RECENT ADVANCES IN PSYCHIATRY FROM PSYCHO-NEURO-IMMUNOLOGY RESEARCH: AUTOIMMUNENCEPHALITIS, AUTOIMMUNE-ENCEPHALOPATHY, MILD ENCEPHALITIS

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RECENT ADVANCES IN PSYCHIATRY FROM PSYCHO-NEURO-IMMUNOLOGY RESEARCH: AUTOIMMUNENCEPHALITIS, AUTOIMMUNE-ENCEPHALOPATHY, MILD ENCEPHALITIS

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Growing evidence derived from cerebrospinal fluid (CSF), neuropathological, imaging, genetic, and epidemiological studies link neuroinflammation and immune dysregulation to a subset of individuals with a variety of severe mental disorders (SMDs), including affective and non-affective psychotic disorders. Further, the recent discoveries of neuronal surface antibodies (NSAs) in autoimmune encephalitis (AE) presenting with diverse neuropsychiatric disorders such as psychosis and cognitive decline, among many others, provides further support to the notion that CNS autoimmunity and neuroinflammation can contribute to the neurobiology of psychiatric disturbances. Further, these immune mechanisms may contribute to a subset of patients currently diagnosed as having treatment-resistant SMDs such as schizophrenia and major depressive disorder. Additionally, mounting data indicate that various infections can serve as an immunological trigger of aberrant immune responses, presumably by causing release of excess neural antigen, thereby giving rise to NSAs or aberrant immune cellular responses to give rise to primary or secondary psychiatric disorders such as schizophrenia and those associated with AE, respectively. Collectively, these findings support the "mild encephalitis" hypothesis of SMD. The significant overlap among AE-associated psychosis, systemic autoimmune disorder-associated psychosis, and psychotic disorders associated with pathological processes involving inflammation and immune dysregulation has also prompted some authors to adopt the term "autoimmune psychosis" (AP). This term reflects that this psychosis subtype is mechanistically linked to complex neuroimmune and inflammatory signalling abnormalities that can be responsive to early immunomodulatory treatment. It also suggests that a subset of AP might represent an incomplete or "forme fruste" subtype of AE presenting with dominant or pure psychiatric symptoms mimicking primary psychiatric illnesses. Because data indicate that delayed diagnosis and treatment may lead to permanent sequelae, early recognition of AP utilizing neurodiagnostic workup (e.g., CSF analysis, neuroimaging, and EEG) and its early treatment with appropriate immunotherapy are paramount to a meaningful recovery.

This eBook will provide an overview of the current knowledge and research areas from epidemiology, risk factors and diagnosis to the management of these conditions,

in this rapidly emerging field, helping to bridge the gaps in knowledge that currently exist in the disciplines of Psychiatry, Neurology, and Neuroimmunology.

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EDITORIAL
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Editorial: Recent Advances in Psychiatry From Psycho-Neuro-Immunology Research: Autoimmune Encephalitis, Autoimmune Encephalopathy, and Mild Encephalitis

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Editorial on the Research Topic

Recent Advances in Psychiatry from Psycho-Neuro-Immunology Research: Auto immunencephalitis, Autoimmune-Encephalopathy, Mild Encephalitis

The precise etiopathophysiology of most severe mental disorders, particularly schizophrenia spectrum and affective spectrum disorders, remains unclear but is thought to be a product of the intricate interplay among a number of risk factors. These include interactions between genetic, developmental and environmental factors, such as infections. Indeed, the evidence from many epidemiological studies carried out in different countries, especially those from Denmark, strongly associates both early and the frequency and severity of life-time infections with an increased risk of psychosis in later life, possibly through diverse immunological mechanisms. More recently, the continued discovery of antibodies against various neuronal cell surface proteins, such as anti-N-methyl-d-aspartate receptor (NMDAR) and gamma-aminobutyric acid beta receptor (GABAßR), directly links central nervous system (CNS) autoimmunity with dysregulation of glutamatergic and GABAergic neurotransmitter pathways to the neurobiology of acute psychosis in individuals with autoimmune encephalitis (AE). These findings, along with the rapidly emerging evidence of many other immunological abnormalities in most severe mental disorders, have made it clear that the nexus of psychiatry, neurology, and neuroimmunology represent a fruitful coalescence in understanding the pathogenesis of psychiatric disease. The interactive presentations on the link between immune dysregulation and various mental disorders during the 13 and 14th Psychoimmunology expert meetings in 2016 and 2018 at Ulm University in Germany (www.psychoimmunology-experts.de) generated widespread interest and discussion among the participants, which led to identifying and planning this research topic. It is comprised of 23 peer-reviewed articles, including original research, reviews, opinion pieces, and case reports.

Here, we provide a brief summary of the main findings of the papers included in this research topic that link inflammatory and immunological mechanisms to the neurobiology of psychiatric symptomatology.

Herken and Pruess reviewed the neurological and psychiatric presentations of 100 patients recruited from the Charité Centre for autoimmune encephalitis in Berlin. In this cohort, about

60% of the individuals presented mainly with psychiatric symptoms that remained dominant throughout the clinical course. About one-third of those patients were initially hospitalized for psychiatric evaluation and treatment. All patients with anti-NMDR encephalitis exhibited behavioral disturbances, hallucinations, delusion, short-term memory deficits, or catatonia. Individuals with other neuronal antibodies were also frequently admitted with psychosomatic diagnosis. The authors identified so-called "red and yellow flags" to facilitate early recognition of patients with autoimmune encephalitis presenting with neuropsychiatric disturbances, highlighting the usefulness of incorporating cerebrospinal fluid (CSF) analysis in the standard diagnostic workup.

Al-Diwani et al. Oxford UK, proposed using a syndrome-level taxonomy for isolated psychiatric syndromes associated with neuronal antibodies that can overlap with autoimmune encephalitis presenting with neuropsychiatric presentations; synaptic and neuronal autoantibody-associated psychiatric syndromes or "SNAps." This pragmatic approach can serve as a reminder to consider early autoantibody screening for diagnosing SNAps, potentially permitting early diagnosis and management of these rare forms of autoimmune encephalitis that can mimic intractable severe mental disorders. Additional research is needed to investigate the therapeutic implications of SNAps and validate their presence as distinct clinical entities.

Ellul et al. provided a thorough review of the clinical evidence supporting the existence of autoimmune psychosis as a distinct clinical entity among individuals with new-onset psychosis, highlighting the diagnostic challenges and therapeutic implications associated with this entity. The authors reviewed the clinical and biological features of autoimmune psychosis, including peripheral biomarkers of autoimmune dysfunction from dysbiosis to autoantibodies such as NMDAR antibodies, discussing the interplay between environmental and genetic factors.

Najjar et al. summarized the clinical and experimental findings suggestive of the potential contribution of neurovascular unit dysfunction and blood brain barrier hyperpermeability to the neurobiology of schizophrenia. These include neuroinflammation- and oxidative stress-related neurovascular changes including endothelial dysfunction, leading to increased cross interactions between brain innate and peripheral adaptic immunities, thereby perpetuating harmful inflammatory and immune responses in the CNS. The authors concluded that these findings provide additional support for the mild encephalitis (ME) hypothesis of schizophrenia.

Borroto-Escuela et al. reviewed the molecular data supporting the volume transmission hypothesis with specific relevance to NMDAR and its pathological allosteric receptor-receptor interactions that can lead to increased internalization and decreased NMDAR signaling. Combined with the triplet puzzle theory it is suggested that mild neuroinflammation is associated with formation of D2R heteromers, which in turn can enhance D2R promoter signaling, leading to schizophrenia-like symptoms.

de Haan et al. reviewed the chronic self-sustaining immunological and inflammatory changes associated with neurodegenerative and psychiatric disorders. The findings link the cascading effects of neuroinflammation and autoimmunity to disturbances of cholinergic, dopaminergic, glutamatergic, histaminic, and serotonergic functions, relevant to the pathophysiology of neuropsychiatric disturbances associated with neurodegenerative and psychiatric disorders. Thus, these related neuropsychiatric disorders might benefit from novel immunotherapies.

De Picker et al. presented a meta-review of recent quantitative systematic reviews and meta-analyses from 2010 to 2017 investigating the functional relevance of microglial activation-related immune signaling and brain plasticity to the pathophysiology of acute psychosis and schizophrenia. This review included data derived from translocator protein (TSPO) positron emission tomography, CSF analysis, and post-mortem studies, coupled with the results of clinical trials pertaining to the efficacy of various immuno-modulatory agents. They suggested that microglial activation and its downstream effects on the immune processes and neuroplasticity can influence the clinical presentation and the course of schizophrenia. However, the functional relevance of the cross talks between systemic and brain inflammation is less clear.

Riedmüller and Müller and Müller and Riedmüller discussed the potential ethical implications of the ME hypothesis of schizophrenia that includes shifting our perspective on schizophrenia from being an incurable psychiatric syndrome to a chronic but a treatable neurological disease. This will lead to a newer theoretical conceptualization of schizophrenia that necessitates interdisciplinary care teams to diagnose and manage new-onset psychosis and schizophrenia. Moreover, this reform will have potential repercussions for the pharmaceutical industry and legal implications surrounding current compulsory treatment orders. It might also limit social isolation and decreases the burden and stigma associated with severe mental disorders.

Kočovská et al. summarized the role of vitamin D deficiency as a potential environmental risk factor for three etiologically distinct disorders; multiple sclerosis, schizophrenia, and autism. The data suggest that vitamin D deficiency has a much more robust role in MS, compared with that in schizophrenia and autism. Further, Endres et al. investigated the prevalence of vitamin D deficiency in a 1-year cohort of adult inpatients with schizophreniform ($n = 60$) and autism spectrum syndromes ($n = 23$) at German tertiary care hospital, compared with that in control group ($n = 3{,}917$). Severe deficiencies (<10 ng/ml) were found more frequently in the schizophreniform (38.3%) and autism spectrum groups (52.2%), compared to that of control group (16.3%). These findings justify the need for a more frequent assessment of serum vitamin D levels in these disorders, and advocate for additional randomized clinical trials investigating the effectiveness of vitamin D supplementation in ameliorating psychiatric and behavioral symptoms.

Endres et al. investigated the prevalence of CSF inflammatory abnormalities in a seven-year cohort study at the Freiburg University clinic. In 63 patients with bipolar disorder, CSF abnormalities, suggestive of mild inflammation, were found in 19% of patients. These include increased albumin quotients (12.9%), increased immunoglobulin (Ig) G indices (3.2%),

oligoclonal bands (OCBs) in 1.6%, increased white blood cell count (1.6%). These findings further support the ME hypothesis of schizophrenia and autoimmune encephalopathy masquerading as severe mental disorder.

Vogels et al. verified the previously reported findings of T-cell deficits and monocyte immune gene activation in a well-controlled cohort of 97 individuals with largely euthymic bipolar disorder. Notably, within the decreased T-cell populations, the counts of T-helper 17 and T-helper 2 were increased whereas T regulatory cell counts were decreased. Moreover, the circulating monocytes demonstrated an increased frequency of anti-inflammatory phenotypes. The authors conclude that T-cell deficits are likely a trait phenomenon while pro and anti-inflammatory factors are state dependent in individuals with bipolar disorder.

Krause et al. showed significantly lower serum kynurenine level, and higher quinolinic acid/kynurenine (Qui/Kyn) ratio in a cohort of 32 individuals with major depression, compared to 20 healthy controls. Higher baseline kynurenine/tryptophan (KYN/TRP) ratio at baseline was predictive of remission and lower Qui/Kyn ratio following an add-on treatment with celecoxib correlated positively with remission. This study suggests that the measurements of above biomarkers might be useful in selecting individuals with major depression who are more likely to respond to anti-inflammatory agents.

Ajdacic-Gross et al. analyzed epidemiological data from the PsyCoLaus, a large cohort study ($n = 3{,}720$) in Switzerland, to explore the potential association between various early-onset anxiety disorders and the age at the onset of common viral childhood illnesses such as chickenpox, measles, and mumps. The authors found that only social phobia among early anxiety disorders was associated with delayed-onset viral infections, suggesting that common viral childhood infections of a delayed onset may increase the risk of social phobia, speculatively by infection-induced behavioral changes.

Rahman et al. investigated the developmental effect of maternal immune activation (MIA), during early and late gestation, on glutamatergic signaling via NMDAR-related molecular changes in various brain regions (cortex, hippocampus, and striatum) in adult rat offspring. The authors found that MIA can alter NMDAR indices, such as increased NR2A expression in cortical and hippocampal regions. This was more prominent in male compared with female offspring, irrespective of MIA gestational timing. They concluded that MIA-induced developmental molecular changes including increased NR2A expression in male offspring might trigger enduring vulnerability to impaired neuroplasticity and its consequent behavioral changes.

Sommer et al. investigated the neural effects of the hydrogen sulfide (H_2S, a gaseous molecule), which is endogenously produced by enzymes utilizing cysteine in the peripheral and central nervous systems. The authors also conducted another experiment to analyze the effects of various antipsychotics on the expression of H_2S forming enzymes in human cell lines. Local sodiumhydrogensulfide (NaHS) infusion alone into the hippocampus resulted in a significant increase in the hippocampal glucose and lactate levels, as well as glutamate release. Pretreatment with peripheral inflammatory lipopolysaccharide (LPS) was associated with a further increase in lactate, but without altering glutamate levels. While NaHS infusion was associated with a significant increase in hippocampal free radical formation, by contrast, LPS pretreatment was associated with reduced radical formation. Additionally, neuroleptics exhibited differential effects on the H_2S forming enzymes, a finding that may be relevant to understanding the diverse functional effects of antipsychotic drugs.

Mack et al. reported a case of a young woman presenting with a 25 years history of dominant psychiatric symptoms, ranging from depression to typical schizophrenia with fluctuating psychotic features. The psychiatric symptoms were poorly responsive to various traditional psychotropic interventions and required multiple prolonged psychiatric hospitalizations. Throughout the course of the illness, she had intermittently exhibited mild skin rashes thought to be related to possible underlying mixed connective tissue disorder. Indeed, one of her later relapses was associated with severe generalize exanthema that resolved rapidly with steroid and azathioprine. KB (the editor above) suspected that the psychosis is likely related to an underling systemic immune disorder for which treatment with azathioprine was maintained, resulting in a complete remission of the psychiatric syndrome for approximately 16 years. However, upon azathioprine discontinuation due to pregnancy, the patient developed a severe relapse of psychosis accompanied by severe diffuse skin rashes that required a 2 years inpatient psychiatric care. Treatment with various immunosuppressants, including belimumab, resulted in a full remission of both psychosis and skin rashes. The combination of systemic biomarkers for immune activation and mild CSF inflammatory abnormalities including the presence of OCBs led to the final diagnosis of atypical Lupus erythematosus with CNS involvement presenting with dominant psychiatric manifestations mimicking schizophrenia.

Endres et al. report a case of steroid-responsive chronic psychosis associated with autoimmune thyroiditis mimicking antipsychotics-resistant schizophrenia, in the context of several clinical red flags that collectively pointed to underlying organic etiopathogenesis. These were intermittent electroencephalogram (EEG) slowing, mild temporal atrophy, and elevated thyroid antibodies. This case illustrates the importance of considering autoimmune psychosis in the differential diagnosis of secondary (organic) psychosis and to complete the relevant diagnostic workup to early identify this subgroup of immune-responsive psychosis. Endres et al. report another case with surprisingly rapid improvement of chronic schizophrenic symptoms under newly introduced antiepileptics after having identified respective suggestive EEG signs.

Ong et al. presented a case of primary Sjögren's syndrome with dominant severe obsessive-compulsive together with depressive symptoms requiring psychiatric hospitalization. The diagnosis was eventually made based on the presence of serum biomarkers of Sjögren's syndrome coupled with CSF findings of mild inflammation. A few months of treatment with various immunosuppressants together with plasmapheresis resulted in a complete remission of all neuropsychiatric symptoms. The

authors suggest that the clinical approach to the management of psychotropic–resistant obsessive-compulsive disorder should also include a careful search for biomarkers of inflammation and autoimmunity.

Greenberg reports a case of severe psychotropic-refractory pediatric neuropsychiatric syndrome mimicking bipolar disorder thought to be linked to previously unrecognized infection, likely Bartonellosis. The presentation and the clinical course did not meet "pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections" (PANDAS) criteria. The patient exhibited a clinically meaningful response to antibiotics targeting Bartonella infection. This case illustrates that prior clinically unrecognized infections can serve as immunological triggers of secondary psychiatric illnesses.

Klein provided a thorough review of the clinical and experimental evidence supporting the "viral hypothesis of schizophrenia." The author suggests that HSV-1 infection of specific limbic brain regions such as hippocampal dentate gyrus among other factors is potentially mechanistically linked to psychosis in a subset of individuals with schizophrenia. The mechanisms potentially linking viral infections to the neurobiology of schizophrenia were highlighted. The author suggests that silencing the viral elements such as HSV-1, via either administering antiviral treatment or suppressing environmental factors that can influence viral expression such as stress can thereby potentially be curative in a subgroup of individuals with schizophrenia.

In summary, the data from these reports strengthen the evidence linking immune dysfunction, autoimmunity, and neuroinflammation to various primary psychiatric illnesses including schizophrenia and affective spectrum disorders. Alternatively, they also show that brain autoimmune disorders such as autoimmune encephalitis and autoimmune encephalopathy can also present with diverse neuropsychiatric syndromes masquerading as severe mental disorders such as primary psychotic disorders unresponsive to traditional psychotropic and behavioral interventions. Thus, it is critical to obtain a detailed history, perform a thorough examination, recognize clinical features (red flags) suggestive of organic causes, and complete a relevant diagnostic workup to include screening for relevant neuronal antibodies in serum and CSF to exclude other organic causes, in order not to overlook immunological causes of new-onset secondary (organic) neuropsychiatric syndromes. Collectively, the above data also support the ME hypothesis of schizophrenia and severe mental illness (1, 2) and the concept of autoimmune psychosis (3) mechanistically linking the neurobiology of a subset of psychosis to underlying inflammatory and immunological changes in the brain. Additional research studies investigating the prevalence of autoimmune psychosis among individuals with new-onset psychosis are needed. Further, development of an expert consensus on the evidence-based clinical practice guidelines addressing the diagnostic challenges and therapeutic dilemmas of new-onset psychosis of suspected immune origin is warranted.

AUTHOR CONTRIBUTIONS

KB prepared the intitial version of the Editorial, which was corrected and supervised and completed by SN and DB.

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Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients

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Autoimmune mechanisms causing diverse psychiatric symptoms are increasingly recognized and brought about a paradigm shift in neuropsychiatry. Identification of underlying antibodies against neuronal ion channels or receptors led to the speculation that a number of patients go misdiagnosed with a primary psychiatric disease. However, there is no clear consensus which clinical signs in psychiatric patients should prompt further investigations including measurement of anti-neuronal autoantibodies. We therefore aimed to analyze the presenting symptoms in patients with autoimmune encephalitis and the time between symptom onset and initiation of antibody diagnostics. For this, we recruited 100 patients from the Charité Center for Autoimmune Encephalitis between May and October 2016, including all types of autoimmune encephalitides. Psychiatric abnormalities were the most common clinical symptoms and were the presenting sign in 60%. One-third of patients were initially hospitalized in a psychiatric ward. All patients positive for antibodies against the *N*-methyl-D-aspartate receptor showed behavioral changes, hallucinations, memory deficits, catatonia, or delusions. Patients positive for antibodies against other cell surface or intracellular antigens were often hospitalized with a psychosomatic diagnosis. The time between occurrence of first symptoms and antibody testing was often alarmingly prolonged. In patients with symptom onset between 2013 and 2016, the mean delay was 74 days, in cases diagnosed between 2007 and 2012 even 483 days, suggesting though that increased awareness of this novel disease group helped to expedite proper diagnosis and treatment. By analyzing the medical records in detail, we identified clinical signs that may help to assist in earlier diagnosis, including seizures, catatonia, autonomic instability, or hyperkinesia. Indeed, reanalyzing the whole cohort using these "red flags" led to a 58% reduction of time between symptom onset and diagnosis. We conclude that the timely diagnosis of an autoimmune psychiatric disease can be facilitated by use of the described clinical warning signs, likely enabling earlier immunotherapy and better prognosis. Also, the threshold for cerebrospinal fluid analysis and autoantibody testing should be low.

Keywords: autoimmune encephalitis, schizophreniform syndrome, cerebrospinal fluid analysis, anti-neuronal autoantibodies, immunotherapy

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INTRODUCTION

The growing number of newly described autoimmune encephalitides has drawn a remarkable link between immunology and psychiatry within the last several years (1–3). Since the pioneering discovery of *N*-methyl-D-aspartate receptor (NMDAR) autoantibodies (4), various further antibodies against receptors and ion channels were identified in patients with psychiatric abnormalities, such as against AMPA, GABA, glycine receptors, metabotropic glutamate receptor 5 (mGluR5), and dopamine-D2 receptors (**Table 1**), not only in humans (5). Patients are often first hospitalized in psychiatric departments before being transferred to a neurology ward (6, 7), stimulating the intriguing question of whether a subset of patients may go misdiagnosed with a primary psychiatric disease (1, 2, 8, 9). Recently, a high prevalence of cerebrospinal fluid (CSF) abnormalities including the detection of anti-neuronal autoantibodies has been observed in 54.4% of psychotic patients (10), highlighting their potential role in psychiatry and underlining the need for increased clinical and scientific awareness in order to not overlook treatable etiologies.

Antibody-mediated encephalitides can be categorized based on the presence of anti-neuronal antibodies targeting (i) neuronal cell surface antigens and (ii) intracellular antigens (11, 12). Autoantibodies directed to cell surface proteins are more frequently found in patients with psychiatric abnormalities, likely due to a suspected direct pathogenic effect (12–14). The demonstration of specific effects of NMDAR antibody-containing CSF *in vivo* convincingly substantiates the link between autoantibodies and the schizophreniform syndrome seen in these patients (15). Most recent work using CSF-derived human monoclonal NMDAR antibodies showed that the antibody is sufficient to change NMDAR expression and electrophysiology (16). Thus, the presence of this antibody alone represents a risk factor for neuropsychiatric symptoms, supporting the need for sufficiently aggressive immunotherapy in affected patients.

Such a clear causative role of autoantibodies on psychiatric symptoms has yet to be shown for further surface-directed antibodies. Nonetheless, psychotic symptoms are common in numerous other autoimmune encephalitides (**Table 1**). For example, patients with antibodies against the voltage-gated potassium channel complex (VGKCc) often present with hallucinations, depression, and memory deficits (13, 14, 17). Neuropsychiatric symptoms were found in 44% of VGKCc antibody-positive patients, occasionally treated for primary psychiatric diagnoses (14). Less well known, patients with antibodies against intracellular targets can also present with psychiatric symptoms (18).

The prognosis of autoimmune encephalitides largely depends on the rapid initiation of immunotherapy. Any delay in diagnosis causes costs and morbidity, while early immunotherapy results in substantial recovery in 70–80% of the patients (6, 19–23). This is especially striking considering the often severe course of the disease, sometimes requiring prolonged episodes of intensive care unit treatment and mechanical ventilation (6). Delayed

TABLE 1 | Classification of encephalitis groups in the present study and commonly associated clinical features.

Encephalitis groups of the present study	Antibodies	Number of patients	Psychiatric symptoms	Additional symptoms	Typical patient
(A) NMDAR encephalitis (*n* = 53)	NMDA receptor	*n* = 53 (53%)	Psychosis, schizophreniform illness, catatonia, hallucinations, aggression	Epileptic seizures, dyskinesia, autonomic instability, speech dysfunction, decreased consciousness	Young women, association with ovarian teratomas
(B) Non-NMDAR cell surface antigens (*n* = 24)	Caspr2	*n* = 4 (4%)	Insomnia, panic attacks, schizophreniform illness, depression	Morvan syndrome, neuromyotonia, muscle spasms, fasciculations	Middle age or elderly patients, may be associated with thymoma
	LGI1	*n* = 14 (14%)	Amnesia, confusion, memory deficits, depression	Limbic encephalitis, faciobrachial dystonic seizures, hyponatremia	Middle age or elderly patients, male:female (2:1), may be associated with thymoma
	Metabotropic glutamate receptor 5	*n* = 2 (2%)	Behavioral changes, emotional instability, memory deficits	Limbic encephalitis, Ophelia syndrome	Young adults, may be associated with Hodgkin's lymphoma
	Glycine receptor	*n* = 1 (1%)	Behavioral changes, schizophreniform syndrome	Stiff-person syndrome (SPS) or progressive encephalomyelitis with rigidity and myoclonus, hyperekplexia	middle age or elderly patients, may be associated with thymomas and lymphomas
(C) Antibodies against intracellular antigens (*n* = 23)	Synaptic antigens: anti-GAD antibodies	*n* = 9 (9%)	Schizophreniform illness, autism, attention-deficit/hyperactivity disorder	Limbic encephalitis, seizures, SPS, brainstem dysfunction, ataxia	Middle age or elderly patients, might be associated with small-cell lung cancer
	Onconeuronal antigens: anti-Yo, -Hu, -CV2, -Ri, -Ma2 antibodies	*n* = 14 (14%)	Behavioral changes	Limbic encephalitis, cerebellar degeneration, sensory neuropathy	Elderly patients, often with malignant tumors (small-cell lung carcinoma, Hu; testicular seminoma, Ma2)

NMDAR, N-methyl-D-aspartate-receptor; LGI1, leucine-rich glioma inactivated 1; Caspr2, contactin associated protein 2.

recognition of the disease can also result in inadequate use of neuroleptics, which in patients with NMDAR encephalitis frequently worsens the symptoms, leading to the working diagnosis of a neuroleptic malignant syndrome (7).

We therefore aimed to retrospectively ascertain the time and frequency of delayed diagnosis of autoimmune encephalitides and asked whether specific clinical signs can assist in earlier recognition, antibody testing, and proper diagnosis of the disease. Indeed, a number of warning signs ("red flags") can help to facilitate the timely diagnosis of an autoimmune psychiatric disease, likely enabling earlier immunotherapy and better prognosis.

MATERIALS AND METHODS

Patient Selection

$N = 100$ patients with different forms of autoimmune encephalitides were recruited in the Charité Centre for Autoimmune Encephalitis from May to October 2016. Patients were grouped in three categories (**Table 1**):

(A) Anti-NMDAR encephalitis ($n = 53$), defined by a compatible clinical picture and positive IgG-NMDAR antibodies in the CSF (**Figure 1A**).
(B) Non-NMDAR surface antibodies ($n = 24$), including patients with antibodies against the neuronal cell surface antigens LGI1 ($n = 14$), CASPR2 ($n = 4$), mGluR5 ($n = 2$, **Figure 1B**), glycine receptor ($n = 1$) and against an unknown epitope determined on brain section immunofluorescence testing ($n = 3$).
(C) Antibodies against intracellular epitopes ($n = 23$), including patients with GAD antibodies ($n = 9$) or onconeuronal antibodies, such as Yo, Hu, Ri, or CV2 ($n = 14$, **Figure 1C**).

Informed Consent

Written informed consent was received from participants at the Charité Department of Neurology or their representatives prior to inclusion in the study, and analyses were approved by the Charité University Hospital Institutional Review Board.

Clinical Data Collection

Most patients were hospitalized in the Charité Department of Neurology during the disease course. Medical charts were retrospectively analyzed, and clinical and para-clinical information was collected during follow-up visits in the outpatient clinic or *via* email/telephone interviews. The following information was systematically retrieved from medical records: age, sex, date of disease onset, neurological and psychiatric symptoms during initial clinical presentation, psychiatric and neurological signs during follow-up, department of initial hospitalization, details of psychiatric hospitalization, symptoms that led to determination of antibodies, date of diagnosis, and time from first symptoms to diagnosis.

RESULTS

Demographic Data

Median age in our cohort was 41 years (range 14–92 years) and 71% were female. Patients positive for NMDAR antibodies were younger (mean age 30 [14–57] years) and mainly women (91%). In contrast, patients with antibodies against non-NMDAR surface antigens were predominantly of male gender (67%) and older (mean age 53 [29–78] years). Patients positive for antibodies against intracellular proteins were predominantly female (65%), mean age was 56 (37–92) years.

Initial Hospitalization in a Psychiatric Department

In order to estimate the overlapping symptoms with primary psychiatric disorders, we analyzed the frequency of patients initially hospitalized in a psychiatric department and the frequency of psychotic symptoms at first evaluation and during follow-up. $N = 31$ patients (31%) were initially hospitalized on a Psychiatry ward, commonly for psychotic or suspected psychosomatic symptoms. Almost two-thirds of all patients ($n = 60$; 60%) showed psychotic symptoms at the beginning of the disease, even if hospitalization was not required, 7% presented with psychosomatic symptoms.

