentration of TNFRSF21 (chr 17:64950980-65032018, 143 SNPs, z=5.2, *P*=1.1e-7), and *PLIN5* was associated with the CSF concentrations of CLEC1B (chr 19:4520043-4545208, 46 SNPs, z=4.7, *P*=1.4e-6). With respect to genetic risk variants that previously have been associated with bipolar disorder (11), one locus proximal to *ITIH1* (rs2336147-T) was significantly (*P*<0.05/64) associated with CSF concentrations of both TNFRSF21 (β=-0.31, SE=0.09, *P*=3.0e-4) and testican-1 (β=-0.28, SE=0.08, *P*=5.2e-4).

Insert Figure 2 here

DISCUSSION

In the largest CSF study of bipolar disorder to date, we analyzed 92 proteins targeted for CNS processes in two independent case-control cohorts comprising a total of 346 included individuals. Our primary analysis comparing cases with controls identified two proteins not previously associated with bipolar disorder that replicated in both cohorts: testican-1 and CLEC1B. When restricting the patient sample to bipolar type 1, these two findings were strengthened, and two additional proteins replicated in both cohorts: draxin and TNFRSF21 (both lower in type 1). None of the case-control associated proteins were associated with drug use after correction for multiple testing. Further, we conducted gene-based analyses where the CSF concentration of TNFRSF21 and CLEC1B were significantly associated with *CACNG4* and *PLIN5*, respectively. These novel findings provide insights to CNS processes implicated in bipolar disorder and may serve as groundwork for identification of biomarkers and/or development of disease-modifying pharmaceutics.

The CSF concentration of testican-1 was lower in bipolar disorder than controls. Testican-1 is a CNS-enriched highly conserved (34) calcium-binding extracellular matrix protein believed to be involved in neuronal cell-cell and cell-matrix interactions, and thereby involved in various neuronal differentiation, growth, and migration processes (35) in, *e.g.*, the Wnt/beta-catenin signalling pathway (36, 37) where lithium acts (38). The CSF concentrations of testican-1 and TNFRSF21 were further associated ($P<0.05/64$) with one of the 64 risk loci identified for bipolar disorder (rs2336147-T, chromosome 3 proximal to *ITIH1*) (11). The biological relevance of these association are however not evident. Testican-1 has previously been implicated in neurodegenerative disorders (39, 40) and brain injury (41), but not in bipolar disorder.

Conversely, the CSF concentration of CLEC1B (aka. CLEC-2) was higher in cases than controls. CLEC1B is a platelet receptor involved in lymphocyte interactions (42), but is not known to be expressed in the human brain (43). In PCA analyses, CLEC1B showed strong loadings for PC3 which was highly correlated with both CSF/serum albumin ratio and BMI. Indeed, the peripheral origin together with the PCA profile suggests that the CSF concentration of CLEC1B was dependent on blood-CSF-barrier integrity, which we previously have reported to be impaired in bipolar disorder in the SBP-S cohort (44) (not replicated here in SBP-G). Further, 4 out of the 5 highest values of CLEC1B in SBP-S came from samples with suspected blood contamination. Still, our analyses were adjusted for relevant covariates and the association persisted in sensitivity analyses; hence, what role CLEC1B could play in bipolar disorder neuropathology remains unclear.

It is noteworthy that most case-control associated proteins seemed to be driven by bipolar type 1. Indeed, comparing only bipolar type 1 with controls strengthened the two primary case-control associations (testican-1 and CLEC1B), identified two additional replicated associations (draxin and TNFRSF21), and strengthened statistics for several other CNS proteins where the *P*-value fell just short of replicated statistical significance (ADAM 23, CADM3, tenascin-R, plexin-B1, RGMB and brorin). Draxin is involved in axon guidance during development of forebrain commissures (45, 46), and in projections that have been linked to bipolar disorder in mice single-cell transcriptomics (47). This axon guiding process depends on glycogen synthase kinase-3 (48) which is inhibited by lithium (38). Draxin deficiency is also known to promote apoptosis of neuroblasts in neurogenic areas of hippocampus (49).

TNFRSF21 (aka. death receptor 6) is a CNS-enriched transmembrane receptor highly expressed in immature oligodendrocytes where it regulates maturation (43, 50). It is critical in the formation of neuronal connections in the developing brain, as it regulates neuronal apoptosis and axonal pruning. Moreover, the CSF concentration of TNFRFS21 was associated with genetic variants in *CACNG4*, a subunit that regulates the activity of *CACNA1C*-containing L-type voltage-dependent calcium channels (51). Although the mechanism of interaction remains obscure, the association is notable as *CACNA1C* polymorphisms are among the most frequently reported genetic associations with bipolar disorder (52, 53). The expression of *CACNG4* is enriched in the brain (43). TNFRSF21 has clinically been linked to astrocytoma (54) and multiple sclerosis (55), but has not previously been associated with any psychiatric disorder.