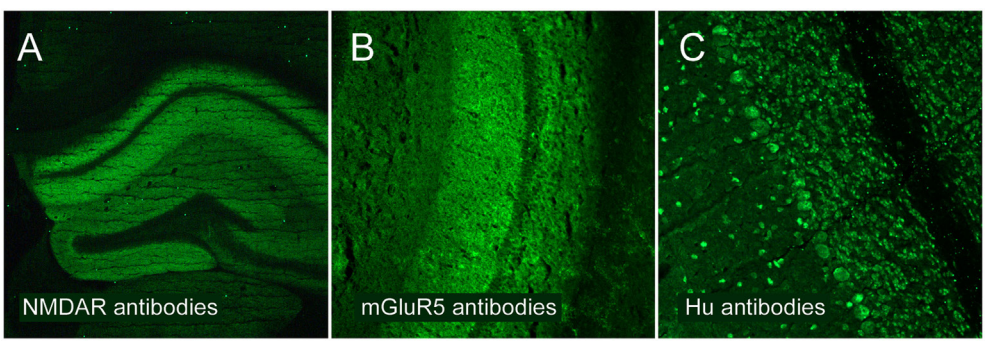

FIGURE 1 | Classification of encephalitis groups analyzed in the present study. Underlying autoantibodies show different patterns of brain binding using immunofluorescence testing. **(A)** Patients with NMDAR encephalitis and high-level autoantibodies against the NR1 subunit of the NMDAR. **(B)** Patients with non-NMDAR antibodies targeting neuronal surfaces, such as antibodies against the metabotropic glutamate receptor 5 (mGluR5). **(C)** Patients with antibodies targeting intracellular epitopes, such as anti-Hu antibodies.

Psychiatric symptoms were not equally distributed across the three encephalitis groups. All patients with NMDAR antibodies ($n = 53$) showed psychotic symptoms. In patients positive for antibodies against other neuronal surface or intracellular antigens, psychosomatic symptoms were common at presentation: 8/24 in the non-NMDAR group (33%), 5/23 in the intracellular antigens group (22%). However, psychotic symptoms did also occur: 6/24 in the non-NMDAR group (25%), 4/23 in the intracellular group (17%). Of the NMDAR antibody-positive patients, 21/53 (40%) were seen by a psychiatrist at first evaluation, while this was the case for only one patient positive for intracellular protein antibodies (4%).

Initial Symptoms

The frequency of first clinical signs was again not equally distributed between the encephalitis groups (**Table 2**). Patients positive for NMDAR antibodies typically presented with psychiatric symptoms and either developed a spectrum of neurological abnormalities, such as seizures, movement, or speech disorders, or already showed them at first evaluation. Their initial psychiatric symptoms were acute behavioral changes ($n = 46$; 87%), hallucinations ($n = 23$; 43%), paranoid delusions ($n = 13$; 26%), and memory deficits, especially short-term memory loss ($n = 11$; 21%). Also, mutism ($n = 8$; 15%), catatonia ($n = 10$; 19%), and depressive symptoms ($n = 10$; 19%) were commonly seen at presentation. One young woman got initially hospitalized with the clinical picture of anorexia. First symptoms in some patient were neurological, consisting of epileptic seizures ($n = 10$; 19%), speech dysfunction such as pressured speech and verbal reduction ($n = 10$; 19%), dyskinesia ($n = 7$; 13%), and headache ($n = 9$; 17%).

Patients of the non-NMDAR group presented also with psychiatric symptoms in most cases, such as acute behavioral changes ($n = 7$; 29%), aggression/confusion ($n = 6$; 25%), or memory deficits ($n = 8$; 33%). Hallucinations and paranoid delusions were also seen (**Table 2**). The neurological symptoms of this group were more characteristic and included faciobrachial dystonic seizures (FBDS, in patients with LGI1 antibodies) ($n = 7$; 29%) and sensorimotor deficits ($n = 7$; 29%).

Patients positive for intracellular epitope antibodies presented less frequently with psychiatric symptoms, including acute behavioral changes and memory deficits. The majority of symptoms in this group were neurological, such as sensorimotor deficits ($n = 13$; 57%), cerebellar ataxia ($n = 7$; 30%), movement disorders ($n = 3$; 13%), and generalized tonic-clonic seizures ($n = 3$; 13%).

In most patients of all three groups, both psychiatric and neurological symptoms occurred during the first month of disease. Interestingly, $n = 13$ (13%) of all patients presented with a depressed mood, in four cases leading to the diagnosis of major depression. Appearance of additional neurological symptoms led to reclassification of diagnosis.

Which Clinical Features Led to Examination of Autoantibodies?

We next determined which clinical symptoms, routine laboratory findings, or imaging abnormalities triggered the testing for autoantibodies in all 100 patients, the results of which finally allowed the firm diagnosis of autoimmune encephalitis (**Table 3**). Indeed, several clinical constellations of neurological and psychiatric symptoms were more common than others to stimulate antibody testing. We semi-quantitatively classified these constellations as

TABLE 2 | Presenting clinical symptoms in all 100 patients.

Initial signs and symptoms	All patients (100)	NMDAR (53)	Non-NMDAR (24)	Intracellular antigens (23)
Psychiatric				
Acute behavioral changes	56 (56%)	46 (87%)	7 (29%)	3 (13%)
Hallucinations (visual, auditory)	25 (25%)	23 (43%)	1 (4%)	
Memory deficits (retro- and anterograde amnesia)	22 (22%)	11 (21%)	8 (33%)	4 (17%)
Confusion/aggression	18 (18%)	11 (21%)	6 (25%)	1 (4%)
Paranoid delusions	17 (17%)	13 (26%)	2 (8%)	1 (4%)
Depressed mood	13 (13%)	10 (19%)	4 (16%)	1 (4%)
Catatonia	10 (10%)	10 (19%)		
Mutism	8 (8%)	8 (15%)		
Anorexia	1 (1%)	1 (2%)		
Any of the above symptoms	65 (65%)	53 (100%)	14 (58%)	7 (30%)
Neurological				
Sensorimotor deficits	30 (30%)	8 (15%)	7 (29%)	13 (57%)
Seizures		10 (19%)	2 (8%)	5
Generalized tonic-clonic	13 (13%)	9 (17%)	1 (4%)	3 (13%)
Focal	4 (4%)	1 (2%)	1 (4%)	2 (9%)
Faciobrachial dystonic seizures	7 (7%)		7 (29%)	
Speech dysfunction (pressured speech, verbal reduction)	15 (15%)	10 (19%)	4 (16%)	
Movement disorders	11 (11%)	7 (13%)	1 (4%)	3 (13%)
Headache	12 (12%)	9 (17%)	1 (4%)	2 (9%)
Reduced levels of consciousness	7 (7%)	5 (9%)	2 (8%)	
Paralysis	7 (7%)	4 (8%)	1 (4%)	2 (9%)
Cerebellar ataxia	10 (10%)	1 (2%)	3 (12%)	7 (30%)
Diplopia	7 (7%)	3 (6%)		4 (17%)
Any of the above symptoms	67 (67%)	39 (74%)	20 (83%)	20 (87%)

TABLE 3 | Clinical symptoms and constellations that led to the determination of anti-neuronal antibodies in all 100 patients.

Symptoms	All patients (100)	NMDAR (53)	Non-NMDAR (24)	Intracellular antigens (23)
Epileptic seizures	14 (14%)	10 (19%)	2 (8%)	2 (8%)
Cerebrospinal fluid (CSF) abnormalities[a] and absent evidence for infectious encephalitis	13 (13%)	12 (27%)	1 (4%)	
Abnormal postures or movements	4 (4%)	4 (7%)		
Reduced levels of consciousness	4 (4%)	4 (7%)		
Aphasia or dysarthria	3 (3%)	3 (6%)		
Lack of improvement with antipsychotics	5 (5%)	4 (7%)	1 (4%)	
Autonomic instability	2 (2%)	2 (4%)		
Suspicious MRI or EEG findings	10 (10%)	3 (6%)	5 (20%)	2 (8%)
Steroid-responsive autoimmune thyroiditis	3 (3%)	2 (4%)		1 (4%)
Lack of improvement with antiepileptic medication	2 (2%)	1 (2%)	1 (4%)	
Focal neurological deficits	3 (3%)	1 (2%)	1 (4%)	1 (4%)
Sensory deficits	3 (3%)	1 (2%)	2 (8%)	
Rapidly progressing psychosis	4 (4%)	1 (2%)	2 (8%)	1 (4%)
Suggested by patients or families	3 (3%)	3 (6%)		
Positive effect of *ex juvantibus* immunotherapy	2 (2%)		1 (4%)	1 (4%)
Faciobrachial dystonic seizures	3 (3%)		3 (12%)	
Neuromyotonia	1 (1%)		1 (4%)	
Cerebellar ataxia	8 (8%)		2 (8%)	6 (26%)
Hyponatremia	2 (2%)		2 (8%)	
Paresthesia or malignant tumor[b]	7 (7%)			7 (30%)

[a]*Increased white blood cell count or CSF-specific oligoclonal bands.*
[b]*Small-cell lung cancer, testicular seminoma.*

"yellow flags" and "red flags," depending on their power to predict the presence of autoantibodies in such patients (**Table 4**).

In the NMDAR encephalitis group, viral encephalitis was a common working diagnosis, often suggested by the clinical picture, acute neurological changes, and CSF pleocytosis. NMDAR autoantibody testing was often initiated once the search for a viral or bacterial pathogen remained negative ($n = 12$; 27%). In all three groups, the occurrence of epileptic seizures frequently initiated CSF investigation including determination of antibodies ($n = 14$; 14%). Suspicious MRI and EEG were another reason for antibody testing, in particular in patients with non-NMDAR surface antibodies ($n = 5$; 20%), but much less in NMDAR antibody-positive patients ($n = 3$; 6%). Patients were frequently transferred from a psychiatric to a neurological ward at this point. Similarly, in patients hospitalized for a schizophreniform syndrome, detection of abnormal neurological signs resulted in antibody testing. These deficits included decreased levels of consciousness ($n = 4$; 7%), abnormal postures or movements ($n = 4$; 7%), and aphasia or dysarthria ($n = 3$; 6%) in patients of the NMDAR encephalitis group. Focal neurological signs were the trigger for antibody testing in one patient each of the NMDAR (2%), non-NMDAR surface antibody (4%), and intracellular epitope antibody (4%) groups (**Table 3**).

Non-NMDAR antibodies testing was performed in several cases because of the occurrence of FBDS ($n = 3$; 12%), sensory deficits ($n = 2$; 8%), or the detection of hyponatremia in the context of unexplained neuropsychiatric symptoms ($n = 2$; 8%). In two patients with non-NMDAR surface antibodies, antibody testing was initiated because of a rapidly progressing psychosis ($n = 2$; 8%). A common reason to test for antibodies against intracellular epitopes was the occurrence of paresthesia in the context of a malignant tumor ($n = 7$; 30%) or clinical deficits resulting from cerebellar symptoms ($n = 6$; 26%).

TABLE 4 | Warning signs pointing to an autoimmune etiology in new-onset psychosis.

Yellow flags

- Decreased levels of consciousness
- Abnormal postures or movements (orofacial, limb dyskinesia)
- Autonomic instability
- Focal neurological deficits
- Aphasia or dysarthria
- Rapid progression of psychosis (despite therapy)
- Hyponatremia
- Catatonia
- Headache
- Other autoimmune diseases (e.g., thyroiditis)

Red flags

- Cerebrospinal fluid (CSF) lymphocytic pleocytosis or CSF-specific oligoclonal bands without evidence for infection
- Epileptic seizures
- Faciobrachial dystonic seizures
- Suspected malignant neuroleptic syndrome
- MRI abnormalities (mesiotemporal hyperintensities, atrophy pattern)
- EEG abnormalities (slowing, epileptic activity or extreme delta brush)

"Red flag" criteria should always prompt determination of anti-neuronal autoantibodies in psychiatric patients. "Yellow flag" criteria should raise suspicion of an autoimmune etiology and include autoimmune encephalitis in the differential diagnoses, in either case if several findings are present.

We further identified seven cases in which the lack of clinical improvement after antipsychotic ($n = 5$; 5%) or antiepileptic therapy ($n = 2$; 2%) led to the suspicion of an autoimmune encephalitis. Another two patients with psychotic symptoms

and cognitive impairment had the working diagnosis of steroid-responsive encephalopathy with autoimmune thyroiditis (SREAT), which triggered antibody testing that resulted in positive NMDAR ($n = 2$; 4%) and onconeuronal ($n = 1$; 4%) antibodies (**Table 3**). Finally, in one case, the patient's family suggested the diagnosis of autoimmune encephalitis after internet research, prompting the testing of NMDAR antibodies which returned positive.

Taken together, several clinical symptoms and abnormalities repeatedly led to antibody testing, bringing about the correct diagnosis of autoimmune encephalitis. We consider these warning signs as "red flags" (**Table 4**) which might facilitate earlier diagnosis of autoimmunity in psychiatric symptoms.

Time from First Symptom to Diagnosis

Given that the prognosis in patients with autoimmune encephalitis depends on the rapid initiation of immunotherapy, we next analyzed the time between symptom onset and diagnosis. For this, patients who were treated primarily in a psychiatry department ($n = 35$) were divided in two groups. In the first group, symptoms started between 2007 and 2012. Here, the delay was very prolonged with a mean time of 483 days (**Figure 2A**). In the second group with symptom onset between 2013 and 2016, the mean time between disease onset and diagnosis was 74 days (**Figure 2B**). The reduction was obvious in both groups for which data were available, namely the NMDAR encephalitis (reduction from 475 to 40 days) and non-NMDAR antibody group (reduction from 509 to 150 days). It seems likely that increased awareness of this new disease group after 2012 and a lower threshold for antibody testing in clinical routine helped to markedly reduce the delay, even though there is an obvious need and opportunity for further improvement.

Earlier Diagnosis of Autoimmune Encephalitis in Psychiatric Patients Using the "Red Flags"

Having established warning signs ("yellow flags" and "red flags") that may guide clinicians in the indication for autoantibody testing in patients with different autoimmune encephalitides (**Table 4**), we then retrospectively applied these criteria to our cohort of encephalitis patients hospitalized in a psychiatric ward. In this way, we aimed to estimate the potential reduction in delay between symptom onset and diagnosis of autoimmune encephalitis. Indeed, reanalysis of the medical records showed that most patients had well-documented evidence of "yellow flag" and "red flag" criteria in their medical records, long before an autoimmune etiology and antibody testing was considered. As a typical example, a patient with a schizophreniform syndrome developed catatonia and autonomic instability (both are "yellow flags") 4 weeks after the symptom onset, but only an epileptic seizure 10 weeks after symptom onset prompted autoantibody testing and revealed positive NMDAR antibodies. We then calculated the time from symptom onset to diagnosis, hypothetically assuming that the first documentation of a "red flag" in the medical chart would have resulted in the determination of autoantibodies. In this example, using the "yellow flag" and "red

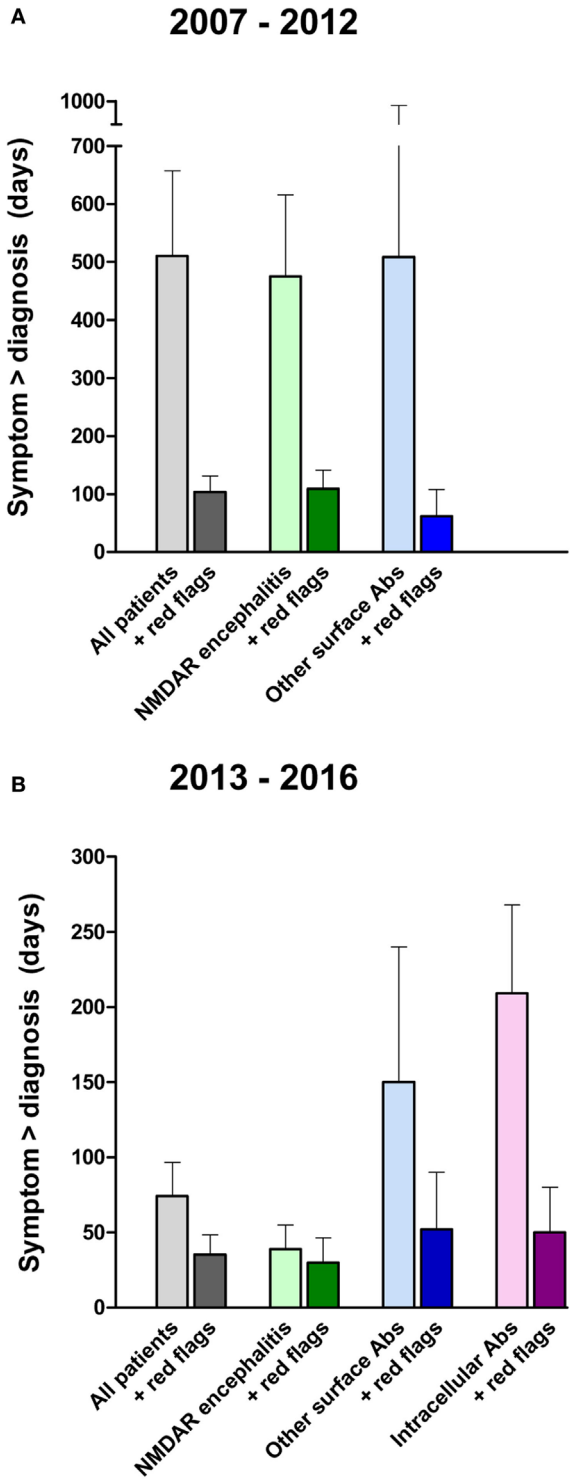

FIGURE 2 | Time between onset of clinical symptoms and diagnosis of antibody-associated encephalitis. Comparing patients with disease onset between 2007 and 2012 **(A)** versus 2013 to 2016 **(B)**, the delay from symptom onset to the diagnosis of autoimmune encephalitis (light colors) has been reduced within the last years, likely due to increased awareness (please note the different *y*-axes). Applying the "red flag" criteria to the same patients by reanalyzing medical records resulted in a marked hypothetical reduction of the delay until antibody testing and encephalitis diagnosis (dark colors).

flag" criteria reduced the delay from symptom onset to diagnosis from 10 to 4 weeks.

Indeed, the analysis of our cohort showed a marked reduction in the time until diagnosis (**Figure 2**). For the more recent patients with symptom onset between 2013 and 2016, a reduction of 58% from 74 to 31 days was detectable. In detail, time between appearance of first symptoms and final diagnosis was reduced from 40 to 10 days (75%) in patients with NMDAR encephalitis, 150 to 52 days (65%) in patients with non-NMDAR surface antibodies, and 209 to 50 days (76%) in patients with antibodies against intracellular epitopes (**Figure 2B**).

DISCUSSION

In accordance with recent publications (7, 8, 24), our results confirm that a broad spectrum of psychiatric symptoms frequently are the first complaints in patients with autoimmune encephalitis. While psychosis typically led to hospitalization of patients with NMDAR encephalitis, a psychosomatic disorder was often suspected in patients with surface non-NMDAR and intracellular epitope antibodies. The "psychosomatic" symptoms included, for example, FBDS in LGI1 antibody-positive patients, muscle spasms, and fasciculations in Caspr2 antibody-positive patients or sensory deficits in patients with onconeuronal antibodies. Interestingly, most patients in all three encephalitis groups showed additional neurological symptoms during the first month of disease.

Analysis of the present cohort of 100 encephalitis patients showed that several clinical symptoms or laboratory findings eventually led to the suspicion of an autoimmune etiology and the determination of autoantibodies. These "yellow flags" and "red flags" are summarized in **Table 4**, classified based on their predictive value to point to an underlying autoimmune encephalitis in the clinical workup of patients with psychiatric abnormalities. Given that systematic controlled trials and systematic reviews of cohort or case-control studies are lacking due to the novelty of this field and the relative rarity of autoimmune encephalitides, this case series analysis can only represent level 4 of evidence. Generally, some constellations are very typical for a given form of encephalitis, e.g., the presence of new-onset psychosis in young women with ovarian teratomas indicating NMDAR encephalitis or the combination of amnesia, hyponatremia, and the pathognomonic FBDS (brief repetitive stereotyped movements predominantly affecting the arm and ipsilateral face) indicating LGI1 antibody encephalitis. Clearly, typical features can be absent and delay the proper diagnosis (11, 25–27). Also, future work will likely add further or modify the proposed criteria.

The most common triggers for autoantibody diagnostic were CSF abnormalities in the absence of an infectious disease. The symptom overlap with viral encephalitis is remarkable regarding neurological and psychiatric changes (28, 29), suggesting that autoantibodies should always be determined, at the latest if virus diagnostic (using PCR) remains negative. CSF is abnormal in almost all patients with NMDAR encephalitis during the disease course (11, 24), underlining the relevance of routine CSF testing in psychiatric patients. This is also valid for the other forms of encephalitis, although patients with LGI1 antibodies have a lower frequency of CSF pleocytosis (41%) or elevated protein (47%) and rarely have intrathecal LGI1 antibody synthesis (25).

The occurrence of epileptic seizures in a psychotic patient was another common reason to reassess the working diagnosis of a primary psychiatric disease and test for antibodies. EEG changes not explained by medication are almost always present in autoimmune encephalitis. The alterations are rarely specific, showing focal or diffuse slow activity frequently associated with one or several foci of epileptic activity, eventually revealing subclinical seizures (27). However, the pattern referred to as "extreme delta brush" in NMDAR encephalitis is quite disease-specific (30). Suspicious MRI findings led to the correct diagnosis in relatively few cases in the present cohort (10%), which is likely explained by the fact that brain MRIs are unremarkable in more than 50% of patients with NMDAR encephalitis (11, 23, 28). If present, however, MRI abnormalities should always prompt autoantibody investigation, even though other diseases might cause similar imaging changes, such as gliomas (25, 28, 31).

Lejuste et al. observed a very high rate of patients with NMDAR encephalitis in which intolerance to antipsychotic drugs led to transfer to a Neurology department or intensive care unit (7). In line with their findings, the combination of autonomic instability and increased creatine kinase levels after neuroleptic therapy in several cases led to the suspicion of a malignant neuroleptic syndrome. Therefore, we included progression under antipsychotic therapy, suspected malignant neuroleptic syndrome and autonomic instability to the "red flag" criteria (**Table 4**). Finally, the presence of an autoimmune thyroiditis together with psychotic symptoms and cognitive impairment resulted in antibody investigation in three cases in the present cohort. It was shown recently that serum thyroid antibodies were elevated in 24.7% of 180 psychotic patients (10). Beneficial effects from steroids suggest the less well-defined constellation of SREAT (32). However, occurrence of specific brain-directed antibodies in our cohort (e.g., NMDAR antibodies) support the idea that SREAT represents increased susceptibility to autoimmunity, rather than that antithyroid antibodies are directly pathogenic. Findings of elevated thyroid peroxidase and thyroglobulin antibodies in psychotic patients should nonetheless raise suspicion and guide autoantibody testing.

Apart from the clinical application of the here proposed criteria, the present study reinforces the recent discussion that autoantibodies may participate in the development of psychiatric disorders, such as schizophrenia, in greater extend than previously assumed. For example, the reduction of NMDAR-specific currents and consecutively impaired glutamatergic neurotransmission is well known under the NMDAR hypofunctionality hypothesis of schizophrenia (33). In parallel, synaptic and extrasynaptic reduction of NMDAR by autoantibodies in NMDAR encephalitis leads to the typical schizophreniform symptoms seen in these patients (34). While internalization of NMDAR after contact with autoantibodies has been established as an important disease mechanism (16, 35), further pathologies are likely to happen in parallel, such as chemokine transfer

from immune cells to NMDAR-bearing neurons *via* volume transmission (36). It seems that these novel synaptic and extrasynaptic autoimmune disorders have brought about a paradigm shift in neuropsychiatry, and further research is urgently needed to clarify the detailed mechanisms of how autoimmunity and inflammation cause or modify neuropsychiatric diseases.

An important finding of our study was the alarmingly long delay between first symptoms and the final diagnosis of autoimmune encephalitis in many cases. It is known from the literature that patients with autoimmune encephalitis have often been misdiagnosed with a sole psychiatric disease despite the presence of neurological comorbidities (7). We could show here that the identification of encephalitis patients occurred much faster in more recent cases (2013–2016) compared to earlier patients, likely due to increased awareness of this novel disease group. The data collectively suggest that continuing increase in disease awareness will lead to further shortening of the time until diagnosis. This is needed as early and sufficiently aggressive immunotherapy is required for a better prognosis (22, 23, 25, 26). Using the here proposed "yellow flag" and "red flag" criteria will likely facilitate the timely diagnosis of an autoimmune psychiatric disease, as demonstrated by the hypothetical reanalysis of our cohort for the presence of such clinical signs. Finally, we conclude that CSF analysis should become clinical routine in patients with new-onset psychosis for several reasons. First, CSF abnormalities were the major indicator for an autoimmune encephalitis in psychotic patients. Second, some antibodies including NMDAR antibodies can be present in CSF only and would therefore be overlooked in serum (37). Third, recent data suggest that the rate of CSF abnormalities can be >50%, thus being much higher than previously thought and an important step to identify patients with treatable etiologies (10). Taken together, the threshold for CSF analysis and autoantibody testing should be low, in particular, when "red flags" are present.

AUTHOR CONTRIBUTIONS

JH and HP initiated the study and conducted the data analyses, wrote the paper, performed the data collection, read and approved the final version of this manuscript.

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Synaptic and Neuronal Autoantibody-Associated Psychiatric Syndromes: Controversies and Hypotheses

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Autoimmune encephalitis (AE) mediated by antibodies against synaptic and neuronal surface targets frequently presents with a psychiatric syndrome. In these patients, removal of autoantibodies treats the disease and outcomes are closely linked to early intervention. The discovery of these autoantibodies in isolated psychiatric syndromes has raised the possibility that these patients may derive similar benefits from immunotherapy, a potentially transformational approach to the treatment of mental illness. Although open-label case series suggest impressive therapeutic outcomes, the pathological relevance of these autoantibodies outside of canonical presentations is debated. The advent of diagnostic criteria for AE attempts to facilitate its prompt identification but risks prematurely neglecting the potential scientific and clinical significance of isolated syndromes that do not satisfy these criteria. Here, we propose using a syndrome-level taxonomy that has occasional, but not necessary, overlap with AE: synaptic and neuronal autoantibody-associated psychiatric syndromes or "SNAps". This will prevent confusion with AE and act heuristically to promote active investigation into this rare example of psychopathology defined on a molecular level. We suggest that this concept would have application in other autoantibody-associated syndromes including seizure, cognitive, and movement disorders, in which similar issues arise. We review putative direct and indirect mechanisms and outline experimentally testable hypotheses that would help to determine prospectively in whom autoantibody detection is relevant, and as important, in whom it is not. We summarize a pragmatic approach to autoantibody testing and management in severe mental illness in order to promptly diagnose AE and advocate a research-orientated experimental medicine paradigm for SNAps, where there is greater equipoise. We conclude that SNAps remains a nascent area of clinical neuroscience with great potential and in ongoing need of psychiatry-led basic and clinical research.

Keywords: autoimmune diseases of the nervous system, mild encephalitis, glutamatergic neurotransmission, blood–brain barrier disruption, immunotherapy, schizophrenia, bipolar disorder, major depression

INTRODUCTION

> The serum of patients with functional psychoses contains abnormal globulins … I have previously suggested that an autoimmune mechanism might be involved … any interpretation of their meaning is completely speculative and must be approached with the greatest caution.
>
> W. J. Fessel, Autoimmunity and Mental Illness, 1962 (1).

Antibodies that bind to cell surface neuronal, glial, or synaptic targets, collectively known as neural surface antibodies (NSAbs), have attracted significant attention in neurology and psychiatry (2). Their detection in a patient presenting with psychiatric symptoms raises the possibility both of a causal or disease-modifying role and of clinical improvement with immunotherapy (IT). This would represent a major step forward from current largely symptom-targeted psychotropic medications and has been met by clinicians and researchers with enthusiasm.

Although this iteration of autoimmune psychiatry is in its infancy (3), here we argue that there already exists ample evidence to warrant an expanding research program, focusing on robustly establishing the prevalence and relevance of NSAbs in what would otherwise appear to be primary psychiatric disorders. We discuss the controversies in applying knowledge of autoimmune encephalitis (AE) to such psychiatric disorders and suggest experiments by which these controversies may be resolved.

ARE AE AND PSYCHIATRIC SYNDROMES ASSOCIATED WITH NSAbs RELATED?

Given the prominence of psychiatric symptoms in many types of AE, and the known importance of receptor targets of NSAbs to psychopathology in psychiatric disorders, there have been extensive efforts to define disease-relevant associations between NSAbs and isolated psychiatric syndromes such as first-episode psychosis (FEP) (4). The development of assays able to detect specific antibodies against central nervous system (CNS) neural surface antigens in combination with careful clinical phenotyping has made this possible. Primary psychiatric disorders, such as schizophrenia, are far more common than AE. If a proportion of these were to have an NSAb-related etiology, then applying the lessons from the rare disease could stand to benefit a far larger group of patients.

Differences in acuity of sample timing, serum testing without paired cerebrospinal fluid (CSF), and variations in assay method have led to a lack of consensus on prevalence of NSAbs in psychiatric illness. Initially, higher rates of autoantibody prevalence were found early in psychotic illnesses compared to the chronic phase (5), but some evidence makes this distinction less clear (6). Live, non-permeabilized cell-based assays (CBAs) largely find higher NSAb prevalence in patients compared to controls (7, 8), but this has not been wholly true of fixed permeabilized CBAs (9–11). The use of CBAs in isolation has been criticized for lacking disease relevance (12); however, some studies supplementing CBA with immunohistochemistry and/or neuronal staining do in fact detect differences between cases and controls in certain psychiatric syndromes such as postpartum psychosis (13).

The rapid development of knowledge on AE, and the increasing and potentially confusing "phenotype spread" associated with NSAbs, have inspired diagnostic criteria for AE (14). These focus on early detection prompting timely IT. Diagnosis is deemed more likely with multiple symptoms, symptoms spanning both neurological and psychiatric domains, and supportive imaging, electroencephalogram (EEG), and CSF changes. Less emphasis is placed on NSAb detection as AEs tend to present stereotypically and can be recognized with readily available investigations, whereas NSAb results may take several weeks to process. However, by definition, psychiatric syndromes with serum NSAb positivity on routinely used CBAs will be incompatible with this system. To illustrate, for NMDAR antibody positivity to be relevant by these criteria, it either needs to be detected in CSF or in serum with multiple assay methods, and "relevance" equates only to a diagnosis of NMDAR-AE. Given that NMDAR antibodies are the most commonly detected NSAb in isolated psychiatric syndromes (9), and that NMDAR-AE occasionally does not progress beyond psychosis, this has implications for much of the field. NSAbs found in isolated psychiatric syndromes, which are currently considered of research interest and potential clinical relevance, would be scientifically neglected or "orphaned." The reality that CSF is often difficult to obtain in these patients, and multiple assay methods to evaluate serum are rarely available outside specialist centers, compounds this issue.

Furthermore, a negative CSF result should not automatically render NSAb seropositivity irrelevant. First, some NSAbs, such as LGI1, are detected less frequently in CSF, in this case in half or less of LGI1 AE cases (15). Second, if NSAbs are pathogenic in some of the isolated psychiatric presentations in which they are detected, it is likely that they occur at lower titers than in fulminant AE. Furthermore, the brain parenchyma can act as an "immunoprecipitator" of NMDAR antibodies (16), so unless the parenchyma is "saturated" by an extremely high concentration of antibody, it is plausible to expect that NSAbs may not be found in the CSF while still directly disrupting brain function.

We propose that these challenges for the field can be addressed by use of a syndrome-level taxonomy. Patients with isolated psychiatric symptoms and detectable NSAb can be characterized as "synaptic and neuronal autoantibody-associated psychiatric syndromes," abbreviated to "SNAps" (**Figure 1A**). SNAps deliberately have less stringent criteria for onset time, antibody class, and assay criteria. This enables a broad category, agnostic to the precise pathogenic role of the NSAb. In real time, additional serum antibody tests, identification of CSF antibody, or further investigations (e.g., MRI, EEG) may reveal abnormalities that meet the diagnostic criteria for AE, and presuming that new symptom domains do not evolve, these patients can then be characterized as having SNAps-AE (see **Table 1**). Patients with clear features of AE would continue to be classified as such. We suggest that this model, in which an NSAb-associated isolated clinical syndrome and AE partially overlap, can be extended to other syndromes such as epilepsy (17) and cognitive impairment

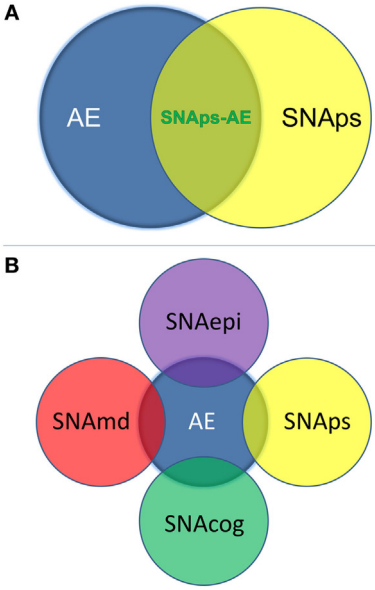

FIGURE 1 | **(A)** Patients with isolated psychiatric symptoms and a detectable neural surface antibody can be characterized as having a "synaptic and neuronal autoantibody-associated psychiatric syndrome," abbreviated to "SNAps." This distinguishes these patients from the majority of patients with autoimmune encephalitis (AE), which is normally a multi-symptom disorder with specific associated clinical and paraclinical features: diagnostic criteria for AE have been outlined in a recent position paper (14). Some patients with isolated psychiatric symptoms will also meet criteria for AE—these patients are here referred to as SNAps-AE and are clinically atypical for AE by virtue of their monosymptomatic presentation. **(B)** The distinction between an isolated symptomatic presentation and a polysymptomatic AE presentation can usefully be extended to non-psychiatric presentations. This scheme recognizes that there will be areas of overlap where a monosymptomatic presentation meets paraclinical criteria for AE e.g. imaging, electroencephalogram, or cerebrospinal fluid parameters. Here 'md' stands for movement disorder, 'cog' is cognitive disorder, and 'epi' is epilepsy.

(18) (see **Figure 1B**). **Table 2** demonstrates that many of the signs and symptoms of AE are in fact seen (often to an attenuated degree) in patients with so called "isolated" psychosis, suggesting that the overlap between these conditions may be even greater still. The nascence of the field would suggest this system to be clinically pragmatic and also heuristic, promoting the delineation of biologically defined disease sub-classes and, equally as important, the signals that imply lack of disease relevance.

ARE NSAbs IN SNAps DIRECTLY PATHOGENIC, INDIRECT MARKERS, OR INNOCENT BYSTANDERS?

Most NSAbs appear to share a common mechanism of action in their ability to cause cross-linking and internalization of their target antigen (4). Where the target is a neurotransmitter ion channel receptor, it is likely that the resulting receptor hypofunction results in impaired neurotransmitter signaling, as demonstrated by abolition of currents on some neurophysiological assays and alterations in synaptic plasticity (31, 32). Although not proven, the rationale for this causing the signs and symptoms of NSAb-associated disease is compelling and the "receptor hypofunction" model resonates well with many current theories of psychiatric disorders (33). Importantly, pathogenic potential as indexed by effects on receptor internalization and postsynaptic currents *in vitro* has been demonstrated for NMDAR antibodies taken from patients with schizophrenia (10, 34) and to a lesser extent bipolar disorder (35), demonstrating that pathogenicity is not restricted to NSAbs found in encephalitic presentations.

Synaptic dysfunction affecting glutamatergic neurotransmission has been proposed as a mechanism in schizophrenia, bipolar disorder, and major depression (36–38). We consider that antibody-mediated glutamatergic synaptic dysfunction, if relevant to psychiatric symptomatology, is likely to have relevance that cuts across traditional diagnostic boundaries. For example, the psychiatric phase of NMDAR-AE can include a diverse range of symptoms including affective and anxiety in addition to psychosis (39) and prevalence estimates detect NMDAR antibodies across traditional diagnostic boundaries (40). If NMDAR antibodies are pathogenic outside of encephalitis, then we could expect the psychiatric manifestations to have similar clinical heterogeneity. Nonetheless, only further clinicopathological correlation of SNAps will help determine an accurate picture. Such study may help validate or suggest new directions for receptor-based models of idiopathic psychiatric syndromes.

Alternatively, NSAbs in SNAps may not be directly pathogenic but still are part of the primary disease process, for example, as part of a broader immune and/or inflammatory syndrome which may be IT responsive. A randomized controlled trial could interrogate this, but to be robust would need to be designed and powered to test multiple forms of IT and detect partial responses.

Another possible role for NSAbs in SNAps is as a prognostic marker, thereby allowing disease stratification. This may include likely response to antipsychotic medication, or illness trajectory. Cohort studies would best assess this hypothesis. Suitable populations might include those at ultra-high risk or in the prodromal phase of a psychiatric disorder, in whom such biomarkers are already sorely needed.

A final potential role of NSAb is as part of a secondary process, following a separate primary disease process. The presence of NSAbs in disorders such as herpes simplex encephalitis, Alzheimer's disease, Creutzfeldt–Jakob disease, and other dementia types (18, 41–43) strongly suggests that NSAb production sometimes occurs following neuronal destruction. Nonetheless, "secondary" antibodies can still have pathogenic potential: in dementia, NSAbs may confer a higher risk of psychosis (43), and in patients who have had herpes simplex encephalitis, NSAbs associate with greater cognitive impairment (44).

It is plausible that NSAbs associated with psychiatric disease fall into this "phenotype-modifying" category. For example, NMDAR antibodies may be found in psychosis because the primary pathology has rendered NMDARs immunogenic. Other "immunizing" conditions could include late pregnancy and parturition, around which time numerous immunological rebound changes are understood to occur (45). Indeed NSAbs have been

TABLE 1 | Comparison of position statement on diagnosis of autoimmune encephalitis (AE) and proposed SNAps concept.

	Position paper AE diagnosis (14)	Proposed SNAps concept	
	Definite anti-NMDA receptor encephalitis	**Synaptic and neuronal autoantibody-associated psychiatric syndrome (SNAps)-AE**	**SNAps**
Reasonable exclusion of other disorders			
Clinical features	*Onset <3 months* of 1 of 6 symptom groups 1. Abnormal (psychiatric) behavior or cognitive dysfunction 2. Speech dysfunction 3. Seizures 4. Movement disorder, dyskinesias, or rigidity/abnormal postures 5. Decreased conscious level 6. Autonomic dysfunction or central hypoventilation	*Onset <3 months* of 1 symptom group 1. Abnormal (psychiatric) behavior	*Any onset* of 1 symptom group 1. Abnormal (psychiatric) behavior
Cerebrospinal fluid (CSF)	With or without Pleocytosis OR oligoclonal bands	With or without Pleocytosis OR oligoclonal bands	Absent
Electroencephalogram (EEG)	With or without Focal/diffuse slow or disorganized activity OR epileptic activity OR extreme delta brush	With or without Focal/diffuse slow or disorganized activity OR epileptic activity OR extreme delta brush	Absent
Magnetic Resonance Imaging (MRI)	With or without Changes suggestive of encephalitis	With or without Changes suggestive of encephalitis	Absent
Autoantibody			
• Class • Target	IgG NMDAR GluN1	IgG NMDAR GluN1	IgG OR IgM OR IgA NMDAR GluN1 NMDAR GluN1 + 2 $GABA_AR$ $GABA_BR$ AMPAR LGI1 Caspr2 OR unknown target
Sample required			
• Cell-based assay (CBA) only • CBA and confirmatory test (live neurons or immunohistochemistry)	CSF ± serum Serum	CSF ± serum Serum	Serum ± Absent

NMDAR antibodies are the most frequently identified neural surface antibody in isolated psychiatric syndromes; therefore, NMDAR-AE offers the most useful paradigm. It is possible to make a diagnosis of NMDAR-AE if an isolated psychiatric syndrome of subacute onset is associated with CSF NMDAR (NR1) antibody or in serum if a CBA result is confirmed by testing on neuronal cultures or immunohistochemistry.

A case would be characterized as SNAps if there was a psychiatric syndrome of any speed of onset with serum antibody of any class against a central nervous system neuronal surface target detected on CBA. We argue that cases of NMDAR-AE with only a psychiatric syndrome share aspects of both and could be considered "SNAps-AE." This reflects the isolated clinical syndrome atypical of AE, but paraclinical features typical of AE.

detected in the serum of postpartum psychosis cases (13). These women responded to usual psychiatric care and clinically did not present as having AE, but even in these situations, the value of IT remains to be decided by rigorous trials.

The autoimmune encephalitides have largely been described in association with Abs of the IgG isotype, and typically only IgG is screened for clinically. While the majority of autoimmune disorders are indeed IgG-mediated, numerous instances of IgA- and IgM-mediated diseases exist outside of the CNS, such as IgA pemphigus and autoimmune hemolytic anemia. IgA and IgM NSAbs have been consistently reported in psychiatric disorders (9, 46, 47). Importantly, there is *in vitro* evidence of their pathogenicity (18, 34, 35), and IgA and IgM seropositivity does appear to associate with clinical phenotype in some non-encephalitis conditions (18). We suggest that it is premature to dismiss *a priori* non-IgG NSAbs as irrelevant to the disease. For the purposes of our categorical model, IgA or IgM seropositivity is included in the definition of SNAps, but not of SNAps-AE, implying possible, but not definite, causal relevance.

Animal models could potentially demonstrate the pathogenicity of NSAbs in SNAps, but animal models of AE have been slow to develop and often recapitulate a limited facet of a complex phenotype (48, 49). The abnormal movements, spontaneous seizures, autonomic instability, or psychosis-like behaviors associated with AE are notably absent. Nonetheless, the animal model of NMDAR encephalitis of Planaguma and colleagues (48) appears to be an unintended but plausible model

TABLE 2 | Clinical overlap between symptoms and signs of autoimmune encephalitis (AE) and psychotic disorders.

Clinical symptom/sign	In which AE syndrome?	Observations in psychotic disorders
Seizures	Observed in AE associated with most NSAbs	Epilepsy overrepresented in patients with schizophrenia (odds ratio 11.1) (19)
Cognitive dysfunction	Observed in AE associated with most NSAbs	Observed in schizophrenia across a range of domains. Associated with poor function and clinical outcome (20)
Movement disorders	Observed in AE associated with most NSAbs	9% of antipsychotic-naive patients with schizophrenia have spontaneous dyskinesias; 17% have spontaneous parkinsonism (21)
Catatonia	Most marked in NMDAR-AE but observed in cases of AE associated with VGKC complex antibodies and $GABA_AR$ antibodies	Prevalence in psychiatric patients ranges from 7.6% to 38%. 10–15% of patients with catatonia have a schizophrenia diagnosis (22)
Language disorders	Most marked in NMDAR and AMPAR AE. Catatonic speech signs such as echolalia and palilalia are also common	"Formal thought disorder" is a cardinal feature of psychotic disorders and manifests in disordered speech, sometimes called "schizaphasia"—in some cases not distinguishable from neurological dysphasia (23)
Autonomic dysfunction	Observed in AE associated with most NSAbs	Ambulatory patients with schizophrenia have mean reduced body temperature of 0.2°C (24). Meta-analytical evidence of reduced heart rate variability in psychotic disorders (25)
Hyponatremia	Observed in cases of AE associated with VGKC complex antibodies, particularly LGI1	Occurs in 6% of chronic psychiatric patients (26); polydipsia present in 3–17% of psychiatric patients (27); 40% of psychotic patients admitted with unexplained hyponatremia are not taking antipsychotic medication (28)
Antipsychotic sensitivity including rhabdomyolysis	Observed in NMDAR-AE	Neuroleptic malignant syndrome (rigidity, catatonia, confusion, hyperthermia and rhabdomyolysis) occurs in up to 0.07–2.2% of patients taking antipsychotics (29). Rhabdomyolysis can occur with water intoxication and hyponatremia
Sleep dysfunction	Observed in AE associated with most NSAbs. Particularly marked in NMDAR-AE- and IGLON-5-associated encephalopathy	Reported in 30–80% of patients with schizophrenia. Consistent findings include increased sleep onset latency, diminished slow wave sleep time, and decreased REM latency (30)

of NMDAR antibody-mediated depressive behavior. Future work on an animal model of SNAps should attempt to integrate passive NSAb transfer experiments with established psychiatric animal endophenotypes. For example, in a potential model of psychosis, multiple behavioral and neurophysiological indices such as latent inhibition and mismatch negativity could be assessed alongside the neuronal effects of antibodies (50).

Ultimately, *in vivo* human studies of SNAps will be necessary to elucidate whether NSAbs differentially impact brain function in psychiatric presentations. Functional MRI and magnetic resonance spectroscopy offer equivalent promise in restricted psychiatric presentations as for more fulminant neurological presentations (51, 52). With the development of PET and SPECT ligands for *in vivo* measurement of microglial activation (53, 54) and the function of individual receptor types [e.g., NMDAR (55) and GABAR (56)] or neurotransmitter synthesis capacity (57), it is likely that this methodology can offer insights into the molecular pathology of SNAps. Correlation of clinical improvement with improvement in disease-relevant biomarkers (for example, a perturbed glutamate MRS signal or ketamine-like functional dysconnectivity) following antibody removal would strengthen an argument for *in vivo* pathogenicity in SNAps. Conversely, an absence of differences from seronegative patients with psychiatric disorders on such neuroimaging measures would make it less likely that NSAbs directly affect brain function.

Does the Blood–Brain Barrier (BBB) Have a Role?

As a number of studies have reported similar seroprevalences in NSAbs in individuals with multiple psychiatric diseases and healthy controls, some authors have postulated that disruption of the BBB must be present for NSAbs to be pathogenic (9).

Beyond defining what "BBB disruption" actually means (disruption of tight junctions, permeability to macromolecules, and hyper- or hypofunction of transporter mechanisms have all been suggested), it is difficult to demonstrate *in vivo*. Serum markers, such as calcium-binding glial protein S100B, appear to correlate well with BBB disruption in some conditions but not others (58). Until recently, neuroimaging approaches were only able to reveal gross BBB disruption, but newer dynamic contrast-based techniques may allow for the identification of more subtle impairments (59). The need for a simultaneous lumbar puncture and blood test makes the gold standard test, CSF/serum albumin ratio (Qalb), difficult to obtain in psychiatric practice.

Proxy markers of BBB disruption may have to suffice. Work by Ehrenreich and colleagues have demonstrated that only in the presence of a history of birth complications and "neurotrauma" does NMDAR seropositivity in schizophrenia predispose to more severe neurological symptoms (10). Additionally, drawing on animal work demonstrating that ApoE4 carriers have a chronically

1. RCTs will show antibody-depleting therapies will improve symptoms in SNAps.
2. A proportion of patients with SMI will demonstrate neuronal reactivity and further characterisation of the NSAb will reveal antigenic targets relevant to the pathogenesis of the respective disorder against as yet unidentified targets.
3. Psychiatric phenotype in SNAps will be determined by BBB properties as indexed by parameters such as Qalb.
4. Human in vivo neuroimaging of NMDAR antibody-positive SNAps patients will reveal a perturbation of brain function consistent with NMDAR hypofunction, as indexed by an increased brain glutamate on MR spectroscopy, or a ketamine-like functional dysconnectivity on resting-state fMRI.
5. Cases of SNAps associated with NSAbs of IgM or IgA isotype will show similar evidence of pathogenicity to cases associated with IgG antibodies, including symptomatic response to antibody-depleting immunotherapies.
6. SNAps will respond specifically to drugs that target the affected molecular system compared to matched seronegative cases e.g. glutamatergic modulators in NMDAR-Ab+ SNAps.

FIGURE 2 | Experimentally testable hypotheses relating to the pathogenicity of neural surface antibodies (NSAbs) in synaptic and neuronal autoantibody-associated psychiatric syndromes (SNAps).

"leaky" BBB, Hammer and colleagues demonstrated that patients with schizoaffective disorder show a higher than expected co-occurrence of NMDAR Abs and ApoE4 carrier status compared to patients with other psychiatric diagnoses and healthy controls, suggesting that in seropositive individuals, a leaky BBB confers susceptibility toward a schizoaffective phenotype (60).

We suggest that Qalb or dynamic contrast-enhanced imaging is used in prospective studies of SNAps where possible, to explore the potentially disease-mediating role of the BBB (see **Figure 2**).

HOW CAN WE INTEGRATE THESE FINDINGS INTO PSYCHIATRIC PRACTICE?

Who Should Be Tested for NSAbs, and Which Tests Should Be Requested?

The need to identify cases of possible AE at an early stage in psychiatric practice is not controversial. However, the breadth of screening is a matter of active discussion. Many cases of definite AE will, even early in their course, have clinical features that prompt presentation to medical services where the likelihood of neurological evaluation including NSAb testing is higher. However, in diseases such as NMDAR-AE, psychiatric symptoms may predominate for the first weeks (or sometimes months) of the illness (61) and occasionally may not progress any further (62) (this is a situation that we have referred to as SNAps-AE in **Figure 1** and **Table 1**).

It is likely that many of these cases satisfy current criteria for "possible AE" as per Graus et al. (14), and some that eventually go on to receive a diagnosis of "probable" or "definite" NMDAR-AE are initially diagnosed as having a primary psychiatric disorder and are at risk of suboptimal management.

Although prior to the discovery of AE it is likely that some cases spontaneously remitted (63–65), there is clear, albeit necessarily observational, evidence that short- and long-term medical and neuropsychiatric prognosis is linked to early clinical identification of AE and timely instigation of IT, frequently before the antibody status is known (66). Therefore, we suggest that cases of FEP or severe mood disturbance such as mania or severe depression, here grouped as "severe mental illness" (SMI), with subacute onset (less than 3 months), should be regarded as "yellow flag" cases, at risk of AE, and undergo testing for a serum NSAb panel. Cases with both yellow and additional "red flag" clinical features suggestive of AE should be more obvious to differentiate clinically, and likelihood of detection of relevant NSAb detection is high (see **Figures 3** and **4**).

For cases of SMI with (a) an onset of longer than 3 months, (b) in relapse, or (c) in a chronic phase—referred to as "gray flag" cases—there is growing evidence to suggest that many of these cases will have NSAbs without differentiating clinical features (67), and that many of these may stand to benefit from IT (68). However, blanket screening of such cases outside of clearly defined research programs risks generating uncertainty insofar as antibody-positive patients may be identified before a clear and evidence-based understanding of their optimal management is known. Therefore, we would strongly advocate screening in these cases to be based within research settings (see **Figure 5**).

How Should Psychiatrists Understand a Positive Test?

Local experience has found that NSAb screening in subacute onset first-episode presentations and more long-standing instances of serious mental illness (SMI) helps to identify cases of AE that will benefit from IT. However, as with any screening test, there will be positive results of less clear clinical significance. For example, in

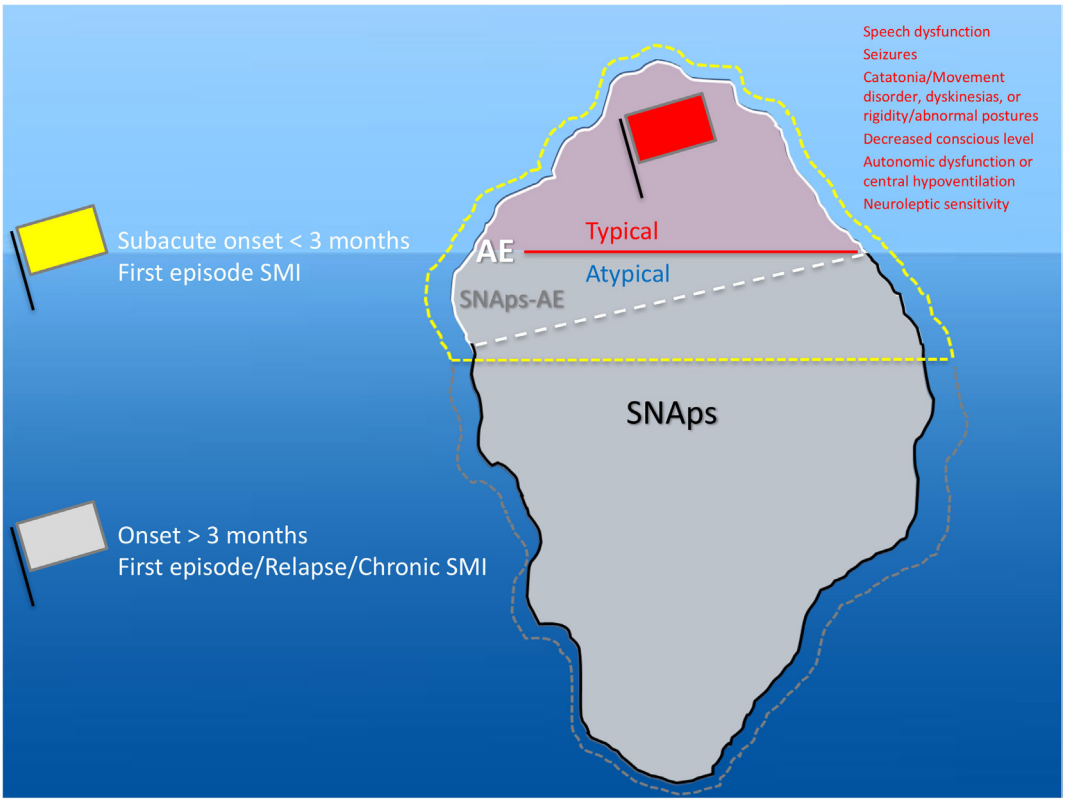

FIGURE 3 | Relationship between severe mental illness (SMI) and the neural surface antibody (NSAb) seropositivity iceberg: psychiatrists will see both autoimmune encephalitis (AE), and synaptic and neuronal autoantibody-associated psychiatric syndromes (SNAps), and overlap areas. In practice, this means that all first-episode SMI with a subacute onset should be regarded as a yellow flag for AE and should be screened for relevant NSAbs (see **Figure 4**). If these cases have red flag clinical features, then there should be a low threshold for further investigations and liaison with neurology colleagues (see **Figure 5**). Screening cases of SMI with a longer onset or treatment resistance will yield cases with NSAbs; however, the management of these cases is less certain. There is an imperative for further well-designed research studies to characterize the biology and immunotherapy responsiveness of these cases.

Yellow Flag ± Red Flag cases:

CBA:

NMDAR
LGI1
Caspr2
$GABA_BR$
$GABA_AR$
AMPAR

Consider:

IHC and/or neuronal staining if available and if fixed, permeabilised CBA system

FIGURE 4 | Initial serum neural surface antibody panel recommended in subacute onset first-episode severe mental illness, at risk for autoimmune encephalitis, and to consider in cases with longer onset.

those in whom illness does not clearly satisfy criteria for AE, or is spontaneously remitting, or is responding well to psychological or psychotropic approaches.

We would advocate that, where possible, cases of SNAps are assessed in detail in a specialist joint neurology–psychiatry clinic. Such services will often be based at research-associated regional neuroscience centers with access to neuropsychology, brain imaging (MRI and increasingly PET), and lumbar puncture/CSF analysis expertise.

We suggest taking a pragmatic approach incorporating available resources and tolerability to investigation. We hope this could prompt clinicians to consider how psychiatric services could incorporate the developments from this rapidly growing field. Specialist clinical work closely allied with multidisciplinary research units will be central in identifying biomarkers of future classificatory and clinical relevance (summarized in **Figure 5**).

Who Should Receive NSAb-Modifying IT?

While the evidence for short- and long-term benefits for IT in AE is clear, clinicians managing SNAps have greater equipoise. If NSAbs in these cases are indeed pathogenic, care must be taken to minimize the duration of untreated autoimmune CNS disease. However, the medical risks of IT are not trivial, and pathogenicity of NSAbs in SNAps has not been demonstrated definitively. Although open-label data (68) and many case reports (7, 69–71) show promising effects of IT in cases of psychosis, these results do not exclude placebo response or regression to the mean. Duration of adequate treatment and optimal treatment of relapses is not clear.