The high covariance together with the weak statistics for cis-regulatory pQTLs (Supplementary Figure 5) is notable. In the absence of distinct disease associations, the CSF protein panel seems instead influenced by common regulatory mechanisms as indicated by the extent of variance explained by PC1 and the substantial protein-protein correlations. Age and blood-CSF-barrier integrity – both previously known to impact the CSF proteome composition (12) – were together with sex associated with PC1 but explained only part of the covariance (~30%). Interestingly, PC2 captured a cluster of CNS-unique proteins that were not influenced by any of the tested covariates.

The strengths of this study include the broad protein discovery panel run in CSF from two independent case-control cohorts, but there are also limitations to consider. First, although the total sample size of 351 individuals is remarkable in CSF-based proteomics, the study could lack power to explore small effect sizes. Low statistical power is also a major limitation for

our genome-wide association analyses. Second, in the absence of a drug-naïve subgroup we were not able to fully disentangle case-control associated effects from drug-related effects. Thus, both case-control associations and drug-protein associations must be interpreted cautiously. Third, the sampling of CSF from bipolar disorder individuals preceded controls in SBP-S. Long-term storage may impact protein concentrations (56) but no protein showed consistent correlation with CSF sampling date. Fourth, SBP-G samples were pre-analytically centrifuged before aliquoting and storage, whereas most SBP-S samples were not (no case-control difference). Normally CSF contains very few cells, still replications between centrifuged and non-centrifuged samples could be problematic for specific proteins. One notable mention is kynureninase, an intracellularly located enzyme involved in tryptophan metabolism, which was highly significant in SBP-S but did not replicate in SBP-G. Finally, our findings should be seen as associated with, but not specific to, bipolar disorder as genomic, transcriptomic and proteomic studies have repeatedly reported a shared neuropathological architecture across psychiatric traits (57-59).

In conclusion, we present four novel CSF protein associations with bipolar disorder (testican-1, CLEC1B, draxin, and TNFSF21) that replicated in two independent cohorts. The reported proteins are involved in pathways of importance for lithium's mechanism of action and have roles in neuronal cell-cell and cell-matrix interactions, possibly of specific relevance for the developing brain.

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CONFLICT OF INTEREST

HZ has served at scientific advisory boards for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. ML declares that he has received lecture honoraria from Lundbeck pharmaceutical. JJ declares that he was an employee at AstraZeneca pharmaceutical 2017-2019, and JHL declares that she is a current employee at AstraZeneca pharmaceutical. Other authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Forrest plot showing odds ratio (OR) and 95% confidence interval (CI) from case-control association tests. Point estimates from the secondary analysis comparing bipolar type 1 with controls are added as unfilled circles. Showing proteins with a significant association in at least one cohort.

Abbreviations: BD1/BD2/BDspect = Bipolar disorder type 1, type 2, and bipolar spectrum other than type 1 or type 2. BMI = Body mass index; CSF = Cerebrospinal fluid; PCA = Principal component analysis. ADAM 22/23 = Disintegrin and metalloproteinase domain-containing protein 22/23, CADM3 = Cell adhesion molecule 3, CLEC1B = C-type lectin domain family 1 member B, KYNU = Kynureninase, NAAA = N-acylethanolamine-hydrolyzing acid amidase, NCAN = Neurocan core protein, NMNAT1 = Nicotinamide/nicotinic acid mononucleotide adenylyltransferase 1, Nr-CAM = Neuronal cell adhesion molecule, RGMB = RGM domain family member B, SIGLEC1 = Sialoadhesin, TNFRSF21 = Tumor necrosis factor receptor superfamily member 21.

Figure 2. Regional association plot from the genome-wide association analyses for a) the CSF concentration of tumor necrosis factor receptor superfamily member 21 (TNFRSF21) in association with *CACNG4*, and b) the CSF concentration of C-type lectin domain family 1 member (CLEC1B) in association with *PLIN5*. c) Boxplots showing the effect of the most significant risk allele within *CACNG4* and *PLIN5*, respectively. Showing CSF protein concentrations in heterozygous (1) and homozygous (2) carriers, compared with individuals without the risk allele (0).

TABLES

Table 1. Demographics and clinical characteristics.

Demographics	SBP-S			SBP-G		
	Cases	*Controls*	*P*	*Cases*	*Controls*	*P*
N	133	88	-	70	55	-
Sex, n (%) males[1]	52 (39)	41 (47)	0.27	25 (36)	25 (45)	0.27
Age, median (IQR) [2]	36.0 (29.0, 51.0)	35.5 (27.8, 44.8)	0.45	38.5 (29.2, 49.0)	45.0 (32.0, 52.0)	0.077
BMI, median (IQR)[2]	24.8 (22.2, 27.8)	23.4 (21.7, 25.5)	0.008	25.0 (23.0, 28.4)	25.0 (22.2, 26.7)	0.18
Nicotine use, n (%)[1]	56 (46)	21 (24)	<0.001	30 (45)	14 (25)	0.027
CSF/serum albumin ratio, median (IQR)[2]	5.2 (4.2, 6.6)	4.8 (3.7, 6.0)	0.018	4.6 (3.7, 6.5)	4.6 (3.4, 5.5)	0.47