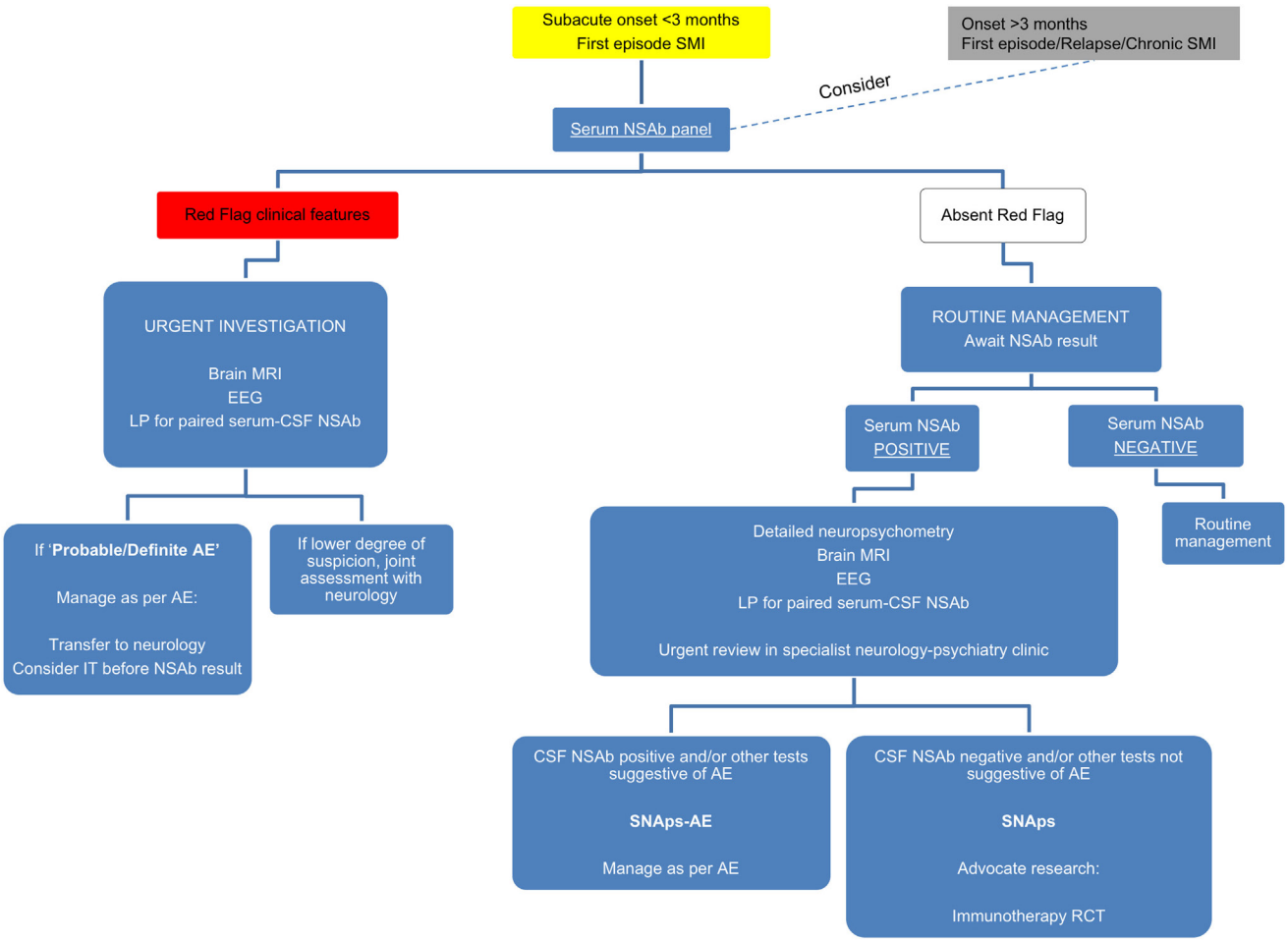

FIGURE 5 | Suggested algorithm for neural surface antibody (NSAb) testing and further management in the context of severe mental illness (SMI).

In our experience, there is another potential hazard that can arise when discussing the possible relevance of NSAbs with seropositive patients: in some cases, the patient and/or their carer may develop unhelpful biomedically reductionist illness beliefs (e.g., "I don't have a mental health problem. I have encephalitis. That's what the antibody test shows.") which prevent engagement in vital psychosocial interventions.

Given such equipoise, there is a clear and pressing need for adequately powered and robustly designed randomized controlled trials to determine whether SNAps cases benefit from IT.

What Other Treatments Should Be Considered?

Identifying SNAps may also allow better targeting of existing treatments. For example, sensitivity to antipsychotic medication and propensity to neuroleptic malignant syndrome-type complications occur relatively commonly in AE and overlap areas (72, 73). In some cases of AE, patients experience treatment-resistant psychiatric symptoms despite IT, and several reports note the efficacy of electroconvulsive therapy (ECT) in this situation (74). The mechanism of this, in the context of an identified molecular pathology, is intriguing. From a clinical perspective, it implies that ECT may be a specific intervention worth considering early in SNAps.

Also intriguing is early evidence of SNAps acting as a paradigm for intervention with rational molecular-based psychopharmacology. For example, Heresco-Levy and colleagues found that the NMDAR co-agonist D-serine could improve psychopathology in a case of chronic treatment-resistant schizophrenia with NMDAR antibodies (75). This approach could be widened to other antibodies implicated in SNAps.

CONCLUSION

The description of AE syndromes caused by NSAbs has profoundly impacted neurological practice and has invigorated neuroimmunology as a basic and clinical science. The extent to which the paradigm—of autoantibodies affecting brain function and behavior—has relevance for clinical psychiatry is a matter of considerable debate.

We have introduced the concept of SNAps as an attempt to clarify some of the issues in this sometimes confusing field. By

making the distinction between AE and isolated psychiatric presentations associated with NSAbs, we hope to encourage the latter as an important focus of research in its own right. It is now generally accepted that many psychiatric disorders may be comprised of subgroups that are phenotypically similar but heterogeneous in their etiologies. We believe that the category of SNAps offers a well-defined candidate for one such subgroup, and we have offered suggestions as to how this hypothesis may be tested.

Research on SNAps will of course be informed by the AE literature to date, particularly in terms of pathogenic mechanisms. In this article, we have outlined the increasingly compelling evidence that NSAbs may have pathogenic potential in SNAps: we are clear however that considerable further work needs to be done in this area before generalized statements can be made about pathogenicity. Further, we would suggest that much of this work needs to be led by psychiatrists. The nuances of psychiatric signs and symptoms are often ignored within the neurological literature, and while this remains the case, a "psychiatric phenotype" associated with NSAbs will remain elusive.

The possibility that the SNAps concept may delineate a subgroup of psychiatric patients with a differential treatment response (including a potential IT response) remains an exciting focus of future research. In an area of medicine where novel therapies are relatively rare and where immune therapies are increasingly under the spotlight, SNAps represent a focus for therapeutic studies that could be potentially transformative for the field.

AUTHOR CONTRIBUTIONS

AA-D, TP, AL, and BL conceived the article. AA-D, TP, and AL drafted the text. AA-D and TP prepared the tables and figures. BL revised the text, tables, and figures. All the authors read and approved the final manuscript.

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OPINION
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The Clinical Challenge of Autoimmune Psychosis: Learning from Anti-NMDA Receptor Autoantibodies

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INTRODUCTION

Schizophrenia is a heterogeneous and complex psychiatric disorder affecting up to 1% of the population worldwide (1). Although the precise development of schizophrenia is not yet fully understood, it is now admitted to be underpinned by the entanglement of genetic, environmental, and immuno-inflammatory factors. Among schizophrenic patients, it is assumed that at least 30% will not respond to conventional antipsychotics (2). These data underlie the importance of precision medicine in psychiatry, in other words, the need to identify subgroups of patients with specific signatures who will benefit from treatment targeting these specific biological pathways. Reviving an area of exploration older than a century, recent and abundant literature emphasized the importance of the immune system in the pathophysiology of schizophrenia [for review, see Ref. (3)].

In psychiatry, the link between psychotic disorders, particularly schizophrenia, and immune system deregulations, including autoimmunity, is an old concept that regained strong support; thanks to the better characterization of brain inflammation-induced psychotic symptoms and autoimmune encephalitis (3). Moreover, recent epidemiological studies evidenced a high prevalence of multiple autoimmune diseases in schizophrenic patients (4). In a recent meta-analysis, autoantibodies against neuronal receptors have indeed been identified in the circulation of patients with neuropsychiatric disorders, constituting, today, one of the hottest topics in psychiatry (5–10). This new era fosters debate on (i) how to explain the increased burden of autoimmunity in schizophrenia, (ii) what could be the precise target(s) and the pathogenic implication(s) of the autoantibodies on the disease onset and development, (iii) how to define patient subgroups carrying such autoantibodies to facilitate their diagnosis, and (iv) how should we treat these patients using appropriate protocols such as immunotherapy (i.e., corticotherapy or plasmapheresis). Several neurological autoimmune diseases are, for instance, efficiently treated once autoantibodies against neurotransmitter receptors and ion channels have been identified (11, 12). The discovery of the autoimmune encephalitis due to anti-*N*-methyl-D-aspartic acid receptor (NMDAR) has greatly revived the relationship between autoimmunity and psychosis. Indeed, directed against the NMDAR *N*-methyl-D-aspartate receptors antibodies (NMDAR-Ab), the autoantibodies are directly responsible for the psychotic symptoms and catatonia, followed by profound neurologic deterioration (13, 14). In patients with schizophrenia, the prevalence and clinical significance of circulating NMDAR-Ab remains controversial with detection prevalence rates varying considerably between studies (15–27). Inspite of such imprecisions, defining and isolating seropositive patients, suffering from "autoimmune psychosis," is a major challenge for appropriate treatments. In this review, we focus our attention on the potential elements possibly helping to define an "autoimmune psychosis" subgroup of schizophrenic patients. Furthermore, we outline some of the specific clinical presentation of these patients that will be of great importance to optimize the diagnostic and subsequent therapies.

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AUTOIMMUNITY AND PSYCHOSIS: ROOTS

Autoimmune disorders occur after the failure of self-recognition processes with consequent production of pathogenic autoantibodies directed against specific or multiple organs. They are heterogeneous disorders, representing more than 80 different diseases. Several risk factors contribute to the high prevalence of autoimmunity including genetic and environmental ones and their interplay. Within this context, the "immunogenetic" contribution is largely dominated by the major histocompatibility complex (MHC) genetic diversity and, at a lesser extent, by mutational events affecting cytokines encoding genes (28). On the other side, environmental stressors are also of major importance for the onset of autoimmunity. For example, infections during pregnancy or in childhood are associated with an increased risk of type 1 diabetes (29). Moreover, and within the GxE context, it has been postulated that the risk of autoimmunity is enhanced through the perturbation of gut microbiota or dysbiosis (30, 31). This dysbiosis seems to be the origin of the emergence of different autoantibodies, even if the exact mechanisms involved are still under debate (32).

In psychosis settings, similar epidemiological associations with early infections, autoimmune disorders, and dysbiosis have pinpointed the possible existence of an autoimmune psychosis subgroup in schizophrenia. Maternal exposure to influenza or toxoplasmosis during pregnancy has been associated with schizophrenia. Childhood autoimmune diseases as well as inflammatory diseases, such as asthma, are known to be associated with an increased number of psychotic experiences in adolescence but also with an increased incidence of schizophrenia in the adulthood. Moreover, in patients with autoimmune conditions, the risk to develop schizophrenia increases linearly with the number of severe infectious episodes (4). The other way around, patients with schizophrenia and their first degree relatives, also exhibit a higher prevalence for autoimmune disorders (33). Last, associations between autoimmunity, gastrointestinal symptoms, and dysbiosis are starting to emerge (34). These data, along with the strong association between the interindividual immunogenetic background and the whole array of brain and peripheral autoantibodies, in at least a subgroup of schizophrenic patients, led us to propose the concept of "autoimmune psychosis." Accordingly, our goal is to review the evocative characteristics that should prompt the search of autoantibodies in front of a patient, in particular, in cases of first episode, resistant ones, or schizophrenia with neurological comorbidity.

BIOLOGICAL AND CLINICAL FEATURES OF PATIENTS WITH AUTOIMMUNE PSYCHOSIS

Autoimmune Psychosis: Genetic and Environmental Risk Factors

Several genome-wide association studies (GWAS) confirmed an association between the MHC region (chromosome 6) and psychosis (35, 36). Moreover, a recent landmark GWAS analysis produced by the largest consortium on genetics of schizophrenia has shown that, like in autoimmune disorders, the MHC region was the most strongly associated (best *p*-value: MHC-region: $p = 3.86e{-}32$; 36,989 cases and 113,075 controls) (37, 38). The consequences of these mutations are still to be fully understood because some of them are found in non-coding region. However, in a matter of interest, the MHC region include the human leukocyte antigen (HLA) cluster, which is the most polymorphic and gene-dense genomic part of the human genome (39) encompassing more than 250 genes (4 Mb) and 14,000 alleles as reported to date (IMGT/HLA database; http://www.ebi.ac.uk/imgt/hla). Governing the specific adaptive immune responses, the HLA molecules were widely explored in disease-association studies (40) especially concerning those classified as autoimmune disorders (40–42). Even if more studies are needed to understand the link between immunogenetic and psychosis, disentangling such diversity might help to delineate the concept of autoimmune psychosis, at least on a genetic point of view.

On the other side, although data on gene–environment interactions are scarce, several environmental risk factors have been associated with schizophrenia and would be worth testing with MHC/HLA haplotypes. In particular, the occurrence of infections by pathogens such as, *influenza*, *herpes simplex type 2*, *cytomegalovirus*, and *Toxoplasma gondii* and/or increased C-reactive protein plasma levels during pregnancy are known to be associated with an increased risk of developing schizophrenia in adulthood (43–45). In the same context, hospitalization for infection increased the risk of schizophrenia by 60%, and there is a dose–response relationship between the number of hospital contact with infection and psychosis (46). Altogether, the reported deep intricacies between infection and autoimmunity, either under an additive or a more complex framework, with a consequent risk of psychosis, reinforce the concept of autoimmune psychosis concept (46).

More than separated risk factors, actual studies argue for a complex interaction between genetic risk factors conferring susceptibility to environmental injuries. For example, polymorphisms of the innate system genes, like IL1B, IL6, TNF alpha, or interferon, will lead to a bigger release of pro-inflammatory cytokines in response to environmental stressors (47).

In summary, immunogenetic dissection especially of the MCH/HLA region according to the natural history of deleterious immune processes including early infection and/or autoimmune features might be a promising route to better understand the interactions between gene and environment.

Autoimmune Psychosis: Peripheral Biomarkers from Dysbiosis to Autoantibodies?

Similar to autoimmunity, dysbiosis is found in patients with psychosis (48). The intestinal microbiota seems essential for the development and functioning of the nervous central system, shedding light on the concept of a gut–brain axis (49). Dysbiosis is a well-known cause of increased intestinal permeability (so-called "leaky gut") in schizophrenia (50). This increased intestinal permeability is demonstrated by the high circulating

levels of CD14, a biomarker of bacterial translocation (49). The release of such pro-inflammatory innate sensor in a repetitive manner could allow, under the framework of particular genetic framework (HLA), to the breakdown of immune tolerance with consequent emergence of autoantibodies. Along this line, various autoantibodies have been found in subgroups of schizophrenic patients. For example, increased anti-bovine casein antibodies have been found in psychosis (51). Meta-analysis found threefold to fourfold times more anti-transglutaminase and anti-gliadine autoantibodies in patients with schizophrenia than in general population. Autoantibodies, specifically against the central nervous system, have also been found in schizophrenic patients. These patients have a higher prevalence of circulating antibodies against hippocampus and hypothalamus as compared to healthy control (52). A recent meta-analysis has confirmed and specified these results, showing that schizophrenic patients are three times more likely to have high levels of anti-glutamate receptor antibodies, *N*-methyl-D-aspartic acid receptor (NMDAR), compared to controls (22). The latter being of major importance. For the first time, they might make the bridge between autoimmune psychosis and the glutamate theory of psychosis and, doing so, sheds light on the pathophysiology of autoimmune psychosis.

In summary, there is a whole array of peripheral and central autoantibodies in schizophrenia, which deserve further exploration to explore their pathogenic role and to describe possible associated clinico-biological signatures, helping to more precise the concept of autoimmune psychosis.

Autoimmune Psychosis: Clinical Picture?

We have seen that, among the heterogeneous group of schizophrenic patients, it is possible to hypothesize the existence of an autoimmune psychosis subgroup. The question is now in front of which clinical history or symptoms should we search for autoimmunity (53). The literature is still heterogeneous in the field and some have found no differences between patients (17, 20, 23). However, based on a French cohort of patients with psychiatric symptoms and autoantibodies against NMDA-R, we described clinical characteristics of patients that should lead to search of biological markers of an autoimmune psychosis [for details, see Ref. (54)]. While the mean age of onset in schizophrenia is 25–35 years old, we observed the first episode of autoimmune psychosis to occur around the 24th years of life (55). It is well known that schizophrenia is associated with the presence of neurological soft signs (56). More than that, we have been able to put forward that 50% of the patients with autoantibodies against NMDA-R had neurological symptoms including headaches, disorientation, paresthesia, anterograde amnesia, or abnormal movements. These results are in agreement with others who have also found neurological comorbidities in autoimmune psychosis cases (26, 57). Catatonia is a complex neuropsychiatric syndrome related to schizophrenia in 20% of cases (58). Its exact physiopathology is still unknown but seems underpinned by a deregulation between glutamatergic and GABAergic signaling (59). Catatonia, schizophrenia, and NMDAR-Ab have been extensively associated in the literature, which might indicate catatonia as a sign of autoimmune psychosis (60–62).

In summary, in front of an early age at onset of psychosis, discrete neurological symptoms, and catatonia, search for autoantibodies should be performed.

Treatment Response

More than 30% of schizophrenic patients are resistant to conventional antipsychotics (63). Among them, 41% exhibited biological signs of immune activation (64). For example, treatment-resistant patient has been strongly associated with increased cytokines level (65–69). It has also been reported the specific presence of NMDAR-Ab in treatment-resistant patients (70). Finally, we have also been able to underlie the tight link between presence of NMDAR Ab and neuroleptic intolerance (54). These data seem to indicate a different pathophysiology, not related to the classical dopaminergic hypothesis, in patients with an autoimmune psychosis.

All these arguments have led to propose that all treatment-resistant/intolerant patients should have an autoantibodies screening, and particularly NMDAR-Ab, as a part of the diagnostic process.

In summary, based on epidemiological studies, genetic and biological biomarkers but also environmental risk factors, there are many arguments to suggest, that among schizophrenia, it is useful to ensure the identification of a subgroup of autoimmune psychosis. It is possibly characterized by (i) history of early infections or severe stress, (ii) autoimmune or infections during childhood or early adulthood, (iii) clinical presentation with the presence of gastrointestinal/neurological symptoms, catatonia, and (iv) presence of one or several autoantibodies associated with schizophrenia leading to a resistant form of schizophrenia (**Figure 1**).

PERSPECTIVES FOR APPROPRIATE TREATMENTS

Now that we are able to isolate autoimmune psychosis, future clinical trials should evaluate if different types of immunotherapy may be helpful, in particular, involving those routinely used in immune/autoimmune-related common disorders such as cortisone pulse therapy, intravenous immunoglobulins, plasmapheresis, humanized monoclonal antibodies (e.g., rituximab), or immunosupressor (e.g., cyclophosphamide).

Among the new therapeutic approaches already used with success in autoimmune psychosis, three can be considered (10). The first one is based on the use of immunotherapies from non-selective immunosuppressive ones like minocycline, steroids, plasma exchange, or cyclophosphamide to a more selective one like the anti CD-20 monoclonal antibody Rituximab (71). Schematically, CD-20 is a potent marker of B-lymphocytes. By targeting CD-20, Rituximab will be able to inhibit B lymphocyte and, doing so, to prevent antibodies circulation (72). The second approach also focuses on antibodies and has been proposed by Diamond and colleagues. They propose to use D-peptide in order to prevent pathogenic antibodies to reach their target, theoretically, without affecting receptor function (73). It has been tested in mice model and seems to indeed prevent autoantibodies

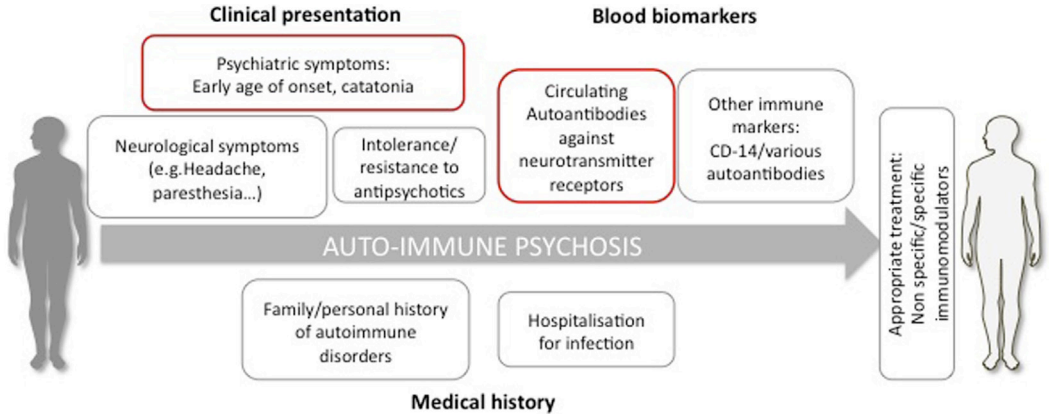

FIGURE 1 | **Diagnostic elements possibly supporting the definition of autoimmune psychosis**. The diagnosis is suspected if the patient presents psychiatric symptoms (e.g., early age of onset, catatonia), neurological signs, resistant or intolerance to antipsychotic treatment, history of autoimmune disorder, and severe infections. The diagnosis relies on the detection in the circulation of autoantibodies and, particularly, directed against neurotransmitter receptors, such as the glutamate NMDA receptor.

effect (73). The last one is more specific and consists in the use of a co-agonist of the NMDAR, the D-serine. It has been used by Heresco-Levy in an open label case study and has shown a dramatic improvement in the psychosis symptomatology (74). The potential mechanism of action behind D-serine is that it will enhance NMDAR activity by increasing the frequency of channel opening to counter act the action of the antibodies.

CONCLUSION

Today, the main problem of the so-called autoimmune psychosis is that patients are not diagnosed. In order to help the physician to evocate it and to consider an autoantibody screening, we propose to gather elements enabling to build a risk score for autoimmune psychosis. This score should take into account the personal and/or familial history of early infections, autoimmune disorders, the demographic and clinical characteristics, and the presence of blood biomarkers such as CD14 and a panel of autoantibodies (anti-bovine casein, anti-transglutaminase, anti-folate receptor, anti-central nervous system, etc.). Of course, such risk score will need to be built and validated, as it should enable to allow early detection of autoimmune psychosis to prevent misdiagnosis with long-term deleterious consequences. Clinical trials targeting specific mechanisms and performed in homogeneous subgroups of autoimmune psychosis will allow to test and to select the most efficient treatment. More than that, the discovery of *N*-methyl-D-aspartate receptors antibodies (NMDAR-Ab) is also of major importance for a better comprehension of the neurobiological basis not only of autoimmune psychosis but also psychosis in general. We hope that, in a few years, personalized psychiatry will become the rule and not the exception anymore.

AUTHOR CONTRIBUTIONS

PE made the bibliography and wrote the article. ML, LG, and RT have corrected the manuscript.

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REVIEW
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Neurovascular Unit Dysfunction and Blood–Brain Barrier Hyperpermeability Contribute to Schizophrenia Neurobiology: A Theoretical Integration of Clinical and Experimental Evidence

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Schizophrenia is a psychotic disorder characterized by delusions, hallucinations, negative symptoms, as well as behavioral and cognitive dysfunction. It is a pathoetiologically heterogeneous disorder involving complex interrelated mechanisms that include oxidative stress and neuroinflammation. Neurovascular endothelial dysfunction and blood–brain barrier (BBB) hyperpermeability are established mechanisms in neurological disorders with comorbid psychiatric symptoms such as epilepsy, traumatic brain injury, and Alzheimer's disease. Schizophrenia is frequently comorbid with medical conditions associated with peripheral vascular endothelial dysfunction, such as metabolic syndrome, cardiovascular disease, and diabetes mellitus. However, the existence and etiological relevance of neurovascular endothelial dysfunction and BBB hyperpermeability in schizophrenia are still not well recognized. Here, we review the growing clinical and experimental evidence, indicating that neurovascular endotheliopathy and BBB hyperpermeability occur in schizophrenia patients. We present a theoretical integration of human and animal data linking oxidative stress and neuroinflammation to neurovascular endotheliopathy and BBB breakdown in schizophrenia. These abnormalities may contribute to the cognitive and behavioral symptoms of schizophrenia *via* several mechanisms involving reduced cerebral perfusion and impaired homeostatic processes of cerebral microenvironment. Furthermore, BBB disruption can facilitate interactions between brain innate and peripheral adaptive immunity, thereby perpetuating harmful neuroimmune signals and toxic neuroinflammatory responses, which can also contribute to the symptoms of schizophrenia. Taken together, these findings support the "mild encephalitis" hypothesis of schizophrenia. If neurovascular abnormalities prove to be etiologically relevant to the neurobiology of schizophrenia, then targeting these abnormalities may represent a promising therapeutic strategy.

Keywords: schizophrenia, blood–brain barrier, neurovascular unit, endothelial cell, neuroinflammation, oxidative stress, nitric oxide synthase, endothelial nitric oxide synthase

INTRODUCTION

Schizophrenia is a pathoetiologically heterogeneous psychotic disorder characterized by delusions, hallucinations, negative symptoms, as well as behavioral and cognitive dysfunction. Current evidence suggests that schizophrenia involves complex interrelated mechanisms that influence immune, inflammatory, oxidative, neurotransmitter, and genetic pathways (1, 2). We previously reviewed the evidence implicating neuroinflammation in the neurobiology of schizophrenia, even during first-episode psychosis (1, 3). Neuroinflammation may contribute to white matter structural and functional disconnectivity, causing symptoms of schizophrenia (3). Neuropathological, biomarker, and genetic studies have documented numerous inflammatory abnormalities in individuals with schizophrenia, including microglial activation and proliferation (MAP), pro-inflammatory cytokine upregulation, and abnormal peripheral immune cell counts (1). Human PET imaging of microglial activation utilizing translocator protein (TSPO), including the second-generation TSPO radiotracer, in individuals with first-episode psychosis and recent-onset schizophrenia has yielded conflicting results. While some studies showed no alteration in TSPO ligand binding or expression (4–6), several others found it to be increased (7–9). The inconsistent findings may result from several factors. First, TSPO expression is not only selective to microglia but also includes other cells such as astrocytes and vascular endothelial cells (10). Thus, the potential negative contribution of astroglial loss or vascular endotheliopathy to TSPO expression in a subset of individuals with recent-onset schizophrenia cannot be totally excluded. Second, we suggest that central TSPO ligand binding may not be a reliable surrogate marker for low-grade neuroinflammation (10) that is typically documented in postmortem brain tissue of subjects with schizophrenia (3). Indeed, reduced TSPO binding in the middle frontal gyrus was found in individuals with recent-onset schizophrenia who were also documented to have elevated pro-inflammatory cytokines levels in both peripheral and central tissues (10). Furthermore, schizophrenia-relevant behavioral abnormalities in infection-mediated neurodevelopmental mouse model were also associated with reduced central TSPO binding despite increased pro-inflammatory cytokine levels (10). Together, these findings suggest that the lack of increased TSPO expression or ligand binding by human PET imaging in first-episode psychosis and recent-onset schizophrenia may not reliably exclude the presence of low-grade neuroinflammatory process (10).

Pro-inflammatory cytokines are thought to contribute to the pathophysiology of primary psychiatric disorders, including schizophrenia (1, 11). A meta-analysis of 40 studies including 2,572 schizophrenia patients and 4,401 controls revealed consistent elevation of serum interferon gamma (IFN-γ), TNF-α, IL-12, and sIL-2R levels in patients with chronic schizophrenia, independent of disease activity (trait markers). In addition, positive correlations were detected between elevated serum IL-6, IL-1β, and transforming growth factor beta levels and disease activity (state markers for acute psychosis) (12). Another meta-analysis found a positive correlation between increased CD4+ T-cell counts and acute psychosis in individuals with schizophrenia (13). More recently, a relative increase in naïve B-cells, natural killer cells, and monocyte counts was reported in those with schizophrenia compared to healthy controls (14). This study also showed a relative decrease in the number of CD4+ memory and human leukocyte antigen (HLA)-DR+ regulatory T-cells, which correlated with the severity of neurocognitive deficits and negative symptoms (14). Furthermore, peripheral blood mononuclear cell cultures derived from individuals with schizophrenia produced higher amounts of IL-8 and IL-1β, either spontaneously or in response to LPS stimulation, suggesting that activation of classical peripheral monocytes can contribute to the pathophysiology of schizophrenia (15).

Oxidative stress occurs in chronic and new-onset schizophrenia (1, 2, 13, 16–18). Oxidative stress markers are found in peripheral blood, neutrophils, red blood cells (RBCs), platelets, cerebrospinal fluid (CSF), and brain tissue (13, 17). Certain oxidative and antioxidative changes in schizophrenia, such as reduced catalase levels in RBCs and plasma, are state dependent and reflect disease progression, whereas others such as decreased soluble superoxide dismutase-1 levels in CSF and RBCs appear to be trait dependent (19). Human and animal studies have indicated a reciprocal cause–effect relationship between oxidative stress and neuroinflammation (1, 3).

Multiple neuropathological and neuroimaging studies have established the effects of neuroinflammation and oxidative stress on the neurovascular unit and blood–brain barrier (BBB), especially in neurologic disorders with comorbid psychiatric symptoms (16). These disorders include epilepsy, stroke, traumatic brain injury, multiple sclerosis, and Alzheimer's disease (16). However, whether neurovascular endothelial dysfunction and BBB hyperpermeability contribute to the neurobiology of schizophrenia, causing behavioral and cognitive symptoms, remains less clear.

There is growing clinical and experimental evidence that vascular endothelial dysfunction and BBB hyperpermeability do occur in a subset of individuals with schizophrenia. However, to date, there has been no systematic attempt to synthesize and analyze the extant literature of neurovascular unit dysfunction and BBB hyperpermeability in schizophrenia. Here, we aimed to characterize the human evidence by performing a systematic review of neuropathological, neuroimaging, serological, CSF, and genetic studies relevant to this effect (**Table 1**).

SEARCH STRATEGY AND METHODS

We performed a systematic electronic search for records indexed within MEDLINE, EMBASE, PsycINFO, or Web of Science to identify potentially eligible published peer-reviewed journal articles studies from January 2009 through February 2017. We included studies that met the following eligibility criteria: (a) neuropathological, neuroimaging, endothelium-dependent flow, cerebral perfusion or flow, serological, CSF, metabolic, and genetic studies that provided data on (b) neurovascular unit function or vascular endothelial function or BBB permeability AND (c) in individuals with schizophrenia (**Table 1**). We also searched for studies that met the following criteria (a) "schizophrenia AND BBB" or "schizophrenia AND neurovascular unit," or

TABLE 1 | Human and experimental data potentially linking neuroinflammation, oxidative stress, and genetic factors to clinical, laboratory, imaging, and pathological findings suggestive of neurovascular unit dysfunction and blood–brain barrier hyperpermeability in schizophrenia.

	Summary of clinical, laboratory, imaging, and pathological findings	Mechanisms		Genetic factors
		Oxidative stress	Neuroinflammation	
Neurovascular unit dysfunction	Cerebral hypoperfusion (51, 59–62) ↓RH-PAT index <1.67 indicative of vascular endothelial dysfunction (24, 26) Epidemiological studies associating schizophrenia with peripheral vascular endothelial dysfunction (23–26) Vacuolar degeneration of neurovascular endothelial cells and astroglial end-feet processes as well as basal lamina abnormalities in postmortem PFC (31)	↓ eNOS activity-dependent oxidative endothelial effects (16, 44, 45, 53–57): – ↓ Endothelial vasodilator NO – ↑$ONOO^-$ – Endothelial oxidative injury ↓ Cerebral blood flow and ↓ vascular reactivity	Astroglial cell activation and loss (1, 3, 16) ↓ AQP4 (16, 30) MAP (1, 3, 7–9, 16, 79, 82–84) Pro-inflammatory cytokines (11, 91–94) ↑ MMPs (18, 85–87)	*eNOS* $T^{-786}C$ (24) COMT Val allele (23–26) MTHFR T allele (26) ↓ Endothelial expression of genes involved in ion transport, cell proliferation, and adhesion (28)
Blood–brain barrier hyperpermeability	↑ S100B in blood, CSF, brain tissue (1, 81) CSF abnormalities (29, 32, 33): – ↑"CSF: serum albumin" – ↑ Intrathecal synthesis of IgG, IgM, IgA – ≥4 OCB – Mild pleocytosis ↑ Serum levels of vascular endothelial adhesion molecules, i.e., sP-selectin, sL-selectin, integrin αIIbβIIIa receptors on platelets (34–36) ↑ serum VEGF (40) ↓ VEGF receptor 2 expression in postmortem PFC (41) ↓ AQP4 expression in postmortem anterior cingulate gyrus (75)	eNOS-independent direct oxidative endothelial injury: – ↑ MMPs (66) – E-cadherin (44) – Altering endothelial tight junction and cytoskeleton proteins (44, 67, 68) – Inducing endothelial NR1 expression? (69) – Impairing mitochondrial oxidation (70)	Astroglial loss (1, 3, 16) ↓ AQP4 (16, 30, 75, 76) MAP (1, 3, 16, 79, 83, 84) Effects of pro-inflammatory cytokines: – Direct endothelial injury (4, 16) – Upregulating MMPs (18, 85–87) – Upregulating endothelial adhesion molecules such as ICAM-1, VCAM-1 (91–94) – Upregulating VEGF (40) – Vascular endothelial mitochondrial oxidative injury (65, 95) ↓ Ndel1 activity (96, 97) ↑ Bradykinin (29, 44, 74, 96–99) ↑ ACE activity in serum, CSF, brain (100–103)	NDST3 polymorphism—gene involved in heparan sulfate metabolism (34, 39)

ACE, angiotensin I-converting enzyme; AQP4, aquaporin 4; CSF, cerebrospinal fluid; COMT, catechol-O-methyltransferase; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1; Ig, immunoglobulin; MAP, microglial activation and proliferation; MMP, matrix metalloproteinase; MTHFR, methylenetetrahydrofolate reductase; Ndel1, nuclear distribution E like-1; NO, nitric oxide; NR1, NMDA receptor subunit 1; OCB, oligoclonal bands; $ONOO^-$, peroxynitrite; PFC, prefrontal cortex; RH-PAT, peripheral arterial tonometry; sP, soluble P-selectin; sL, soluble L-selectin; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

"schizophrenia AND endothelial" AND any of the following key words: (b) neuroinflammation, microglia activation, cytokines, matrix metalloproteinases (MMPs), astroglia, inflammation, adhesion molecules, oxidative stress, reactive oxygen species (ROS), endothelial nitric oxide synthase (eNOS), cerebral perfusion or flow, bradykinin, or angiotensin I-converting enzyme (ACE) (**Tables 1** and **2**). We also present a theoretical integration of human and experimental data that potentially relate oxidative stress and neuroinflammation to neurovascular unit dysfunction and BBB hyperpermeability in schizophrenia (**Tables 1** and **2**). We discuss the relevance of peripheral inflammation to neurovascular endotheliopathy in schizophrenia patients, given the human and experimental data suggesting the potential bidirectional interaction between systemic inflammation and neuroinflammation in schizophrenia (20).

NEUROVASCULAR UNIT DYSFUNCTION

The neurovascular unit consists of the brain's microvessels, pericytes, glial cells (astroglia, microglia, oligodendroglia), and neurons. It is the epicenter of several vital, tightly regulated, dynamic, and complex cellular interactions between glia, neurons, and the cerebral microvascular endothelium (16, 21, 22). Evidence indirectly linking neurovascular dysfunction to schizophrenia is derived from epidemiological data associating schizophrenia with medical conditions involving or resulting from vascular endothelial dysfunction, including cardiovascular disease, type 2 diabetes mellitus, and metabolic syndrome (23–25). About two-thirds of individuals with schizophrenia have comorbid cardiovascular disease (23, 24, 26). Smoking, poor diet, unhealthy lifestyle, chronic use of antipsychotic medication, and metabolic syndrome contribute to increased risk of cardiovascular disease and diabetes mellitus in these patients (25). Metabolic syndrome (abdominal obesity, abnormal glucose metabolism, dyslipidemia, and hypertension) accelerates atherosclerosis-related vascular endothelial dysfunction *via* metabolic, inflammatory, and oxidative pathways, independent of smoking and chronic atypical antipsychotic drug use (24, 26). In addition, some authors have documented primary peripheral vascular endothelial dysfunction in schizophrenia. The non-invasive peripheral arterial tonometry (RH-PAT)-EndoPat 2000 device has been used to assess peripheral arteriole endothelial-dependent vasodilatation (23, 24). Reduced RH-PAT values are considered clinically useful in predicting impaired peripheral

TABLE 2 | Putative mechanisms relevant to schizophrenia neurobiology that are shown in human and experimental studies to disrupt neurovascular unit function and increase blood–brain barrier permeability.

Mechanisms	Human studies	Experimental studies
Oxidative stress		
eNOS uncoupling and decreased endothelial NO levels	Only indirect evidence (16, 23, 24)	(16, 43–45, 48, 50, 71–73)
ROS	(1, 2, 13, 17, 18)	(16, 44, 45, 66–69, 71)
Increased VEGF activity	(40, 41)	(42)
Cerebral hypoperfusion	(16, 51, 59–63)	(16, 53–56, 58, 64, 65)
MMP activation	(18, 85, 86)	(66, 89)
Neuroinflammation		
Astroglial loss and decreased AQP4	(1, 3, 75, 76)	(16, 39)
Microglial activation	(1, 3, 7–9, 79)	(16, 78, 82–84)
Pro-inflammatory cytokines	(1, 12, 94)	(4, 11, 16, 91–93)
Upregulation of adhesion molecules (ICAM-1, VCAM-1)	(34–36, 94)	(38, 91–93)
Bradykinin alteration	(96, 97)	(29, 44, 74, 98, 99)
ACE upregulation	(100, 101)	(100, 102, 103)

ACE, angiotensin I-converting enzyme; AQP4, aquaporin 4; eNOS, endothelial nitric oxide synthase; ICAM-1, intercellular adhesion molecule-1; MMP, matrix metalloproteinase; NO, nitric oxide; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

arteriole endothelial-dependent vasodilatation and may reflect reduced endothelial eNOS-mediated nitric oxide (NO) synthesis (23, 24). Studies utilizing RH-PAT methodology revealed a high prevalence of vascular endothelial cell disturbance among individuals with schizophrenia (23, 24). In a prospective cohort of 83 patients with a schizophrenia spectrum diagnosis, 41 patients (50%) met the criteria for endothelial dysfunction defined as an RH-PAT index less than 1.67 (23). This effect remained statistically significant after adjusting for age, race, gender, smoking status, or atypical antipsychotic drug use (23, 24). Another study documented vascular endothelial dysfunction in medication naïve patients with schizophrenia (27). Genetic factors may also contribute to primary vascular endothelial dysfunction in schizophrenia. One study reported a strong correlation between eNOS genetic variants and endothelial functioning in individuals with schizophrenia; *eNOS* $T^{-786}C$ genotype correlated with lower RH-PAT index regardless of the presence or absence of metabolic syndrome, while CC genotype correlated with a much higher RH-PAT index only in individuals without metabolic syndrome (24). A strong association has been also demonstrated between endothelial dysfunction (RH-PAT index <1.67) and the catechol-O-methyltransferase (*COMT*) Val allele that can influence folate metabolism, regardless of other known risk factors for vascular endotheliopathy such as metabolic syndrome and chronic antipsychotic exposure (23, 24). Schizophrenic individuals carrying at least one *MTHFR* T and/or *COMT* Val risk allele have a lower RH-PAT index, reflective of greater endothelial dysfunction and lower frontal executive functions, compared with *MTHFR* CC and *COMT* Met/Met genotypes (26). These findings suggest that abnormal folate and homocysteine metabolism in association with *MTHFR* and *COMT* risk alleles can contribute to peripheral vascular and cerebrovascular endotheliopathy, thereby constituting an independent risk factor for cardiovascular disease and neurocognitive deficits in individuals with schizophrenia (26). Furthermore, lower endothelial expression of genes involved in ion transport, cell proliferation, and adhesion in schizophrenia individuals compared with healthy controls (28) lends support to the role of genetic factors in endothelial dysfunction in schizophrenia.

BBB HYPERPERMEABILITY

The BBB consists of neurovascular endothelial cells continuously interconnected by highly functional tight junctions, pericytes, surrounding basal lamina extracellular matrix, and perivascular astroglial end-feet processes. BBB integrity is critical for maintaining brain homeostasis and immunoprotection by restricting interactions between innate and adaptive immunity (16, 21, 22, 29). Neurovascular endothelial cells play a critical role in the homeostatic regulation of cerebral microenvironment, both alone and through their complex interactions with surrounding astroglial end-feet processes and other cells (30). They regulate the efflux of toxic substances, the influx of essential nutrients, and brain ionic homeostasis. They also restrict the entry of peripheral inflammatory mediators, neuroactive substances, and water-soluble molecules into the brain (21). There is indirect evidence of BBB breakdown in schizophrenia individuals and that BBB hyperpermeability may contribute to the pathogenesis of schizophrenia (28, 29, 31, 32). This is consistent with clinical observations of increased psychosis in neurological disorders associated with BBB disruption, such as systemic lupus erythematosus, epilepsy, and autoimmune encephalitis (16).

The elevated "CSF:serum albumin ratio" in schizophrenia indicates an increased permeability of the BBB and blood–CSF barrier (29, 32, 33). A study of 63 psychiatric subjects and 4,100 controls revealed that 41% of psychiatric subjects (14 MDD and BPD and 14 schizophrenia) had CSF abnormalities, reflecting BBB hyperpermeability. These CSF abnormalities included increased intrathecal synthesis of IgG, IgM, and/or IgA, up to four IgG oligoclonal bands, and mild pleocytosis (32). Elevated S100B levels in the blood, CSF, and brains of individuals with schizophrenia are considered to be neurobiological consequences of glial activation and/or injury associated with BBB and blood–CSF hyperpermeability (1). Multiple authors have reported increased serum levels of vascular endothelial adhesion molecules such as soluble P (sP)-selectin and sL-selectin and an increased number of integrin αIIbβIIIa receptors on platelets of untreated acute schizophrenic patients compared with healthy controls (34–36). In addition, atypical antipsychotics, such as risperidone, are shown to further impair the vascular endothelial function in diabetic rats *via* activation of vascular endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and sL-selectin (37). The activation of endothelial adhesion molecules and integrins may contribute to increased transendothelial lymphocyte and monocyte migration, which in animal models correlated with cognitive and behavioral changes in response to

systemic inflammation (38). Genetic factors may also influence BBB hyperpermeability and facilitate transendothelial migration of inflammatory cells in schizophrenia. A genome-wide association study has linked a *NDST3* polymorphism to an increased risk for schizophrenia (39). *NDST3* is expressed in the brain and encodes an enzyme involved in the metabolism of heparan sulfate (34). Heparan sulfate is a component of basal lamina extracellular matrix that is vital to BBB integrity. Therefore, genetically predetermined heparan sulfate abnormalities may increase BBB hyperpermeability and facilitate transendothelial leukocyte migration in some individuals with schizophrenia (34). Recent studies have documented elevated serum levels of vascular endothelial growth factor (VEGF) (40) and significantly reduced expression of VEGF receptor 2 in the prefrontal cortex (41), which likely reflects its accelerated destruction by increased levels and activity of VEGF in individuals with schizophrenia. VEGF regulates angiogenesis and increases BBB permeability (40). VEGF activation in animal models of ischemia promotes BBB disruption through endothelial endocytosis (42). These findings indicate that VEGF upregulation may contribute to BBB hyperpermeability and cerebral hypoperfusion in schizophrenia. Further support for BBB disruption comes from ultrastructural studies showing vacuolar degeneration of neurovascular endothelial cells and astroglial end-feet processes, together with thickening and irregularity of the basal lamina in the prefrontal and visual cortices of postmortem brains from schizophrenia subjects (31).

THEORETICAL INTEGRATION OF OXIDATIVE AND NEUROINFLAMMATORY MECHANISMS

Oxidative Stress

Reactive oxygen species minimize tissue injury and facilitate recovery at lower levels, but at high levels, they induce tissue injury by oxidizing biological macromolecules, such as DNA, proteins, and lipids (16). Common ROS include superoxide $\left(O_2^-\right)$ and peroxynitrite ($ONOO^-$). The biological effects of NO are dependent on its sources. NO produced by non-endothelial sources can be harmful and induce vascular endothelial injury through oxidative stress and inflammation (16, 43). When combined with O_2^-, NO produces highly reactive oxidant $ONOO^-$, which damages the vascular endothelium and disrupts BBB integrity (44, 45). Non-endothelial NO production is mediated by neuronal NO synthase that is regulated by Ca^{2+} influx (46) and inducible NO synthase that is positively regulated by nuclear factor-kappa B signaling (47) and pro-inflammatory cytokines (48). In contrast, endothelial-derived NO is beneficial and exerts protective effects on vascular endothelial cells (16, 43). *In vitro* studies showed that endothelial-derived NO can increase cerebral blood flow by enhancing endothelium-dependent vasodilation (44, 45), inhibiting platelet aggregation by increasing endothelial cyclic guanosine monophosphate levels (44, 45), and downregulating the synthesis of vasoconstrictors such as 20-hydroxyeicosatetraenoic acid (16, 49). Endothelial-derived NO can also ameliorate vascular endothelial oxidative injury by scavenging cellular free radicals (44, 45). Endothelial eNOS mediates endothelial NO production *via* oxidative conversion of L-arginine to L-citrulline. activity of eNOS is influenced by several factors, including endothelial Ca^{2+} levels, its substrate arginine (50), and its cofactor tetrahydrobiopterin (BH_4) (16). Reduced eNOS activity can decrease endothelial NO levels resulting in (a) reduced cerebral blood flow, (b) increased platelet aggregation, which may contribute to an increased risk of cardiovascular disease, and (c) decreased vascular reactivity due to oxidative injury of the vascular endothelium (16).

There is limited evidence for uncoupling and reduced activity of endothelial eNOS in schizophrenia (**Figure 1**). Reduced RH-PAT values in individuals with schizophrenia are considered indirect clinical indicators of reduced endothelial eNOS-dependent endothelial NO synthesis (23, 24). Several genetic studies have shown a significant association between eNOS gene polymorphisms and schizophrenia (24). Among 203 participants with schizophrenia or schizoaffective disorder who were carriers of the TT genotype of the *eNOS* $T^{-786}C$ variant, those without metabolic syndrome, had a lower RH-PAT index (24). A postmortem study from schizophrenia subjects showed an association between increased arginine metabolism, increased arginase II activity, and reduced eNOS expression in the frontal regions of the brain (51). We suggest that oxidation and inflammation associated with schizophrenia can also contribute to uncoupling and reduced activity of endothelial eNOS (16) (**Figure 1**); ROS promotes oxidative conversion of the eNOS cofactor BH_4 to dihydrobiopterin (BH_2), thereby reducing endothelial BH_4 bioavailability, which in turn inhibits eNOS activity (16). Decreased BH_4 and increased BH_2 endothelial levels dissociate or uncouple oxidation of L-arginine from the proton-coupled electron transfer reaction, thus shifting the substrate of eNOS from L-arginine to molecular oxygen, thereby facilitating harmful O_2^- synthesis while reducing the endothelial bioavailability of beneficial NO (16). O_2^- combines with residual NO, to form $ONOO^-$ (52), which can cause vascular endothelial oxidative injury. $ONOO^-$ in turn promotes the oxidative conversion of BH_4 to BH_2, which further lowers eNOS activity in a positive feedback loop (52, 53).

We also suggest that uncoupling and reduced activity of endothelial eNOS may contribute to the increased risk of cardiovascular disease and neurovascular endothelial dysfunction in schizophrenia. In cardiovascular diseases, eNOS-associated endothelial dysfunction may result from (a) increased endothelial O_2^- production through an NAD(P)H oxidase-dependent mechanism, (b) increased $ONOO^-$ synthesis, (c) decreased endothelial BH_4 bioavailability, and (d) a metabolic syndrome-related pro-inflammatory state (53–57). Furthermore, eNOS-independent direct oxidative endothelial injury can impair vasodilation (58). We suggest that similar mechanisms account for the comorbidity of cardiovascular diseases in schizophrenia. However, evidence for the potential contribution of aberrant endothelial eNOS activity to neurovascular endothelial dysfunction in schizophrenia is less direct and more limited. Regionally selective cerebral hypoperfusion abnormalities (51, 59–61), including decreased resting cerebral blood flow (62), have been documented in schizophrenia and were partly attributed to depressed neuronal activities. However, based on the findings described above, cerebral hypoperfusion may also be linked to impaired vasodilation that is mechanistically

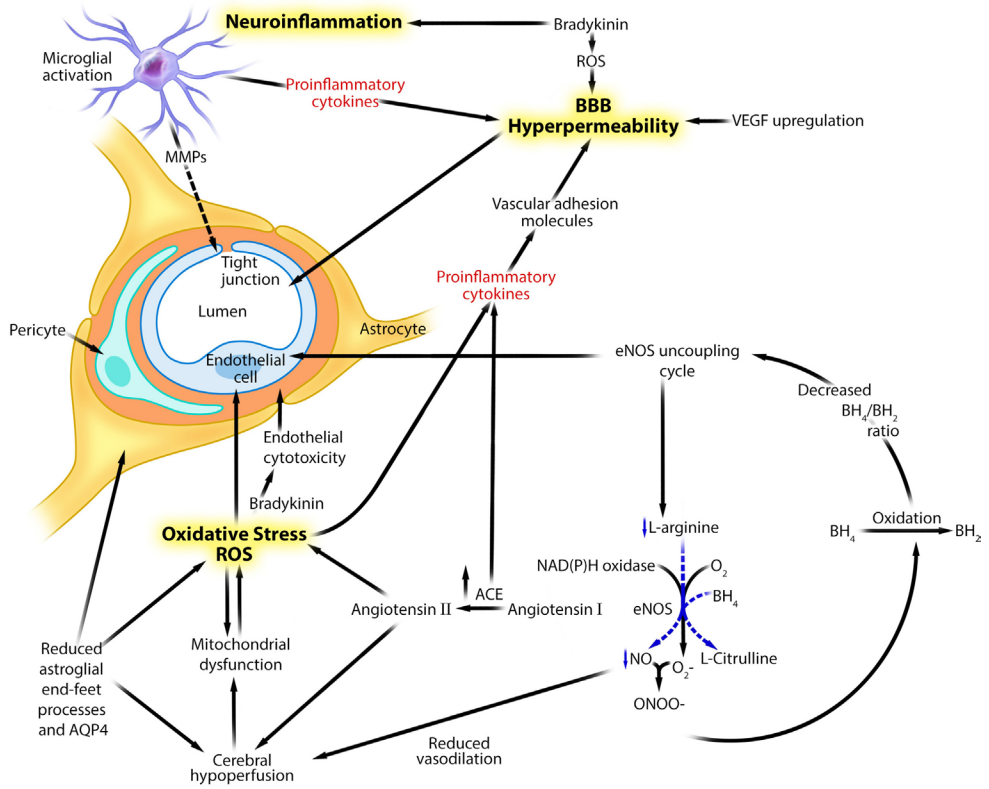

FIGURE 1 | Theoretical integration of human and experimental data linking neuroinflammation, oxidative stress, and genetic factors to neurovascular unit dysfunction and blood–brain barrier hyperpermeability in schizophrenia. Adapted with permission from Abbott et al. (16, 21). This figure describes several putative mechanisms linking neuroinflammation, oxidative stress, and eNOS uncoupling to neurovascular dysfunction and blood–brain barrier hyperpermeability in schizophrenia. ACE, angiotensin I-converting enzyme; AQP4, aquaporin 4; BH_2, dihydrobiopterin; BH_4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; MMP, matrix metalloproteinase; NAD(P)H, nicotinamide adenosine dinucleotide phosphate; NO, nitric oxide, $ONOO^-$, peroxynitrite; O_2^-, superoxide; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.

linked to reduced neurovascular eNOS-dependent NO biosynthesis (16, 51) (**Figure 1**). Pro-inflammatory state associated with metabolic syndrome can also impair endothelial eNOS function (24, 63). Moreover, sustained cerebral hypoperfusion can further compromise endothelial mitochondrial oxidative function, increasing the formation of endothelial ROS (64, 65), which in turn promotes eNOS uncoupling and lowers endothelial NO levels, thereby further reducing cerebral perfusion in a positive feedback loop (53–56).

In animal models, direct oxidative injury of the neurovascular endothelium has been shown to contribute to BBB disruption and cerebral hypoperfusion through several eNOS activity-independent mechanisms (**Figure 1**). These include (a) upregulating MMPs through upregulation of pro-inflammatory cytokines (66), (b) reducing endothelial expression of E-cadherin (44), (c) damaging BBB tight junction proteins by toxic molecules such as phosphatidylinositol-3-kinase γ (44, 67, 68), (d) altering endothelial cytoskeletal proteins, (e) inducing endothelial excitotoxicity by upregulating endothelial NMDA receptor subunit 1 expression (69), and (f) impairing endothelial mitochondrial oxidative metabolism (70). However, the mechanistic relevance of these abnormalities to the pathophysiology of schizophrenia remains unclear.

We suggest that cerebral hypoperfusion related to neurovascular endothelial dysfunction can also contribute to neuronal dysfunction and neurocognitive deficits in schizophrenia. A 99mTc-ECD-single-photon emission computed tomography brain imaging study found that schizophrenia patients with metabolic syndrome had more significant cerebral hypoperfusion associated with substantially lower frontal executive functions, compared with those without metabolic syndrome (63). This suggested a mechanistic link between neurovascular endothelial dysfunction and cognitive deficits in schizophrenia. *In vitro* animal models of selected neurological disorders have shown that reduced eNOS expression may worsen neuronal injury (71, 72). In animal models, reduced eNOS expression was associated with expansion of stroke (73), and increased levels of endothelial $ONOO^-$ correlated positively with BBB breakdown and neurobehavioral deficits in traumatic brain injury (71). Furthermore, treatment with the antioxidant, *S*-nitrosoglutathione, improved neurovascular unit function by decreasing the synthesis of endothelial $ONOO^-$ (71).

Neuroinflammation

Neuroinflammation includes astroglial cell activation and loss, MAP, upregulation of inflammatory mediators, and BBB

disruption with an associated increased transendothelial inflammatory cell migration. Human and animal data suggest that schizophrenia-associated neuroinflammation can disrupt neurovascular function (**Tables 1** and **2**; **Figure 1**). Astroglia regulate cerebral blood flow and volume as well as BBB permeability, among many other critical functions (16, 74). Thus, in schizophrenia, the documented loss of astroglia from functionally relevant areas, such as the subgenual cingulate, anterior, dorsolateral, and prefrontal cortices, as well as the hippocampus and corpus callosum (3), may contribute to reduced cerebral blood flow and increased BBB permeability. Aquaporin 4 (AQP4) is a bidirectional water channel mainly expressed in the perivascular astroglial end-feet processes and is critical to the development and integrity of the BBB and brain water homeostasis. AQP4 expression has been documented to be significantly reduced in the deep layers of the anterior cingulate gyrus in schizophrenia subjects (75). Decreased AQP4 expression can impair astroglial–endothelial interactions that are vital for maintaining cerebral homeostasis and regulating BBB permeability (16, 30). Furthermore, reduced AQP4 expression has been associated with an increased risk for psychiatric disorders such as psychosis (76). Thus, reduced AQP4 expression in schizophrenia individuals may contribute to neurovascular dysfunction and BBB hyperpermeability. More studies are needed to investigate the full effects of reduced AQP4 expression on the functions of neurovascular endothelium and BBB in schizophrenia.

Microglia provide immune surveillance and regulate synaptic pruning in the brain (77). Although transient MAP can limit neuronal injury and enhance recovery, persistent MAP can be harmful and perpetuate neuronal injury (78). Harmful MAP has been implicated in the pathophysiology of schizophrenia (1, 3, 79). Postmortem studies of brains from schizophrenia subjects have consistently documented MAP, including an increased HLA-DR immunoreactivity, in multiple regions compared with healthy controls, particularly in the dorsolateral prefrontal, superior temporal, and anterior cingulate cortices (3, 79). Free-water diffusion tensor imaging showed a significant increase in the extracellular free-water volume of gray and white matter in individuals with first-episode schizophrenia, suggestive of widespread neuroinflammation (80). A more recent systematic review demonstrated consistent evidence for white matter inflammation in schizophrenia individuals, which might contribute to the structural and functional white matter disconnectivity, even during first-episode psychosis (3). Furthermore, white matter inflammation has recently been associated with elevated serum S100B levels in patients with new-onset schizophrenia (81), indicating that white matter inflammation together with glial activation and/or injury as well as BBB hyperpermeability occur in early stages of schizophrenia. In experimental models of neurological diseases such as stroke and trauma, MAP damaged BBB endothelial tight junction proteins and increased BBB permeability through several mechanisms involving activation of inducible NOS (82), promotion of ROS synthesis (83), induction of COX2 expression within the neurovascular unit (1), and upregulation of pro-inflammatory cytokines and MMPs (1). An increased BBB permeability may in turn facilitate interactions between brain innate and peripheral adaptive immunity, thereby perpetuating MAP and synthesis of brain pro-inflammatory cytokines in a positive feedback loop (16). This is further supported by recent evidence linking MAP and activation of peripheral monocytes to the pathophysiology of several psychiatric disorders, including schizophrenia (84).