Cases clinical characteristics	SBP-S	SBP-G
Bipolar disorder subtype, n (%)		
Type 1	65 (49%)	27 (39%)
Type 2	50 (38%)	37 (53%)
Bipolar spectrum (other than type 1 or type 2)	18 (14%)	6 (9%)
Total lifetime mood episodes, median (IQR)	11 (6, 23)	25 (12, 38)
Lifetime (hypo)manic episodes, median (IQR)	4 (2, 9)	10 (4, 20)
Lifetime mixed episodes, median (IQR)	0 (0, 0)	0 (0, 10)
Lifetime depressive episodes, median (IQR)	4 (3, 10)	10 (5, 15)

Ever psychotic, n (%)	67 (52%)	17 (25%)
Duration of illness, median years (IQR)	16 (9, 27)	18 (11, 30)
CGI lifetime, median (IQR)	4 (4, 5)	4 (4, 5)
MADRS at sampling, median (IQR)	4 (0, 11)	3 (1, 9)
YMRS at sampling, median (IQR)	0 (0, 2)	1 (0, 2)
Medication		
Lithium, n (%)	78 (59%)	35 (50%)
Antipsychotics, n (%)	33 (25%)	23 (33%)
Anticonvulsants, n (%)	46 (35%)	35 (50%)
Antidepressants, n (%)	62 (47%)	35 (50%)

[1]Pearson's Chi-squared test; [2]Kruskal-Wallis rank sum test. 15 individuals were missing data for nicotine use and one individual was missing data for BMI. Abbreviation: BM I=Body Mass Index, CGI=Clinical global impression scale, CSF=Cerebrospinal fluid, IQR=Interquartile range, MADRS=Montgomery-Åsberg depression rating scale, YMRS=Young mania rating scale

Table 2. Results from case-control analyses, showing proteins with significantly (*P*<0.05) altered CSF concentrations in at least one cohort.

	Bipolar disorder vs. control						Bipolar type 1 vs. control					
	SBP-S			**SBP-G**			**SBP-S**			**SBP-G**		
	OR	(95% CI)	*P*	OR	(95% CI)	*P*	OR	(95% CI)	*P*	OR	(95% CI)	*P*
ADAM 22	1.17	0.88-1.56	0.29	0.77	0.51-1.16	0.22	1.07	0.76-1.51	0.70	0.57	0.31-0.98	0.049
ADAM 23	0.72	0.52-0.98	0.041	0.78	0.50-1.18	0.24	0.63	0.42-0.91	0.016	0.64	0.36-1.10	0.11
Brorin	0.90	0.66-1.21	0.48	0.71	0.45-1.07	0.11	0.78	0.54-1.11	0.17	0.53	0.29-0.91	0.027
CADM3	0.81	0.60-1.09	0.17	0.56	0.31-0.90	0.032	0.74	0.51-1.04	0.086	0.52	0.29-0.87	0.017
CLEC1B	3.33	1.72-6.85	6.6e-04	1.69	1.08-2.71	0.024	7.40	2.90-21.21	7.4e-05	2.52	1.33-5.31	8.1e-03
Draxin	0.69	0.51-0.94	0.019	0.76	0.50-1.12	0.17	0.57	0.39-0.81	2.5e-03	0.59	0.35-0.97	0.044
Kynureninase	1.98	1.39-2.89	2.5e-04	0.81	0.53-1.20	0.30	1.76	1.18-2.71	7.8e-03	0.75	0.42-1.27	0.30
NAAA	1.41	1.02-1.98	0.041	0.92	0.61-1.39	0.69	1.57	1.06-2.42	0.031	1.07	0.62-1.83	0.81
Plexin-B1	0.86	0.64-1.16	0.33	0.76	0.50-1.14	0.19	0.75	0.53-1.07	0.12	0.58	0.32-0.97	0.047
RGMB	0.81	0.60-1.09	0.17	0.72	0.46-1.09	0.13	0.72	0.50-1.02	0.070	0.54	0.30-0.93	0.032
Sialoadhesin	1.41	1.04-1.94	0.028	0.81	0.52-1.23	0.32	1.31	0.91-1.89	0.15	0.58	0.32-1.01	0.064
Testican-1	0.62	0.45-0.85	4.1e-03	0.60	0.38-0.92	0.023	0.47	0.30-0.70	3.4e-04	0.46	0.25-0.82	0.011
Tenascin-R	0.70	0.51-0.94	0.022	0.77	0.51-1.15	0.20	0.56	0.38-0.82	3.6e-03	0.64	0.35-1.09	0.13
TNFRSF21	0.82	0.61-1.09	0.17	0.64	0.40-0.98	0.047	0.71	0.50-0.99	0.048	0.49	0.27-0.85	0.016

OR=Odds ratio, 95% CI=95% confidence interval. ADAM 22 (23)= Disintegrin and metalloproteinase domain-containing protein 22 (23), CADM3=Cell adhesion molecule 3, CLEC1B=C-type lectin domain family 1 member B, NAAA=N-acylethanolamine-hydrolyzing acid amidase, RGMB=RGM domain family member B, TNFRSF21= Tumor necrosis factor receptor superfamily member 21