Matrix metalloproteinase upregulation may contribute to the pathology of schizophrenia, including neurovascular dysfunction (**Figure 1**). Cumulative evidence suggests that serum levels and activity of MMP-9 are increased in schizophrenia individuals compared with healthy controls (18, 85). Genetic studies have also suggested that MMP-9 may contribute to the pathology of schizophrenia (86). MMP-9 influences synaptic plasticity, thought to be relevant to the neurobiology of schizophrenia possibly by converting pro-brain-derived neurotrophic factor (BDNF) to BDNF (87). Upregulation of BDNF has been associated with resistance to antipsychotic medications (88). MMP-9 also acts *via* non-synaptic mechanisms that may be relevant to schizophrenia pathology. These mechanisms include tissue remodeling, angiogenesis, inflammation, oxidative injury, and BBB breakdown (85). In animal models of acute cerebral ischemia, upregulation of MMP-9 correlated positively with disruption and hyperpermeability of BBB (89). A positive correlation was also shown between serum levels of MMP-9 and the lipid peroxidation marker malondialdehyde in individuals with schizophrenia (18). Serum levels of malondialdehyde correlated positively with increased BBB permeability following acute neurological insults such as neonatal asphyxia (90). However, the correlation between MMP-9 upregulation, lipid peroxidation, and BBB breakdown in schizophrenia remains speculative.

Pro-inflammatory cytokines can damage and increase the permeability of the BBB (11) (**Figure 1**). *In vitro* data have shown that pro-inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) cause a dose-dependent increase in BBB permeability by (a) inducing expression of adhesion molecules such as ICAM-1 and VCAM-1 on the luminal surface of BBB endothelial cells in animals (91–93) and humans (94), which facilitates transendothelial lymphocyte and monocyte migration; (b) causing vascular endothelial oxidative injury by impairing vascular endothelial mitochondrial oxidative metabolism (65, 95); and (c) directly damaging endothelial tight junctions (11, 16). More recently, an association was described between elevated serum IL-6 and VEGF levels in schizophrenia (40), supporting the role of inflammation in inducing BBB hyperpermeability in schizophrenia. More studies are needed to fully explore the relevance of these mechanisms to the onset and progression of schizophrenia pathology.

Bradykinin alterations in schizophrenia patients have received limited attention (96, 97). The bradykinin polypeptide mediates inflammation, prostaglandin synthesis, vasodilation, and increased capillary permeability. The oligopeptidase nuclear distribution E like-1 (Ndel1) modulates several neurodevelopmental processes involved in schizophrenia pathophysiology such as cell signaling, neurite outgrowth, neuronal migration, and cytoskeletal organization (96, 97). It also mediates the breakdown of several neuropeptides including bradykinin, which is thought to contribute to schizophrenia neurobiology

(96, 97). Lower Ndel1 activity has been reported in the plasma of individuals with schizophrenia compared with healthy controls (96, 97), particularly those with treatment-resistant schizophrenia (96, 97). Therefore, lower Ndel1 activity may limit bradykinin catabolism, thereby increasing bradykinin levels in the brains of schizophrenic individuals. Upregulation of bradykinin may contribute to neurovascular endothelial dysfunction and BBB hyperpermeability *via* inflammatory and oxidative mechanisms (**Figure 1**). Activation of bradykinin and its inducible B1 and constitutively expressed endothelial B2 receptors induces inflammation, promotes oxidative injury, and increases BBB permeability (98). *In vitro* human studies have demonstrated that inflammation-induced expression of the bradykinin B1 receptor could increase BBB permeability (98). Bradykinin activation can augment astroglial nuclear factor-kappa B pathway-mediated IL-6 production, which may increase BBB permeability (29, 74). Bradykinin activation can also stimulate phospholipase A2 activity, which in turn enhances arachidonic acid release and metabolism, leading to increased production of malondialdehyde (99) and extracellular NO (44) that can increase BBB permeability. Endothelial B2 receptor activation increases endothelial Ca^{2+} influx, which activates pro-oxidant enzymes involved in ROS synthesis (29, 44, 74). An increased ROS production can further increase BBB permeability and augment its susceptibility to the harmful effects of bradykinin (99). *In vivo* human studies aimed at elucidating the role of bradykinin activation in schizophrenia can be informative.

Angiotensin I-converting enzyme upregulation may also contribute to neurovascular endothelial dysfunction (**Figure 1**). ACE is a central component of the renin–angiotensin system and converts angiotensin I to angiotensin II. Angiotensin II has vasoconstrictive and pro-inflammatory properties. ACE activity is significantly increased in the plasma, CSF, and brains of schizophrenia patients compared with healthy controls (100, 101). An increased ACE activity in schizophrenia patients correlated positively with a significant increase in the serum levels of pro-inflammatory cytokines such as IL-17 and IFN-γ (101) and cognitive deficits including disorganization of thought process (100). The pathological effects of increased ACE activity on brain, including cognitive decline, neurodegeneration and increased BBB permeability, are mediated by angiotensin II-mediated activation of angiotensin type 1 receptors (102). Activation of angiotensin II in animal models can also induce harmful cerebrovascular remodeling through inflammatory and oxidative mechanisms. These findings, collectively, suggest that ACE upregulation is relevant to the neurobiology of schizophrenia, which includes neurovascular endothelial dysfunction and increased BBB permeability (102, 103).

Limitations and Future Directions

The evidence presented in this review suggesting a role of primary neurovascular endothelial dysfunction and BBB hyperpermeability in schizophrenia neurobiology has several limitations inherent to the following assumptions and extrapolations: (1) peripheral inflammation consistently correlates with neuroinflammation and (2) peripheral endothelial dysfunction is consistently associated with or a good surrogate marker of neurovascular endothelial dysfunction. Furthermore, although some studies suggest that neurovascular endothelial dysfunction in schizophrenia can be a primary process, many other studies support the contributory role of confounding vascular risk factors (e.g., age, BMI, smoking, metabolic syndrome, antipsychotics) to vascular endotheliopathy. Accordingly, the potential contribution of neurovascular endothelial dysfunction and increased BBB permeability to the neurobiology of schizophrenia needs to be confirmed by future investigations in animals and humans. Relevant postmortem studies should focus primarily on the neuroanatomical regions wherein astroglial loss and MAP have been consistently reported in schizophrenia, such as the subgenual cingulate, anterior, dorsolateral, and prefrontal cortices, as well as the corpus callosum (3). Future studies should concurrently investigate the potential mechanistic links between oxidative stress, neuroinflammation, aberrant expression and reduced activation of endothelial eNOS, white matter disconnectivity, and neurovascular endothelial dysfunction together with BBB hyperpermeability, in untreated new-onset schizophrenia versus chronic schizophrenia. Voxel-based morphometry could be used together with free-water diffusion tensor imaging to assess the white matter inflammation. Low RH-PAT values can be useful in predicting aberrant endothelial eNOS activity and reduced endothelial NO availability. Findings of these studies need to be correlated with serum biomarkers for inflammation (e.g., IL-6, MMP-9), oxidative stress (e.g., malondialdehyde, total antioxidant status), impaired endothelial–astroglial interaction (e.g., AQP4, S100B), and altered endothelial functions (e.g., VCAM-1, ICAM-1, sP-selectin, sL-selectin, integrin, VEGF). Furthermore, correlating *in vivo* positron emission tomography imaging of MAP using TSPO C11-PK11195 (4, 7) with serum biomarkers of vascular endothelial dysfunction and BBB breakdown may shed additional light on the role of neuroinflammation-related cerebral microvascular endotheliopathy and BBB hyperpermeability in the pathophysiology of schizophrenia.

CONCLUSION

An increasing body of evidence suggests that neurovascular endotheliopathy and BBB hyperpermeability can occur in schizophrenia. Our review provides a theoretical integration of clinical and experimental findings linking neuroinflammation and oxidative stress to cerebral microvasculature abnormalities in schizophrenia. These abnormalities may contribute to the behavioral and cognitive symptoms of schizophrenia *via* several mechanisms involving disruption of BBB integrity, leading to reduced cerebral perfusion and impaired homeostatic processes of cerebral microenvironment. BBB breakdown can also facilitate interactions between brain innate and peripheral adaptive immunity, thereby perpetuating harmful neuroimmune signals and toxic neuroinflammatory responses. Taken together, these findings support the "mild encephalitis" hypothesis of schizophrenia (33). Further investigation into the molecular, functional, and structural neurovascular abnormalities and their contribution to white matter disconnectivity in untreated new-onset schizophrenia versus chronic

schizophrenia, and antipsychotic treatment-responsive versus treatment-resistant schizophrenia, can be informative. If neurovascular abnormalities prove to be etiologically relevant to schizophrenia pathophysiology, then targeting these abnormalities may represent a promising therapeutic strategy for schizophrenia.

AUTHOR CONTRIBUTIONS

SN wrote the manuscript and was responsible for acquisition and interpretation of the data. SP, VS, JS, AN, and DC participated in the data acquisition and interpretation. All authors listed contributed to the final version of the manuscript.

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PERSPECTIVE
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IL1R2, CCR2, and CXCR4 May Form Heteroreceptor Complexes with NMDAR and D2R: Relevance for Schizophrenia

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The mild neuroinflammation hypothesis of schizophrenia was introduced by Bechter in 2001. It has been hypothesized that a hypofunction of glutamatergic signaling *via N*-methyl-D-aspartate receptors (NMDARs) and hyperactivation of dopamine D2 receptors play a role in schizophrenia. The triplet puzzle theory states that sets of triplet amino acid homologies guide two different receptors toward each other and contributes to the formation of a receptor heteromer. It is, therefore, proposed that putative NMDAR-C-C chemokine receptor type 2 (CCR2), NMDAR-C-X-C chemokine receptor type 4 (CXCR4), and NMDAR- interleukin 1 receptor type II (IL1R2) heteromers can be formed in the neuronal networks in mild neuroinflammation due to demonstration of Gly-Leu-Leu (GLL), Val-Ser-Thr (VST), and/or Ser-Val-Ser (SVS) amino acid homologies between these receptor protomers. This molecular process may underlie the ability to produce symptoms of schizophrenia in mild neuroinflammation. In this state, volume transmission (VT) is increased involving increased extracellular vesicle-mediated VT from microglia and astroglia. These vesicles may contain CCR2, CXCR4, and/or IL1R2 as well as their ligands and upon internalization by endocytic pathways into neurons can form heteroreceptor complexes with NMDAR in the plasma membrane with pathological allosteric receptor–receptor interactions involving increased internalization and reduced NMDAR signaling. The triplet puzzle theory also suggests the formation of putative D2R-CCR2, D2R-CXCR4, and D2R-IL1R2 heteromers in mild neuroinflammation in view of their demonstrated sets of Leu-Tyr-Ser (LYS), Leu-Pro-Phe (LPF), and/or Ser-Leu-Ala (SLA) triplet homologies. These D2R heteroreceptor complexes may also contribute to schizophrenia-like symptoms in mild neuroinflammation by enhancing D2R protomer function.

Keywords: receptor–receptor interactions, schizophrenia, neuroinflamation, NMDAR, chemokine receptors, cytokine receptors, heteroreceptor complexes, volume transmission

INTRODUCTION

The mild neuroinflammation hypothesis of schizophrenia was introduced by Bechter (1–3). Recent work supports a role of inflammation in schizophrenia (4) and a relevant cellular basis appears to be microglia, which upon activation release proinflammatory cytokines (4, 5). It is still unclear, however, if the classical antibiotic drug minocycline can be used as an antipsychotic drug in spite of its ability to block microglia activation. It is known that microglia plays an important role in brain development and possesses protective and destructive functions in neuroinflammation (6–8).

CSF studies may be especially informative on brain events, at least in the clinical situation, and can be performed repeatedly even during acute psychotic episodes. Such studies demonstrated, both in affective and schizophrenic spectrum disorders, the prevalence of activated CSF cells similar to the situation in neurological neuroinflammatory disorders (9, 10). In addition, at least three immunological subgroups were found in affective and schizophrenic spectrum disorders as defined by established CSF examination (11) and a subgroup with increased CSF neopterin (12). In another study, all patients investigated demonstrated an increase of IL8 (13). Taken together, between 70 and 100% of severely diseased patients with the affective and schizophrenic spectrum disorder presented certain CSF abnormalities (14, 15). These findings supported the mild encephalitis (ME) hypothesis of these disorders (1, 2, 16). Further support came from the neurological field with the first description of NMDAR autoimmune encephalitis (17, 18). The more general relevance of the ME hypothesis is suggested by epidemiological findings demonstrating that infections and autoimmune disorders are important risk factors in schizophrenia bipolar and depressive disorders (19–21). Furthermore, recent CSF studies in larger groups of similar psychiatric patient groups also supported this view (22–24).

There is an agreement that the *N*-methyl-D-aspartate receptor (NMDAR) hypofunction plays an important role in the schizophrenia disease development (25). Previously it was found that chronic brain inflammation can produce a decline in both hippocampal GluN1 NMDARs and GluN2A and GluN2B subunits of NMDARs which likely is mainly caused by reductions in their transcriptional mechanisms (26, 27). They may be linked to cognitive deficits is schizophrenia.

It is, therefore, of high interest that schizophrenia-like symptoms can often be found in patients with NMDAR antibody induced encephalitis (17, 19, 28–30). The NMDAR autoantibodies have been shown to lead to specific, titer-dependent, reversible loss of NMDARs, the dysbalance within the network function being able to explain a spectrum of symptoms (31). It seems possible that the mechanism can involve disturbances in NMDAR function through interactions with the NMDAR antibody leading *inter alia* to NMDA receptor internalization and breakdown (31). On the other hand, a broad repertoire of antibody-secreting cells is enriched in the CNS during encephalitis producing different types of autoantibodies in parallel in the CSF (32). In addition, for neuronal damage in autoimmune encephalitis cytotoxic, T cells may be responsible not the autoantibodies. Triggers of autoimmune encephalitis may be cancers or virus infections or remain unknown (33). So far, the situation is rather similar to that predicted with the ME hypothesis. The latter is further supported by CSF findings (14, 15) and not least by rare cases of acute psychosis with brain biopsy showing definite but mild neuroinflammation in the cerebral cortex (34–36). Recent evidence for a more general relevance of the ME hypothesis comes also from a postmortem study showing an increased number of immune cells in the brain seemingly linked to a minor blood brain barrier breakdown (37).

Apparently, in classical and ME, one can plausibly expect some general pathological mechanisms but potentially also specific pathological mechanisms to be involved in parallel. There exists no clearcut evidence that specific autoimmunity explains the whole disorder in autoimmune encephalitis (38) nor that it represents an exclusive single pathological mechanism. In multiple sclerosis (MS), there is an early involvement of the cerebral cortex found in both experimental allergic encephalomyelitis (39) and in human MS (40). The interesting findings by Najjar et al. (34–36) in cortical biopsies may similarly represent not only proof for mild local neuroinflammation but may in addition indicate a more distributed mild neuroinflammatory process, the latter indicated by the findings of Bogerts et al. (37).

The current perspective article will discuss the different molecular mechanisms that may underlie the ability of neuroinflammation to produce positive, negative, and/or cognitive symptoms of schizophrenia. It likely involves the release of chemokines and cytokines from activated microglia, astroglia, and monocytes (41, 42), which *via* volume transmission (VT) can target their receptors on glia and neurons (43, 44). There may also exist an increased extracellular vesicle-mediated VT (44) from glia and megacaryocytes. Glial and immune cells may contain receptor proteins and different forms of mRNAs for chemokine and cytokine receptors in mild neuroinflammation. Extracellular vesicles containing mRNA and proteins for these receptors can *via* VT communication have a relevant role for producing schizophrenia-like symptoms by being internalized *via* e.g., cell adhesion receptors into the neuronal component of glia–neuron networks. Extracellular vesicles containing e.g., the cytokine and chemokine receptors may be taken up by an uptake mechanism that depends on proteins located both on the neuronal target cell at extrasynaptic and/or synaptic sites and on the extracellular vesicle (45). The extracellular vesicles are then internalized by a number of endocytic pathways. In this process, the internalized receptors can reach e.g., early endosomes and be rapidly returned to the plasma membrane (46) where they are proposed to interact with extrasynaptic and synaptic NMDARs and D2Rs, indicated to be involved in schizophrenia. This may lead to pathological receptor–receptor interactions in neurons in brain areas with mild neuroinflammation (43, 44).

The allosteric receptor–receptor interactions in D2R heterocomplexes are already indicated to play a role in schizophrenia, especially the antagonistic A2AR–D2R interactions in A2AR–D2R heterocomplexes (47–49). Using the triplet puzzle theory (50), four sets of triplet amino acid homologies were found between the A2AR and D2R protomers which may contribute to the formation of the A2AR–D2R heterocomplexes and to the

development of the antagonistic A2A–D2 receptor–receptor interactions (47–49).

POSSIBLE MOLECULAR MECHANISMS BASED ON THE TRIPLET PUZZLE THEORY CONTRIBUTING TO SCHIZOPHRENIA-LIKE SYMPTOMS IN MILD NEUROINFLAMMATION

Triplet Puzzle Theory Supports the Formation of Glutamate NMDAR–CytokineR/ChemokineR Heteroreceptor Complexes through Gly-Leu-Leu (GLL), Val-Ser-Thr (VST), and Ser-Val-Ser (SVS) Homologies

In 2010, based on a bioinformatic approach, it was possible to indicate that receptor that form heterodimers show triplet amino acid homologies (50). This was not observed in pairs of receptors that do not form heterodimers. It was, therefore, proposed that these triplet homologies participate in the receptor interface and gives a code that facilitates the formation of the heterodimer. It was named the triplet puzzle theory (50, 51). The code formed from the triplet amino acid homologies may assist in guiding the receptors toward each other.

Such protriplet homologies appear to be phylogenetically old mechanisms for protein recognition and are already found in integrins (an alpha–beta heterodimer) of marine sponges (52) and remain in human D2 receptor heteromers (53).

It is of particular interest that the NMDAR shows one protriplet amino acid homology with CCR2 (GLL), C-X-C chemokine receptor type 4 (CXCR4) (VST), and interleukin 1 receptor type II (IL1R2) (SVS) (**Table 1**) as previously observed (43). The GLL protriplet of CCR2 is located in the C-tail and may interact with the GLL of the intracellular part of NR2A (**Table 1**). The VST protriplet of CXCR4 is also found in the C-tail and may interact with the VST protriplet of the intracellular part of NR2A (**Table 1**). The VST protriplet of CXCR4 may also interact with the VST in the NR1-1,4,5 subunits present in the C-tail (**Table 1**). The SVS protriplet is located in the N-terminal of IL1R2 and may interact with the SVS protriplet in the extracellular part of NR2A,B,D (**Table 1**).

Interleukin 1 receptor type II is a decoy receptor that can bind to IL1α, IL1β, and IL1R antagonist. It can also interact with IL1R accessory protein. It should be noticed that the ITGA-ITGB heterodimer shows a SVS protriplet homology and the GABA B receptor (GABAB1-GABAB2 heterodimer) a GLL protriplet homology and the known GABAB1-CXCR4 heterodimer *inter alia* a VST and a SVS homology (**Table 1**). These observations further support the current view that NMDARs can form heteromers with CCR2, CXCR4, and IL1R2.

In mild neuroinflammation, it is proposed that VT is increased involving increased extracellular vesicle-mediated VT from microglia and astroglia (see above). It should, therefore, be considered that these vesicles may contain CCR2, CXCR4, and/or IL1R2, which upon internalization into neurons can form heteroreceptor complexes with NMDAR with pathological allosteric receptor–receptor interactions (**Figure 1**). If these receptor mechanisms lead to a hypofunction of the NMDAR protomer, they represent one mechanism for the schizophrenia-like effects seen in mild neuroinflammation (3). If the mild neuroinflammation takes place in the hippocampus and the cerebral cortex, the pyramidal nerve cells, key nerve cells in the cortical, and hippocampal circuits will also be affected in view of their expression of NMDA receptors. It will lead to deficits in cognitive functions and contribute to negative symptoms of schizophrenia.

TABLE 1 | Example of schizo triplets in the interface of human receptor heteromers.

Receptor heteromer	Reference	Ser-Val-Ser (SVS)	Gly-Leu-Leu (GLL)	Val-Ser-Thr (VST)	Leu-Tyr-Ser (LYS)	Leu-Pro-Phe (LPF)	Ser-Leu-Ala (SLA)
GABAB1–GABAB2	(54–56)	–	#	–	–	–	–
GABAB1–CXCR4	(57)	#	–	#	–	–	–
NMDA–CCR2	Possible heteromer	–	#	–	+	–	–
NMDA-interleukin 1 receptor type II (IL1R2)	Possible heteromer	#	–	–	–	–	–
NMDA–CXCR4	Possible heteromer	+	–	#	+	–	–
MOP–DOP	(58)	–	–	–	#	#	–
CCR2–CXCR4	(59)	–	–	–	#	–	–
DOP–CXCR4	(60)	–	–	–	#	#	–
5HT1A–5HT1B	(61)	–	–	–	–	#	#
5HT1A–5HT7	(62)	–	–	–	–	#	#
ADRA2A–ADRA2B	(63)	–	–	–	–	–	#
ADRB2–ADRB3	(64)	–	–	–	–	#	#
D2–CCR2	Possible heteromer	–	–	–	#	–	–
D2–IL1R2	Possible heteromer	–	–	–	–	#	#
D2–CXCR4	Possible heteromer	–	–	–	#	#	–

#, yes in both receptors and may mediate their interaction; +, yes in both receptors; –, no in any receptor.

Location: ec, extracellular; ic, intracellular; TM, transmembrane; SVS—ic GABAB1 # CXCR4; ec NR2A,B,D # IL1R2(N-terminal); GLL—TM GABAB1 # GABAB2; ic NR2A # CCR2(C-tail); VST—ic GABAB1 # CXCR4 (C-tail), NR2A # CXCR4(C-tail), NR1-1,4,5(C-tail) # CXCR4(C-tail); LYS—TM MOP, DOP, CXCR4, D2; LPF—TM MOP, DOP, 5HT1A,B, 5HT7; TM CXCR4 # D2(TM'ec); ec IL1R2 # D2(TM'ec); SLA—TM 5HT1A,B, 5HT7, ADRA2A,B, ADRB2,3; TM IL1R2 # D2. The highlighted text in red color represent the new postulated heteroreceptor complexes based on the Triplet Puzzle Theory

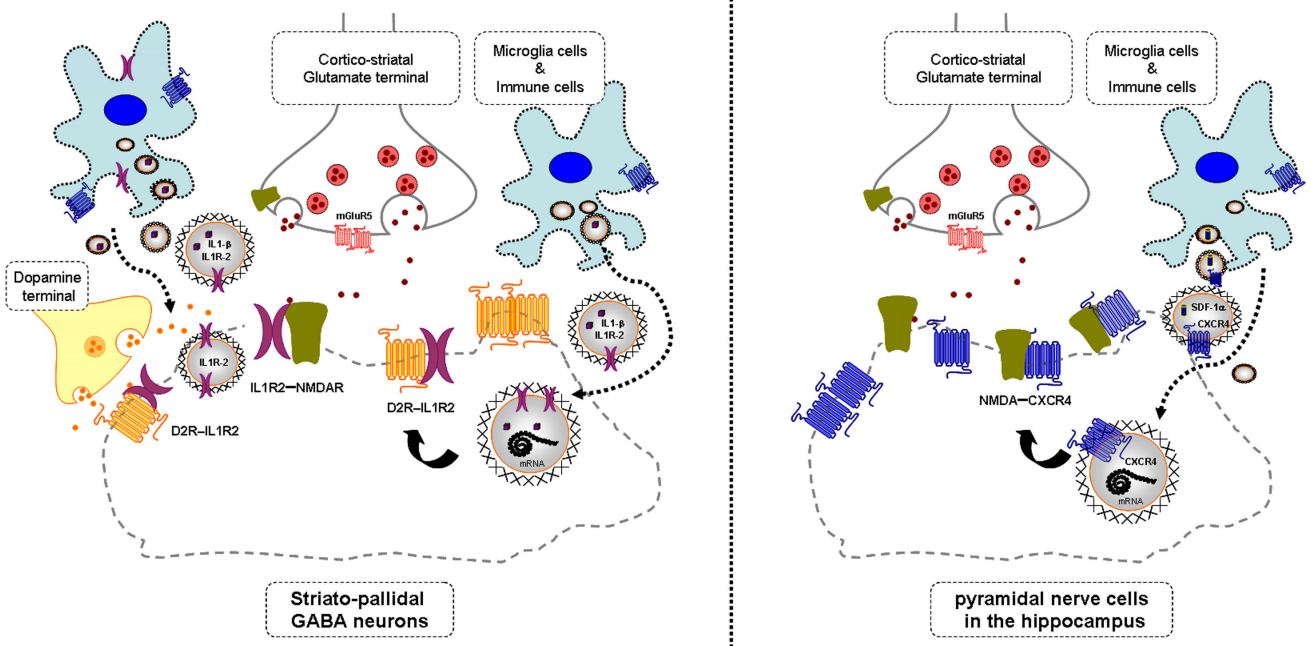

FIGURE 1 | Illustration of the potential chemokine and cytokine receptor transfer *via* extracellular vesicle-volume transmission (VT) from immune and microglial cells to striatopallidal GABA neurons [containing dopamine (DA) D2Rs and NMDARs] and hippocampus pyramidal neurons (containing NMDA receptors) in mild inflammation. One mechanism is shown for how chemokine C-X-C chemokine receptor type 4 (CXCR4) and cytokine receptor IL1-R2 including their mRNAs can produce schizophrenia-like symptoms in neuroinflammation. These receptors may be transferred *via* extracellular vesicle-mediated VT from immune cells, activated microglia, and/or astroglia to nerve cells containing DA D2R and NMDA receptors. Upon internalization, the receptors CXCR4 and IL1-R2 can according to the triplet puzzle theory (see **Table 1**) form complexes with DA D2R and NMDAR as illustrated here. **(A)** is shown the D2R-interleukin 1 receptor type II (IL1R2) heteromers in striatopallidal GABA neurons and how they reach the plasma membrane NMDAR *via* early endosomes and recycling endosomes. **(B)** is shown the NMDAR-CXCR4 heteromers in the pyramidal nerve cells in the hippocampus with microglial extracellular vesicles containing CXCR4 and also SDF-1alpha reaching the NMDAR in the plasma membrane *via* internalization and endosomes in the pyramidal cells. Through the development of novel allosteric receptor–receptor interactions in such heteroreceptor complexes, D2R and NMDAR signaling may become pathologically altered contributing to schizophrenia-like symptoms.

These disturbances may become enhanced by an increase in the glia release of the endogenous cytokine and chemokine ligands for these receptors. The agonist induced receptor–receptor interactions can amplify the reduction of NMDAR function in these heterocomplexes and thus help develop the schizophrenia episodes. It will be of high interest to test if the putative NMDAR-CCR2, NMDAR-CXCR4, and NMDAR-IL1R2 heteroreceptor complexes in fact exist using the *in situ* proximity ligation assay in brain tissue. It is postulated that a reduced NMDAR signaling develop in these heteroreceptor complexes through the agonist activated chemokine and cytokine receptor protomers reducing the NMDAR signaling *via* allosteric mechanisms in these heterocomplexes. This may also lead to increases in the internalization of these heterocomplexes to late endosomes and lysosomes with a reduction in the density of NMDA receptors. It is unknown if the cytokine and/or chemokine-activated receptor protomer involves a negative allosteric modulation of the glutamate-binding site on GluN2 and/or a negative allosteric modulation of the glycine modulatory site on GluN1 (25).

C-C chemokine receptor type 2 with its ligand CCL2 plays a major role in immunobiology and neurobiology (65). It is mainly located on monocytes and is involved in systemic and brain inflammation. CXCR4 and its ligands CXCL12 are involved in the pathogenesis of brain disease and participate in neuron–glial interactions and in neurotoxicity (66, 67). The IL1R2 exists both in soluble and membrane bound forms, shows no transmembrane signaling, and is regarded as a decoy receptor for IL1 signaling However, it can also act as a binding protein in the membrane and interact with the IL1R accessory protein (68). This accessory protein also exists as an alternatively spliced brain-specific isoform having a significant role in homeostatic sleep (69).

According to the triplet puzzle hypothesis, it can bind to the NMDAR [**Table 1**; see also Ref. (51)] and form a NMDAR–IL1R2 complex in which NMDAR function is postulated to become reduced. Previous work (70) indicated that this receptor can participate in Alzheimer's disease, but its biological function is unknown. It may participate in anti-inflammation and help keep the neuroinflammation at a low level.

Triplet Puzzle Theory Supports the Formation of Dopamine (DA) D2R–Cytokine Receptor/Chemokine Receptor Heteroreceptor Complexes through LYS, LPF, and SLA Triplet Homologies

There exists dysregulation of the DA neurons in the pathophysiology of schizophrenia (71). The therapeutic effects of typical and atypical antipsychotic drugs are mainly mediated *via* blockade of the DA D2 receptors (72, 73) located postjunctionally in the mesolimbic-cortical DA neurons (74–76). The D2Rs are mainly located outside the DA synapses and targeted by extracellular DA VT (76). Over the last decades, the discovery was made that D2Rs participate in many different types heteroreceptor complexes in which D2R protomers directly interact *via* allosteric mechanisms with other receptor protomers to integrate biological signals changing D2R signaling (47, 49). Some of them offer new targets for the therapeutic effects of D2R antagonists. In other D2R heteroreceptor complexes, the blockade of the D2R protomers by antipsychotic drugs may instead produce side-effects.

It is of particular interest that the NR2B subunit of the NMDAR interacts with the D2R in the glutamate synapses (77), which leads to an antagonistic allosteric receptor–receptor interaction reducing NMDA signaling in the heteroreceptor complex. Thus, DA through VT may diffuse into the glutamate synapses, activate D2R protomers, and reduce NMDAR-mediated synaptic glutamate transmission, which should enhance schizophrenic symptoms according to current hypotheses. The D2Rs and their heteroreceptor complexes are mainly located in the ventral striatopallidal GABA anti-reward neurons. Their enhanced inhibition by enhanced D2R signaling leading to reduced NMDAR signaling should markedly bring down anti-reward activity in this pathway, which can contribute to a malfunction of salience in schizophrenia with all stimuli becoming relevant and disturbing ongoing behavior (49).

It is, therefore, of high interest that, according to the triplet puzzle theory, D2Rs can form heteroreceptor complexes with CCR2, IL1R2, and CXCR4 as indicated for the first time in the current article (**Table 1**). Three "schizo triplets" were found: LYS, LPF, and SLA. The possible D2R-IL1R2 heterodimer had two sets of triplet homologies, LPF and SLA. LPF is located in the TM6/extracellular region of the D2R and in the extracellular region of the IL1R2 (**Table 1**). SLA is instead located in TM2 of the D2R and in the TM region of IL1R2 (**Table 1**). The possible D2R-CXCR4 heterodimer also shows two sets of triplet homologies, one is again LPF, this time located in the interface between TM6-TM2 with the LPF located in the TM2 (**Table 1**). The other triplet is LYS, which is located in the TM7 of the D2 and in TM3 of the CXCR4 (**Table 1**) indicating that TM7 and TM3 of the D2R and CXCR4, respectively, can also participate in this interface. The possible D2R-CCR2 heterodimer only exhibits one set of triplet homology that may help mediate the interaction. Also, in this case, the LYS triplet is found and here present in the TM1 of CCR2 that may interact with the LYS triplet in TM7 of D2R.

It is proposed that D2R-CCR2, D2R-IL1R2, and D2R-CXCR4 heteromers can be formed upon mild neuroinflammation in the brain, especially in the ventral striatum. This may contribute to positive schizophrenic symptoms by enhancing D2R inhibitory function in critical brain circuits like the ventral striatopallidal GABA anti-reward pathway leading to exaggerated salience development (**Figure 1**). Both chemokine and cytokine receptors appear to be involved in forming complexes with the DA D2Rs as is the case with NMDARs. It should also be underlined that the NMDARs interact with the same chemokine and cytokine receptor subtypes as the D2Rs but using different sets of triplet amino acid homologies.

Other GPCR Heteroreceptor Complexes with LYS, LPF, and SLA Homologies

It is of interest that a number of 5HT1A isoreceptor complexes and α2- and β-adrenergic isoreceptor complexes (78) also possess sets of LYS, LPF, and SLA protriplets that may assist in the formation of these isoreceptor complexes (**Table 1**). Previous work demonstrated also crosstalk between opioid and chemokine receptor subtypes (79) and extensive formation of heteromers take place between opioid and receptor subtypes according to the triplet puzzle theory (80). In **Table 1**, we report some results from this study showing that LYS and LPF protriplets may also participate in opioid and chemokine isoreceptor complexes as well as in the delta opioid-CXCR4 heterodimer. Their possible role in schizophrenic symptoms in neuroinflammation remains to be explored, but they may have an impact on pain and reward mechanisms (80).

Hypothesis: Glial Cytokine and Chemokine Receptor Subtype Transfer into Neurons in Mild Neuroinflammation Can Produce Novel Dysfunctional NMDAR and D2R Heteroreceptor Complexes Contributing to Schizophrenia Development

This hypothesis is based on the existence of not only soluble VT signals but also of extracellular vesicle-mediated VT signals (44). In 2006, it was found that exosomes can be released from cortical neurons in culture (81). In 2012, cell cultures were demonstrated to transfer GPCRs *via* extracellular vesicles to other cells and also form GPCR heteromers in the recipient cells by direct interactions with their GPCRs and A2AR-D2R heteromers developed (82). Glial cells, especially the microglia, are activated in inflammation and can release a number of chemokines and cytokines as soluble VT signals and produce a panorama of cytokine and chemokine receptors (6, 83). They may communicate as soluble VT signals (ligands) and *via* extracellular vesicle-mediated VT as to receptors and their receptor mRNAs (43, 44). The extracellular vesicles may then *via* cell adhesion receptors become internalized into neurons and their cargo released. In the neurons, CCR2, CXCR4, and IL1R2 can according to the triplet puzzle theory (see above) interact with NMDARs, known to be disturbed in schizophrenia, and as discovered in the current paper with D2Rs, the major target for currently used antipsychotic drugs.

The hypothesis states that the NMDAR protomer develops a hypofunction in mild neuroinflammation due to antagonistic receptor–receptor interactions produced by activation of the

CCR2, CXCR4, and/or IL1R2 protomers in the plasma membrane. Their agonist ligands are released by the microglia and/or immune cells and/or astroglia into the extracellular fluid to activate these chemokine and cytokine receptor protomers in the plasma membrane. The current findings based on the triplet puzzle theory indicate that the CCR2, CXCR4, and/or IL1R2 can also interact with D2Rs through such mechanisms. Based on the antipsychotic actions of D2R antagonists, it is proposed that enhancing allosteric receptor–receptor interactions develop in the D2R-CCR2, D2R-CXCR4, and D2R-IL1R2 heteromers upon agonist activation of these chemokine and cytokine receptors leading to increases in D2R protomer signaling with development of schizophrenia-like symptoms.

It will be of high interest to test in cellular models if, in fact, the NMDAR and D2R heteroreceptor complexes with CCR2, CXCR4, and/or IL1R2 protomers are formed using the BRET methodology and the postulated allosteric receptor–receptor interactions develop. We will also test if these NMDAR and D2R heteroreceptor complexes exist in the ventral and dorsal striatum in models of neuroinflammation using the *in situ* proximity ligation assay and if the proposed allosteric receptor–receptor interactions occur in these brain regions upon neuroinflammation with or without agonist ligands for the CCR2, CXCR4, and/or IL1R2 protomers.

CONCLUDING REMARKS

It is proposed that the following mechanisms can contribute to schizophrenia-like symptoms in mild neuroinflammation:

- Extracellular vesicle-mediated VT with receptor and ligand transfer from glial networks to neuronal networks involving distinct cytokine and chemokine receptors and their agonist ligands can lead to formation of dysfunctional and separate NMDAR and D2R heteroreceptor complexes containing CCR2, CXCR4, and/or IL1R2 according to the triplet puzzle theory. The agonist ligands for these three receptors may produce allosteric receptor–receptor interactions in these dysfunctional complexes reducing NMDAR and increasing D2R signaling in the plasma membrane. Schizophrenia-like symptoms may, therefore, develop.
- However, there is no consensus as to which psychopathological symptoms are specific for schizophrenia. There is in fact clear evidence that symptoms in any type of encephalitis are in principle non-specific and variant. As far as the mechanisms proposed in the current paper, they may, from a theoretical perspective, contribute not only to schizophrenia symptoms but may also participate in bipolar disorder and other affective disorders associated with mild neuroinflammation.
- The hypothesis introduced on the formation of distinct NMDAR–cytokine receptor/chemokine receptor and D2R–cytokine receptor/chemokine receptor heterocomplexes with pathological receptor–receptor interactions in the brain upon mild neuroinflammation will primarily be tested as follows: the possible existence of the putative and distinct NMDAR and D2R heteroreceptor complexes will be studied in cellular models and brain models of neuroinflammation using proximity ligation assay and BRET. Then, it will be tested in these models if their allosteric receptor–receptor interactions will lead to a reduction of NMDAR signaling and to increases in D2R signaling in the above heteroreceptor complexes. Finally, if positive results are obtained, the critical role of the demonstrated sets of triplet amino acid homologies for the formation of these heteroreceptor complexes and their receptor–receptor interactions will be tested through mutations of these triplet homologies.

AUTHOR CONTRIBUTIONS

KF made substantial contributions to the conception and design of the work. He participated in the manuscript writing and critically evaluated it. He agreed with the submission to Frontiers of the current version. DOB-E made substantial contributions to the conception and design of the work, specially the design and conception of the figures. He prepared the reference list and participated in the manuscript writing and critically evaluated it. He agreed with the submission to Frontiers of the current version. AOT made substantial contributions to the conception and design of the work, specially the mathematical and bioinformatic analysis of the amino acid protriplets. He participated in the manuscript writing and critically evaluated it. He agreed with the submission to Frontiers of the current version. KB made substantial contributions to the conception and design of the work. He participated in the manuscript writing and critically evaluated it. He agreed with the submission to Frontiers of the current version.

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Autoimmune Aspects of Neurodegenerative and Psychiatric Diseases: A Template for Innovative Therapy

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Neurodegenerative and psychiatric diseases (NPDs) are today's most important group of diseases, surpassing both atherosclerotic cardiovascular disease and cancer in morbidity incidence. Although NPDs have a dramatic impact on our society because of their high incidence, mortality, and severe debilitating character, remarkably few effective interventions have become available. The current treatments, if available, comprise the lifelong intake of general immunosuppressants to delay disease progression or neurotransmitter antagonists/agonists to dampen undesired behaviors. The long-term usage of such medication, however, coincides with often severe adverse side effects. There is, therefore, an urgent need for safe and effective treatments for these diseases. Here, we discuss that many NPDs coincide with subtle chronic or flaring brain inflammation sometimes escalating with infiltrations of lymphocytes in the inflamed brain parts causing mild to severe or even lethal brain damage. Thus, NPDs show all features of autoimmune diseases. In this review, we postulate that NPDs resemble autoimmune-driven inflammatory diseases in many aspects and may belong to the same disease spectrum. Just like in autoimmune diseases, NPD symptoms basically are manifestations of a chronic self-sustaining inflammatory process with detrimental consequences for the patient. Specific inhibition of the destructive immune responses in the brain, leaving the patient's immune system intact, would be the ultimate solution to cure patients from the disease. To reach this goal, the primary targets, e.g., the primary self-antigens (pSAgs) of the patient's chronic (auto)immune response, need to be identified. For a few major NPDs, immunological studies led to the identification of the pSAgs involved in the autoimmune damage of specific brain parts. However, further research is needed to complete the list of pSAgs for all NPDs. Such immunological studies will not only provide crucial insights into NPD pathogenesis but also ultimately enable the development of a new generation of safe and effective immunotherapies for NPDs. Interventions that will dramatically improve the life expectancy and quality of life of individual patients and, moreover, will significantly reduce the health-care costs of the society in general.

Keywords: neurodegenerative disease, psychiatric disease, chronic inflammation, immune tolerance, self-antigen, viral vector, reverse vaccine

THE CAUSE OF DEGENERATIVE DISEASES

The immune system comprises an intricate network of tissues, cells, and molecules responsible for responding to pathogen infection, malignant cells, and wounding, and thus for maintaining the body's homeostasis.

The first line of active immunity, named innate immunity, is activated by general evolutionary conserved pathogen-associated molecular patterns (PAMPs) released from infected cells or damage-associated molecular patterns (DAMPs) released by malignant and wounded cells (1). Cells of the innate immune system carry a variety of cytoplasmic and membrane-bound pattern recognition receptors to sense and respond to PAMPs and DAMPs. Upon binding of these alarm signals, the innate immune cells remove the cognate pathogens, damaged, or malignant cells and, when necessary, activate the second line of immunity, named adaptive immunity. Our adaptive immune system consists of humoral and cellular components. Humoral adaptive immunity is mediated by B lymphocytes (B cells), which secrete antibodies, whereas cellular adaptive immunity is mediated by T lymphocytes (T cells), which secrete cytokines and are capable of destroying damaged, malignant, or infected cells. The adaptive immune system acts *via* the activation of antigen-specific cell clones, which means that each clone of B or T cells bears a different receptor that binds antigenic peptides (epitopes) with a high specificity. Where B cells directly bind antigen epitopes, recognition by T cells requires presentation of antigen epitopes on major histocompatibility complex molecules.

During homeostasis, the adaptive immune system is in the tolerance mode. Immune tolerance is actively maintained by homeostatic interactions between somatic cells and innate immune cells with lymphocytes wherein the lymphocytes have a regulatory role by suppressing immune responses to self and foreign antigens (2, 3).

When PAMPs or DAMPs released by infected, wounded, and malignant cells are perceived by innate immune cells, inflammasomes assemble in the cytoplasm and a local inflammatory response is initiated. Inflammation is a highly orchestrated cascade of protective local and systemic events aimed at confining the pathogen, reducing the cell damage, promoting wound repair, and removing malignant cells (4). When all necroptotic cellular components, PAMPs, and DAMPs are removed, the inflammation stops and the homeostasis is restored. Usually, in healthy individuals, the repair activity of the innate immune system is sufficient to maintain homeostasis.

However, in the case the infection is too widespread, the wound is too big or the malignancy grows too fast, and the innate immune cells are not able to remove all the necroptotic cellular components, PAMPs, and DAMPs in time, cells of the adaptive immune system will infiltrate the inflamed area, locally break the immune tolerance in an antigen-specific manner, and destroy the infected, injured, or malignant cells (5). The destroyed and thus necroptotic cells further enhance the cellular immune response and speed-up the wound repair process (6). Again, after such a normal wound repair response, when all necroptotic cellular components, PAMPs, or DAMPs are removed, inflammation stops and the tolerance toward self will be restored (7).

In the case an adaptive immune response to self for whatever reason remains active, the wound repair process becomes self-sustaining and chronic. Chronic or flaring immune responses to tissue-specific antigens coincide with amyloid plaque formation, hypervascularization, fibrosis, and tissue scarification (jointly named sclerosis) at the site of inflammation. Wound healing is a beneficial process, but chronic self-sustaining wound healing is detrimental to the affected tissue and will result in the development of a degenerative disease (8–11). In this process, the self-antigen-specific chronic or repeated stimulation of a cellular immune response is the cause and driver of pathogenesis and thus disease progression (12).

The incidence of degenerative diseases associated with chronic or flaring immune responses, such as obesity, diabetes mellitus, arthritis, atherosclerotic cardiovascular disease, and NPDs, is increasing rapidly in our aging population. The reason for this increase has remained unclear. With a global population of more than seven billion individuals, we are a highly successful mammalian species. Several hypotheses have been formulated to explain our success. One of them says that we have developed an extremely efficient immune system that enables us to reach a high age under extremely challenging biotic and abiotic environmental conditions. The down-side of having such an efficient or "tensed" immune system is the increased risk of developing undesired immune responses to self-components, resulting in autoimmune diseases, or to harmless non-self-components, resulting in allergies.

THE CAUSE OF THE CAUSE OF DEGENERATIVE DISEASES

The majority of degenerative diseases are sporadic (idiopathic or acquired) diseases, and because the immune responses in patients are directed to tissue-specific self-antigens, these diseases are classified as autoimmune diseases. Examples of well-studied autoimmune diseases include diabetes mellitus type 1 (DM1), rheumatoid arthritis (RA), and multiple sclerosis (MS). For these diseases, the causal relationships between chronic immune activation, pathogenesis, and disease symptom development have been demonstrated in experimentally induced animal models. Autoimmune diseases are the result of an interplay between environmental stimuli, such as diet, lifestyle, trauma, exposure to microbes, pathogens, or toxic compounds (smoking), and the possession of predisposing gene alleles, which ultimately at a certain moment in time "trigger" a sustained loss of self-tolerance resulting in an immune-mediated damage of autologous tissues (13). Frequently, patients with an autoimmune disease have an increased susceptibility to develop other autoimmune diseases. Genes involved in innate or adaptive immunity, such as antigen uptake, processing, presentation, and signaling, represent the strongest predisposing genetic factors (14). This indicates that exposure to environmental trigger stimuli is the cause of aberrant immune responses to self-antigens, which on its turn is the cause of the development of the characteristic disease symptoms.

In patients with a pathogen-induced degenerative disease, such as viral hepatitis and the acquired immune deficiency syndrome, the causal trigger stimuli are chronic infections resulting in a continuous release of PAMPs by the infected and necroptotic cells. This results in chronic self-sustaining immune reactions directed against antigens derived from the pathogen. In case the pathogen-derived antigens mimic self-antigens (molecular mimicry), a chronic immune response will be induced in the tissue that expresses the mimicked self-antigens. There are indications that retroviral elements and chronic infections with herpesviruses such as Epstein–Barr virus or cytomegalovirus are associated with a number of autoimmune diseases, including NPDs with relapsing and remitting phases. Harmless bacteria may elicit an erroneous chronic immune response. In principle, such a response to harmless microbe-derived antigens is an allergic response. When the microbe-derived antigens mimic self-antigens, autoimmune diseases may be induced in predisposed individuals. Examples of bacteria-derived NPDs are the Guillain–Barré syndrome caused by *Campylobacter jejuni* and Lyme disease caused by tick-borne *Borrelia burgdorferi* infections.

The majority of degenerative diseases, however, result from an improper restoration of immune tolerance to specific self-antigens after a normal wound repair response. The incapacity to restore immune tolerance in a particular organ or tissue may occur when DAMPs are over-abundantly released inducing a hyperimmune response, when DAMPs continuously keep emerging, or when lymphocytes and tissue cells interact aberrantly during a wound repair response.

In humans with a degenerative disease, it is almost impossible to identify the primary targets of the activated T cells (the primary self-antigens, pSAgs), since the sustained destructive immune responses manifest often years after the onset, basically as the characteristic disease symptoms resulting from the tissue damage. Most efforts to identify the pSAgs of a degenerative disease have been dedicated to verifying humoral immune responses in patients with the disease. These studies have revealed the presence of autoantibodies in the sera of patients that usually bind to intracellular components which are released in the blood after tissue degeneration. Because the studies are performed with blood samples from patients who have the disease for a relatively long time, many of the identified antibody targets represent secondary self-antigens, which may also be post-translationally modified by the pathogenic process.

Acute autoimmune encephalitis is a rare group of NPDs diagnosed by the presence of autoantibodies in the sera of patients that bind hippocampal self-antigens (15). In approximately half of the cases, the encephalitis is caused by the presence of tumors in patients that express and present neo-antigens that are identical to the hippocampal self-antigens. In the other half of the cases, it is assumed that infections with unknown viruses or microbes are the cause of the encephalitis (16, 17).

For a long time, the role of cellular immunity in the tissue destruction has been underestimated. Only recently, it has become clear that T cells play a predominant role in the tissue destruction in patients with a degenerative disease. For a number of degenerative diseases, studies on the variability of T cells that infiltrate the inflamed tissue of a patient, like, for example, the anterior horns of the spinal cords of amyotrophic lateral sclerosis (ALS) patients and the hippocampus of epilepsy patients, revealed that the infiltrating T cells comprise a single or few cell clones, indicating that a cellular adaptive immune response to one or few self-antigens is responsible for the autoimmune tissue damage (18, 19). Therefore, immunological studies focusing on cellular immune responses to self-antigens, preferably in early onset patients, may be the preferred method to identify the pSAgs of the disease.

A more feasible approach to demonstrate the causal relationship between chronic immunity to a pSAg and disease symptom development is to immunize animals with a pSAg of a degenerative disease. Immunization of animals with a secondary self-antigen does not lead to the induction of disease symptoms, because there is a massive immune tolerance present and actively maintained to these proteins in the non-inflamed body parts. The now classical immunization experiments with pSAgs have not only led to the identification of the pSAgs of degenerative diseases but also to the development of the currently used and most valuable inducible animal models of human degenerative diseases, such as the myelin oligodendrocyte glycoprotein (MOG)/myelin basic protein (MBP)-induced rodent and marmoset experimental autoimmune encephalitis (EAE) models resembling human MS, the proinsulin-induced mouse model of human DM1 and the collagen type 2-induced rodent and marmoset collagen type 2-induced arthritis models resembling human RA. The pSAgs of degenerative diseases identified thus far are tissue-specific extracellular matrix proteins, cytoskeleton-associated proteins, or components involved in intercellular signaling. Secondary self-antigens are generally cytoplasmic proteins present in different tissues and are targeted relatively late by the patients' immune system when the affected tissue is chronically inflamed in a process referred to as epitope spreading.

In analogy to the Koch's postulates for identifying the causal relation between pathogens and infectious diseases, pSAgs of degenerative diseases have to fulfill the following three criteria:

1. the pSAg of a degenerative disease is predominantly or exclusively expressed in the affected tissue.
2. induction of an adaptive immune response to the pSAg in an animal results in the development of the disease.
3. all patients have an adaptive immune response to the pSAg, while these are absent in healthy people.

With the exception of MS, the role of the immune system in NPD pathogenesis is relatively poorly studied. Only for a few NPDs solid immunological studies in animals and patients have been employed to identify the pSAgs of the disease. Immunological follow-up studies for other NPDs are highly desired, because they will not only provide key insights into the pathogenesis of this group of diseases but also result in new targets for drug development.

THE CANDIDATE pSAgs OF NPDs

Neurodegenerative and psychiatric diseases are associated with chronic or flaring inflammation of specific brain areas with infiltration of peripheral immune cells, resulting in mild or severe

brain damage that leads to the development of the characteristic disease symptoms. Based on the type of neurotransmitter used for signaling by the affected neurons the diseases can be categorized in groups (See **Table 1**).

In patients with a familial neurodegenerative disease, such as Huntington disease (HD), and the familial forms of Alzheimer's dementia (AD), Parkinson disease (PD), ALS, and epilepsy with accumulated genetically predestinated cellular defects, DAMPs originating from necroptotic cells continuously emerge in a specific brain part. As a consequence of this, a self-sustaining immune response to tissue-specific self-antigens is induced in the brains of patients. However, a major part of the neurodegenerative diseases (and all psychiatric diseases) is sporadic, meaning that the trigger environmental stimulus is unknown.

Neurodegenerative diseases are the result of the presence of predisposing gene alleles (risk genes) interacting with unknown environmental stimuli, such as trauma, infection, or exposure, to toxic compounds. The immune reactions in neurodegenerative diseases (HD, PD, MS, AD, ALS, and epilepsy) usually are severe and associated with massive amyloid deposition (huntingtin, alpha-synuclein, Abeta, tau, TDP-43, etc.) and sclerosis at the affected brain part, often resulting in a fatal loss of function.

Psychiatric diseases are the result of the presence of risk genes determining the personality interacting with environmental stimuli, such as psychosocial factors, lifestyle, and stress. In contrast to neurodegenerative diseases, the CNS inflammation and immunity in the psychiatric diseases is mild, with barely detectable damage to the affected brain part [attention-deficit hyperactivity disorder (ADHD), addiction, reward deficiency syndrome (RDS), depression, anxiety, autism spectrum disorder (ASD), schizophrenia, and Tic disease (TD)] (20).

Neurodegenerative and psychiatric diseases can be progressive (HD, PD, ADHD, RDS, AD, ALS, and ASD) or relapsing-remitting (anxiety, depression, MS, epilepsy, schizophrenia, and TD). For these diseases, the immune responses flare. During relapses, the immune tolerance is broken and the symptoms are worse and during remissions, the immune tolerance is intact and the disease does not progress.

For the majority of NPDs, the pSAgs involved in the brain damage await identification and there is only indirect evidence what they could be (See **Table 1**, last column). For this latter group of diseases imaging, pharmacological and genetic studies using knockout and knockdown mouse mutants revealed a number of candidate pSAgs, but immunological studies as outlined in the previous paragraph are needed to confirm their role in the disease pathology.

Group 1: Cholinergic NPDs

Multiple Sclerosis

Multiple sclerosis is characterized by chronic inflammation and degeneration of cholinergic myelinated axons in the brain and spinal cord, resulting in functional disability and premature death (21). Analysis of the T cell receptors (TCRs) present on the surface of cytotoxic T lymphocytes (CTLs) that infiltrate the inflamed brain parts revealed that these CTLs have a strongly biased TCR repertoire compared to splenic CTLs. This suggests that the infiltrating CTLs recognize a single or few self-antigens (8, 22). Immunization of animals with MOG or MBP or peptides derived thereof results in the development of EAE that shares similarities with human MS (23). Since MOG and MBP fulfill the three criteria outlined in Section "The cause of the cause of degenerative diseases," these two proteins are candidate pSAgs involved in the autoimmune inflammatory/demyelination of cholinergic neurons in the CNS white matter MS patients.

Alzheimer's Dementia

Alzheimer's dementia is the most common cause of aging-related dementia, associated with chronic inflammation and

TABLE 1 | Candidate primary self-antigens (pSAgs) of neurodegenerative and psychiatric diseases.

Group	Neuron type	Disease	Affected brain part	pSAg
1	Cholinergic	Multiple sclerosis	White matter	Myelin oligodendrocyte glycoprotein, myelin basic protein
		Alzheimer's dementia	Basal forebrain	Neurofilament heavy
		Amyotrophic lateral sclerosis	Motor neurons	Neurofilament light
		Autism spectrum disorder (autism, Asperger syndrome, PDD-NOS)	Striatum, cerebellum	Muscarine acetyl choline receptors
2	Dopaminergic	Huntington disease	Striatum	Huntingtin
		Parkinson disease	Substantia nigra compactum and VTegm	Vesicular monoamine transporter-2
		Attention-deficit hyperactivity disorder	Mesostriatal system	Dopamine transporter-1
		Reward deficiency syndrome (addiction, obsessive–compulsive disorder)	Striatum	Dopamine receptor-1, 2, 3
3	Glutamatergic	Epilepsy (temporal lobe epilepsy)	Hippocampus	metabotropic Glutamate receptor 3
		Schizophrenia	Hippocampus	N-methyl-D-aspartate receptor
4	Histaminergic	Tic diseases (Tourette syndrome)	Basal ganglia	Histamine 3 receptor
5	Serotonergic	Depression	Prefrontal cortex	5-hydroxytryptamine transporter
		Anxiety	Brain stem	5-hydroxytryptamine receptor 2C

The identification of the candidate pSAgs is based on the combination of different lines of research as indicated in Section "The Candidate Primary Self-Antigens of NPDs."

T lymphocyte-mediated degeneration of cholinergic myelinated large axons in the basal forebrain, resulting in severe cognitive impairment and eventually death (24–26). Rats immunized with adjuvated extracts of cholinergic neurons from the electric fish, Torpedo, develop experimental autoimmune dementia (EAD) resembling human AD (27). It was found that the intermediate neurofilament heavy (NFH) protein is the most likely pSAg in these extracts, capable of inducing EAD in rats (28). Almost all diagnosed AD patients have a humoral immune response to NFH, but not to the intermediate neurofilament light (NFL) protein (29, 30). Since NFH fulfills the three criteria outlined in Section "The cause of the cause of degenerative diseases," this neurofilament is a candidate pSAg involved in the autoimmune cortex destruction in AD patients.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis is characterized by progressive muscle weakness (paresis), disability, and eventually death, with a median survival of 3–5 years. The motor cortex and the anterior horn of the spinal cord of ALS patients are chronically inflamed and infiltrated with macrophages and CTLs, resulting in the loss of cholinergic myelinated motor axons (31–35). Immunization of guinea pigs with adjuvated bovine motor neuron extracts induces experimental autoimmune motor neuron disease (EAMD) resembling human ALS (36). Immunization of mice with NFL results in the development of spastic paresis resembling EAMD (37). It has further been reported that almost all diagnosed ALS patients have antibodies against NFL (38–40). Since NFL fulfills the three criteria outlined in Section "The cause of the cause of degenerative diseases," it is believed that neurofilament is a candidate pSAg involved in the autoimmune destruction of motor neurons in ALS patients.

Autism Spectrum Disorders

Autism spectrum disorder, including autism, the Asperger syndrome and the pervasive developmental disorder-not otherwise specified (PDD-NOS) represent a group of NPDs involving cholinergic neurons in the dorsomedial striatum and cerebellum characterized by often severe repetitive behaviors and cognitive inflexibility manifested as an impaired development of social interaction and communication skills and a markedly restricted repertoire of activity and interest. ASD coincides with chronic inflammation and altered immune responses in the affected brain parts (41–43). Studies in rats demonstrated that antagonists of muscarine acetyl choline receptors (mAChRs) increase and mAChR agonists reduce the repetitive behaviors (44, 45). Treatment of mouse models of human ASD with acetylcholine esterase inhibitors resulting in increased synaptic acetylcholine levels leads to an improvement of the behavioral rigidity (46). Destruction of cholinergic neurons in the dorsomedial striatum leads to increased repetitive behaviors that can be alleviated by treatment with M1 mAChR agonists (47), suggesting that the loss of cholinergic signaling by the autoimmune damage to the M1 mAChR-bearing neurons in the dorsomedial striatum results in the development of ASD.

Group 2: Dopaminergic NPDs

Huntington Disease

Huntington disease is a progressive and usually fatal neurodegenerative disease of the striatum associated with the gradual loss of dopaminergic striatal neurons involved in motor coordination and subcortical dementia. The disease is caused by mutations in the gene encoding huntingtin, resulting in the chronic deposition of huntingtin amyloid in the striatum that coincides with a chronic immune response to a striatum-specific self-antigen (possibly huntingtin) and a gradual loss of dopaminergic striatal neurons (48).

Parkinson Disease

Parkinson disease is the most common neurodegenerative disease at younger age. PD symptoms include tremor, uncontrolled movements (bradykinesia), muscle stiffness, impaired posture and balance, loss of automatic movements, addiction, and loss of cognitive functions. PD coincides with chronic inflammation and T lymphocyte infiltration of the dopamine-producing neurons in the substantia nigra compactum and ventral tegmentum (49) and a gradual loss of dopamine-producing neurons (50–52). Immunization of mice or guinea pigs with dopaminergic neuronal cells or substantia nigra neurons results in the development of experimental autoimmune nigral damage with symptoms resembling human PD (53, 54). PD has been associated with the loss of vesicular monoamine transporter-2 (VMAT2) in dopamine-producing neurons resulting in elevated levels of cytoplasmic dopamine, which is highly neurotoxic (55, 56). Injection of animals with the VMAT2 antagonist 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to necroptosis of dopamine-producing neurons due to the accumulation of neurotoxic dopamine in the cytoplasm. The necroptotic dopamine-producing neurons subsequently induce a chronic T cell-mediated and nigral-specific immune response that results in the development of the characteristic PD symptoms (51, 57). MPTP-induced PD in mice and marmosets are generally considered the most accurate animal models of human PD. VMAT2-knockout mice display the pathology and symptoms of PD, whereas VMAT2-knockdown mice are highly susceptible to MPTP-induced PD induction (58). On the contrary, animals overexpressing VMAT2 have increased striatal dopamine levels and are MPTP-resistant (59). These genetic, pharmacological, and immunological studies strongly suggest that VMAT2 expressed at high levels in dopamine-producing neurons is a candidate pSAg involved in the autoimmune destruction of dopamine-producing neurons in PD patients (60).

Attention Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder symptoms include difficulties staying focused and paying attention, impulsivity (difficulty in controlling behavior), and hyperactivity. ADHD patients have inflammation markers in the blood (61) and white matter loss in the striatal, frontal, and parietal, dopamine-innervated brain areas (62–64). Mice with knockout and knockdown mutations in the dopamine transporter-1 (DAT1) gene show severe behavioral changes, including hyperactivity, memory, repetitive behavior, and learning deficits, mimicking human

ADHD (65–68). Immunization of mice with DAT1 results in the development of characteristic ADHD symptoms (64). In addition, ADHD patients have a humoral immune response to DAT1 (69). Since DAT1 fulfills the three criteria outlined in Section "The Candidate Primary Self-Antigens of NPDs," it is believed that transporter is the pSAgs involved in the autoimmune damage of the dopaminergic striatal and cortical neurons in ADHD patients. Furthermore, these studies suggest that DAT1 is predominantly expressed in post-synaptic neurons in the striatum and frontal–parietal brain areas that are innervated by dopamine-producing neurons.

The Reward Deficiency Syndrome

Reward deficiency syndrome comprises substance addiction and the obsessive–compulsive disorder (OCD). Substance addiction resides in the dopaminergic neurons of the nucleus accumbens in the striatum and coincides with increased inflammation markers in the affected compartment (70, 71). The chronic and disabling obsessional thoughts and compulsive rituals of OCD patients are associated with hyperactivity of the ventral cognitive circuit, involving dopaminergic neurons in the striatum (72, 73). OCD coincides with inflammation markers in the blood and the striatum (74–77). Drug use leads to elevated levels of dopamine, increased dopamine receptor-1 (DAR1), and decreased DAR2 signaling in the striatum that results in stimulation of the reward circuitry (78). In drug addicts, striatal DAR2 levels are reduced compared to those in healthy individuals (79). Mutations in the gene encoding DAR2 which render the receptor less sensitive to dopamine are associated with addictive behavior (80). Downregulation of DAR2 in rats promotes the reward deficits resulting in addictive behavior, such as compulsive food-seeking (81). DAR2 knockout mice display elevated DA synthesis resulting in hyperlocomotion and supersensitivity to drugs, such as cocaine (82). DAR2 overexpression in the nucleus accumbens of rats attenuates addictive behavior (83). These studies together with genetic and pharmacological studies on DAR3 (84–86) suggest that a dopamine receptor from the DAR2 family is the most likely candidate pSAg of addiction. Pharmacological studies in mice using dopamine receptor-1 agonists show that these drugs induce complex movement sequences such as grooming, resembling human OCD (87). Overall, the studies on dopamine receptors suggest that they are the candidate pSAgs for the reward-associated NPDs, addiction, and OCD.

Group 3: Glutamatergic NPDs

Epilepsy

Epilepsy is a group of diseases characterized by convulsive or absence seizures, caused by an improper functioning of glutamatergic cortical neuron ion channels. The most common form of epilepsy is temporal lobe epilepsy (TLE) in which the seizures occur in the hippocampus of patients. Repeated seizures in epilepsy patients are associated with immune responses, resulting in hippocampal necroptosis and/or cortical damage at the site of the seizures (88–90). Treatment with the strong mAChR agonist pilocarpine results in N-methyl-D-aspartate (NMDA) receptor (NMDAR)-mediated excitotoxicity of hippocampal glutamatergic neurons (91). The degeneration of glutamatergic neurons coincides with severe epileptic seizures. Pilocarpine-induced epilepsy in rodents is, therefore, generally considered the most accurate animal model of human TLE (92). Patients with severe epilepsy symptoms known as Rasmussen encephalitis show a humoral immune response to metabotropic Glutamate receptor 3 (mGluR3) (93), whereas the affected brain parts are also infiltrated by CTLs (94, 95). The CTLs isolated from the inflamed brain parts of patients show a limited TCR repertoire, suggesting that these CTL's are clonally derived and recognize a single or few self-antigens (19, 96). Agonists of the metabotropic group II receptors (mGluR2 and mGluR3) inhibit glutamate release in synapses of the glutamatergic hippocampal neurons of epilepsy patients and are potent anticonvulsants against motor and absence seizures (97), indicating that a shortage of metabotropic group II receptors may cause the generation of seizures in epilepsy patients. Immunization of rabbits with mGluR3 induces epileptic seizures (98). Immunization of rabbits with mGluR3 (but not mGluR1, 2, 5, or 6) results in the development of seizures and histopathological changes that mimic Rasmussen's encephalitis (99). These immunological and pharmacological studies indicate that mGluR3 is the most likely pSAg involved in the destruction of hippocampal and cortical neurons in epilepsy patients.

Schizophrenia

Schizophrenia patients suffer from delusions, hallucinations, a distorted awareness, and disorganized thinking during psychotic episodes. Schizophrenia is associated with immune responses and lymphocyte infiltrations in the hippocampus and a reduced hippocampal gray matter size (100–106). It has been suggested that reduced striatal glutamate signaling increases the risk of sensory overload and of exaggerated responses in the monoaminergic system, consistent with schizophrenia symptoms. NMDAR antagonists, such as phencyclidine (PCP), induce schizophrenia symptoms, whereas NMDAR agonists are schizophrenia medicines. Mice mutants with reduced NMDAR levels develop symptoms resembling schizophrenia, which can be overcome by treatment with NMDAR agonists (107, 108). This suggests that NMDAR is a candidate pSAg involved in the striatal damage of schizophrenia patients (109).

Among the acute autoimmune encephalopathies anti-NMDAR encephalitis is the most prevalent. Patients generally show epileptic and/or schizophrenic symptoms indicating that hippocampal glutamatergic circuits are affected. It has remained unknown whether NMDAR is the pSAg or a secondary self-antigen of the disease. The encephalitis is treated by removal of the coinciding ovary teratoma that presents hippocampal pSAgs or by using anti-inflammatory drugs. The few remaining therapy-resistant anti-NMDAR encephalitis patients represent epilepsy and/or schizophrenia patients depending on the disease symptoms shown (110).

Group 4: Histaminergic NPDs

Tic Disease

Patients with a tic disease including the Tourette syndrome show spontaneous short muscle contractions resulting in movements

(motor tics) or vocals (vocal tics). Markers of inflammation are found in the basal ganglia and in the peripheral blood. Histaminergic neurons in the basal ganglia are targeted by the patient's immune system (111, 112). Mice with a knockout mutation in the histidine decarboxylase gene that lack the capacity to produce histamine show tic-like stereotypes (113). These genetic and pharmacological studies suggest that the histamine 3 receptor is the pSAg of TD (114).

Group 5: Serotonergic NPDs

Depression

Depression comprises bipolar or manic depression and unipolar or major depression. Patients usually undergo periods of mania and/or often severe depression. Depression is associated with decreased serotonergic activity in the prefrontal cortex, amygdale and/or hippocampus, inflammation markers in the brain and periphery, and a reduced size of the prefrontal cortex (115, 116). Imaging and post mortem studies in patients with severe depression revealed damage of specific parts in the prefrontal cortex (117–119) associated with leukocyte infiltrates from the periphery (120, 121). Depression is caused by a shortage of 5-hydroxytryptamine (5-HT) transporter (5-HTT) activity resulting in overstimulation of glutamatergic neurons in the prefrontal cortex upon stress (122). Indeed, 5-HTT knockout mice and rats show depression-related behavior with impaired neural plasticity (123, 124), suggesting that 5-HTT is the pSAg of depression. Selective serotonin reuptake inhibitors (SSRIs) are widely-prescribed to treat depression (125). The genetic studies using 5-HTT knockout animals, therefore, seem to be in conflict with the pharmacological studies using SSRIs. A possible explanation for this discrepancy could be that SSRIs serve as 5-HT receptor (5-HTR) agonists rather than 5-HTT antagonists (126).

Anxiety

Anxiety includes general anxiety, panic disease, and phobias. Anxiety coincides with decreased serotonergic activities in the raphe nuclei in the brain stem (127, 128). Studies using mouse strains with knockout or conditional knockout mutations in genes involved in serotonin or 5-hydroxytryptamine (5-HT) metabolism and strains overexpressing these genes, combined with pharmacological studies using SSRIs revealed that anxiety is caused by a shortage of 5-HT perception, resulting in defective glutamate signaling in the prefrontal cortex upon stress. These studies suggest that one of the serotonin receptors, probably receptor 2C (5-HTR2C) is the pSAg of anxiety (129).

NOVEL IMMUNOTHERAPIES TO TREAT NPDs

In our aging population, degenerative diseases associated with aberrant immune responses, such as diabetes mellitus, arthritis, atherosclerotic cardiovascular disease, chronic obstructive pulmonary disease, and NPDs, have become highly prevalent (130–132). For only a few degenerative diseases more or less effective treatments have become available. To date, psychiatric diseases are treated with neurotransmitter antagonists/agonists that dampen undesired behaviors. A number of anti-inflammatory drugs, such as cyclooxygenase inhibitors, minocycline, omega-3 fatty acids, and neurosteroids, ameliorate the symptoms of psychiatric diseases, confirming the role of the patient's immune system in the pathogenesis (133). For the neurodegenerative diseases, anti-inflammatory drugs are not sufficient to ameliorate or significantly delay disease progression. Only MS drugs that inhibit the migration of lymphocytes from the periphery to the CNS decrease the frequency of disease relapses.

Overall, all current treatments, if available, non-specifically suppress inflammation and only decrease the frequency of relapses and thus alleviate disease symptoms. Long-term use of non-specific immuno-suppressing medication coincides with often severe adverse side effects and enhances the risk of developing cancer or autoimmune processes in other tissues. There is, therefore, an urgent need for novel treatments of degenerative diseases in general and NPDs in particular that specifically inhibit tissue damage caused by activated cells of the adaptive immune system, leaving the general immune response unaffected. In principle, this should be feasible since autoimmune diseases are acquired diseases by individuals with fully functional immune systems.

Restoration of the immune tolerance to the pSAgs involved in the autoimmune tissue destruction, also named reverse vaccination, has been a longstanding goal in autoimmunity research. This has been attempted by administration of the self-antigens or peptide fragments derived thereof to patients (134–137). However, naked proteins or peptides are rapidly degraded in the body. Linkage of peptides to nanoparticles to improve their stability results in a transient effect with a very narrow spectrum of activity. The results of such protein/peptide-based tolerization approaches pursued so far did not meet the expectations (138).

An efficient way to instruct immune cells for suppressing an autoimmune response is to let them produce the self-antigens involved in the autoimmune tissue destruction by introducing the self-antigen-encoding genes into these cells, in a non-inflammatory or tolerogenic environment, such as the liver.

For the best-studied autoimmune disease animal models (DM1, MS and RA), it has been shown that viral vector-mediated expression of the pSAgs of the disease protects the treated animals from the autoimmune disease both in prophylactic and therapeutic settings (139–142). Viral vector-mediated tolerization, e.g., reverse viral vector vaccination, therefore, has an enormous potential for effectively treating degenerative diseases, including NPDs.

To date, reverse viral vector vaccines have not been tested in the clinic. The main reason for this is the immunogenicity or lack of *in vivo* efficacy of the currently most popular viral vectors, adeno-associated viral vectors derived from adeno-associated virus (AAV), and lentiviral vectors derived from the human immunodeficiency virus type 1 (143, 144). AAV's immunogenicity in humans, and as a result clinical inefficacy, will remain the major challenge for the approval of new AAV-based interventions. Therefore, in order to develop efficient *in vivo* gene therapies and to efficiently restore immune tolerance in patients with a degenerative disease, a new viral gene delivery vector is needed. A new vector suitable for use in reverse viral vector vaccinations

should combine the *in vivo* efficacy of AAV vectors in animals with being non-immunogenic in humans.

Only for the well-studied autoimmune diseases, the pSAgs are known and have been used in preclinical reverse viral vector vaccination studies (139–142). For some NPDs, such as MS, AD, ALS, ADHD and epilepsy candidate pSAgs have been identified (see The Candidate Primary Self-Antigens of NPDs), but for other important NPDs no clear candidate pSAgs involved in disease progression have been identified. All patients with an autoimmune disease have an adaptive immune response to the pSAg of the disease. Immunization of animals with a pSAg of an autoimmune disease results in the development of the characteristic disease symptoms (23, 28, 37, 64, 98, 99). Therefore, in order to verify whether a candidate self-antigen is the pSAg of the disease, immunological studies as described above are needed.

Once the vector immunogenicity and pSAg identification hurdles are taken, reverse viral vector vaccination has the potential to yield a whole new range of therapeutics and even prophylactics addressing today's major diseases, which will dramatically change the medicine landscape.

This is particularly relevant for the highly mortal and severely debilitating NPDs. Effective reverse vaccines will halt neurodegeneration in patients with a neurodegenerative disease and will stop chronic or flaring inflammation in the CNS of psychiatric patients rendering them amenable to existing psychotherapy.

AUTHOR CONTRIBUTIONS

PH wrote the review. HK and BH equally contributed in editing the manuscript.

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REVIEW
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Microglia and Brain Plasticity in Acute Psychosis and Schizophrenia Illness Course: A Meta-Review

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Objective: Schizophrenia poses a tremendous health, social, and economic burden upon patients and society, indicating current treatment options remain inadequate. Recent findings from several lines of evidence have pointed to the importance of immune system involvement in not only premorbid neurodevelopmental but also subsequent symptom generation and aging processes of brain change in schizophrenia. In this meta-review, we use the summarized evidence from recent quantitative systematic reviews (SRs) and meta-analyses of several subspecialties to critically evaluate the hypothesis that immune-related processes shape the symptomatic presentation and illness course of schizophrenia, both directly and indirectly through altered neuroplasticity.

Methods: We performed a data search in PubMed for English language SRs and meta-analyses from 2010 to 2017. The methodological quality of the SRs was assessed with the AMSTAR instrument. In addition, we review in this paper 11 original publications on translocator protein (TSPO) positron emission tomography (PET) imaging in schizophrenia.

Results: We reviewed 26 SRs and meta-analyses. Evidence from clinical observational studies of inflammatory or immunological markers and randomized controlled drug trials of immunomodulatory compounds as add-on in the treatment of schizophrenia suggests psychotic exacerbations are accompanied by immunological changes different from those seen in non-acute states, and that the symptoms of schizophrenia can be modified by compounds such as non-steroidal anti-inflammatory drug and minocycline. Information derived from *post-mortem* brain tissue analysis and PET neuroimaging studies to evaluate microglial activation have added new perspectives to the available evidence, yet these results are very heterogeneous. Each research domain comes with unique opportunities as well as inherent limitations. A better understanding of the (patho-)physiology of microglial cells and their role in neuroplasticity is key to interpreting the immune-related findings in the context of schizophrenia illness exacerbations and progression.

Conclusion: Evidence from clinical studies analyzing patients' blood and cerebrospinal fluid samples, neuroimaging and *post-mortem* brain tissue suggests that aberrant

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immune responses may define schizophrenia illness' course through altered neuroplasticity representing abnormal aging processes. Most findings are however prone to bias and confounding, and often non-specific to schizophrenia, and a multidisciplinary translational approach is needed to consolidate these findings and link them to other schizophrenia hypotheses.

Keywords: schizophrenia, psychosis, microglia, neuroinflammation, neuroplasticity, translocator protein, positron emission tomography, *post-mortem* study

INTRODUCTION

Schizophrenia is a devastating and complex central nervous system (CNS) disorder that affects approximately 1% of the world population. Despite antipsychotic drugs being available for several decades, personal and societal costs remain highly expensive: the World Health Organization estimated that the direct costs of schizophrenia range between 7 and 12% of the gross national product in Western countries (1). Current treatment options are inadequate, both due to side-effects that disable patients and threaten their compliance to treatment (2) as well as the drugs' lack of efficacy evidenced by the absence of clinically relevant improvements on negative and cognitive symptoms, and up to one-third of schizophrenia patients not responding at all (3–5). Acute psychosis is common and relapse prevention represents an important treatment issue in schizophrenia. An estimated 82% of patients have an illness relapse within 5 years after recovery from first-episode psychosis (FEP), and a majority will experience multiple relapses throughout their illness course (6). Furthermore, a high relapse rate is associated with poorer outcomes, including more treatment-resistant symptoms, cognitive deficits, and functional disability (7, 8).

These problems emphasize the urgent need for novel therapeutic approaches implicating the identification of new pathophysiological substrates. The illness is syndromic, of heterogeneous presentation and course and while there are well-established criteria in place for making the diagnosis of schizophrenia, the causes are still under debate. This is partly due to the complexity of this human disease that cannot be wholly replicated in animal models (9), as well as the methodological difficulties related to studying brain functionality in living patients. With increased understanding of heritability and ongoing brain changes, a vulnerability–stress model (sometimes described as a two-hit model) has become a prominent hypothesis to explain the pathophysiology of schizophrenia, in which an interaction of genetic, epigenetic, and environmental risk factors is assumed to result in brain abnormalities that continue to generate abnormal responses throughout life upon exposure to relevant stressors (10). This presents a modification of the original neurodevelopmental hypothesis which proposed that "a fixed lesion early in life interacts with normal brain maturational events that occur much later" (11). Indeed, it now appears that the neurodevelopmental concept should be extended to encompass altered ongoing neuroplasticity (12–14) creating a background of susceptibility for new insults later in life to catalyze neurodegenerative effects.

In the late twentieth century, the investigation of astrogliosis as hallmark for an inflammatory reaction in the schizophrenic brain combined with the expansion of neuropsychiatric genetics shape the debate about the etiology of schizophrenia. The absence of neuroinflammation and the presence of heritable candidate genes were thought to indicate a neurodevelopmental origin and a static rather than a neurodegenerative disease course. Although the earliest study reported the presence of low-grade inflammation (15), subsequent efforts did not replicate this finding. Consequently, the consensus was that reactive astrogliosis is absent in schizophrenia. However, the refinements of immunohistochemistry and modern stereological microscopy have yielded evidence for glial changes and, at the same time, brain imaging studies have increasingly demonstrated that the schizophrenic brain is not static during its clinical course. Over the last decades, immune system involvement has increasingly been implicated in the pathophysiological processes in schizophrenia (16–18). It has been hypothesized to be a driving factor behind both psychotic relapses and the macroscopic brain changes that occur in schizophrenia, including the characteristic enlarged ventricle size and reductions in gray matter volume, whole-brain volume, and white matter anisotropy (14).

The field has now advanced enough for several major meta-analyses and systematic reviews (SRs) to have come out in recent years, each compiling the evidence derived from individual studies and large groups of subjects in a variety of sub-disciplines contributing to this work. This meta-review aims to condense the evidence that immunological factors are critically involved in the schizophrenia illness course—modifying symptomatology and prognosis due to progressive brain changes. We critically appraise the current literature, identifying the opportunities and methodological limitations arising from each of the different lines of evidence. We identify areas where crucial information is still lacking and defend the position that opportunities can emerge by adopting a multidisciplinary translational approach to consolidate current findings and link this field of research to other key hypotheses of schizophrenia research. As the schizophrenia psychoneuroimmunology literature is very large, the scope of this review is necessarily limited. The focus will be on specific topics in the schizophrenia literature on neuroinflammation:

Abbreviations: BPRS, Brief Psychiatric Rating Scale; CNS, central nervous system; CSF, cerebrospinal fluid; FEP, first-episode psychosis; GWAS, genome-wide association study; IL, interleukin; MIA, maternal immune activation; MA, meta-analysis; MRI, magnetic resonance imaging; PANSS, Positive and Negative Syndrome Scale; NAC, *N*-acetylcysteine; PET, positron emission tomography; SAPS, Scale for the Assessment of Positive Symptoms; SR, systematic review; TSPO, translocator protein.

(1) *in vivo* neuroimaging and (2) neuropathological studies of microglial activation; (3) systemic inflammation and its link to psychosis; (4) clinical trials of immunomodulatory drugs as add-on treatment; and (5) neuroplasticity. Other relevant domains such as epidemiological studies, genetic studies, animal studies and clinical studies on changes in related mechanisms such as the kynurenine pathway and oxidative stress markers have their own sub-literature, and will not be dealt with here.

METHODS

We undertook a qualitative "review of reviews" outlining the summarized evidence for immune system involvement in the illness course of schizophrenia. The PubMed database was searched for English-language publications from January 2010 to August 2017. The different search strings employed per domain are detailed below. For each of these, the PubMed filter "Article Type" was set to categories "Systematic Review" and "Meta-analysis." Besides the articles found through the PubMed database, a manual review of reference lists of included articles was performed to identify additional papers. A consensus was reached among authors on the studies retained or discarded on the basis of the following inclusion and exclusion criteria. The included articles were (1) meta-analyses and systematic quantitative reviews; (2) investigating immune-related alterations in the blood, cerebrospinal fluid (CSF), or brain (3) of patients meeting DSM-IV or DSM-V criteria for the schizophrenia spectrum. All included studies are summarized in **Table 1**. We excluded publications focusing on preclinical research, or premorbid immunological risk factors such as epidemiological or genetic background risks. Articles were excluded if they were: (1) narrative or qualitative review articles; (2) reviews on topics outside the scope of this paper (as indicated in the inclusion criteria); (3) reviews of *in vitro* or animal studies; (4) reviews of genome or gene expression studies; (5) reviews of epidemiological risk factors for schizophrenia; or (6) not published in English. We assessed the methodological quality of the SRs using the AMSTAR instrument (19); scores are presented in **Table 1**.

Meta-analyses of immunological or inflammatory changes in the blood, CSF and brain of schizophrenia patients were identified using the following search string: *schizophrenia[Title/Abstract] AND (*inflamm*[Title/Abstract] OR *immune*[Title/Abstract] OR cytokine*[Title/Abstract] OR chemokine*[Title/Abstract] OR *glia*[Title/Abstract])*. 82 articles were retrieved, of which 15 were included. Other studies were excluded because they did not study schizophrenia patients ($n = 15$); concerned genome or gene expression studies ($n = 11$); were narrative or qualitative review papers ($n = 13$); or were outside the scope of this paper ($n = 24$). Two additional articles were added manually after review of the reference list.

To identify meta-analyses pertaining to randomized controlled trials (RCTs) of anti-inflammatory agents in schizophrenia, the following search string was used: *schizophrenia[Title/Abstract] AND (RCT*[Title/Abstract] OR randomized[Title/Abstract]) AND (minocycline[Title/Abstract] OR NSAID[Title/Abstract] OR *inflamm*[Title/Abstract])*. This generated 11 articles, of which 8 were included. Three studies were excluded for the following reasons: does not concern clinical trials ($n = 1$); is a narrative review ($n = 1$); compound is outside the scope of paper ($n = 1$).

Because no meta-analysis or SR could be identified on positron emission tomography (PET) studies of microglial activation in schizophrenia, we chose to add the eleven original studies to this paper, as this is an important sub-discipline in this research field. These papers were identified using a Pubmed and Medline search executed in August 2017 without limitation of time, language, or publication type. The search string combined following terms: *(schizophrenia[Title/Abstract] OR psychosis[Title/Abstract]) AND (TSPO*[Title/Abstract] OR Translocator[Title/Abstract] OR *PK11195[Title/Abstract] OR *PBR*[Title/Abstract])*.

MICROGLIAL ACTIVATION INFLUENCING THE COURSE OF SCHIZOPHRENIA

The hallmark of neuroinflammation is the activation of microglia, the resident immune cells in the CNS (43), which are central to the current immune-related hypothesis in schizophrenia. Microglia are critically involved in the organization of the neuronal network during brain development (44) by primarily pruning excess synapses (45). Experimental models of schizophrenia showed that maternal infection during embryogenesis contributes to the presence of activated microglia in the offspring (46). This suggests that neonatal infections might induce perinatal microglial priming (47), a process in which highly sensitized microglia lead to an exaggerated response by example during the maturation processes occurring at the adolescent age and which can result in behavioral changes (48–50). This has led to the so called "microglia hypothesis" of schizophrenia, positioning exaggerated microglial activation as a key factor in the etiology of schizophrenia (17). In this hypothesis, exposure to inflammatory responses and/or genetically rooted excessive synapse pruning in the perinatal period (51) triggers an activated or primed state of microglia in adulthood (17), leading to ongoing systemic and central abnormal immune responses which drive schizophrenia symptoms such as psychotic episodes, and cause an unfavorable illness course, with progressive loss of function.

The presence of such ongoing microglial activation in schizophrenia patients has been supported both by some *post-mortem* studies as well as a few *in vivo* PET imaging studies using specific ligands (32, 52) that visualize and quantify microglial activation.

Neuroimaging Studies on Microglial Activation

PET radioligands for microglial activation have recently been developed allowing for the first time functional imaging of neuroinflammation *in vivo*. These radiotracers selectively bind to the 18-kDa translocator protein [translocator protein (TSPO), previously called peripheral benzodiazepine receptor], which is expressed on the outer mitochondrial membrane of microglia in their activated states. Interestingly, although the (patho-) physiological function of the TSPO, which is related to cholesterol transport for steroid production in microglia, is not well understood (53), these tracers have been proven very useful and

TABLE 1 | Summarized evidence of immune processes influencing the course of schizophrenia.

	Research methods	MA/SR	*N* studies	*N* patients	AMSTAR score (out of 11)	Outcomes
Clinical studies on peripheral expression of biomarkers and cells	Case–control study on blood and CSF inflammatory markers and immune cells (patients versus controls OR *post-mortem* comparison of patients in different states)	Miller (20): MA blood cytokine studies	40	1,324 SZ; 1,154 C	9	Trait and state increases of inflammatory cytokines; in schizophrenia patients, blood *trait* markers are IL12, IFNγ, TNFα, sIL2R, CRP, CD56 cells; *state* markers are IL1β, IL6, TGFβ, CD4 cells; in unmedicated FEP blood trait markers are TNFα, IL17, and IFNγ, state markers are IL6 and IL2, and possibly IL1β; in CSF increased IL1β, IL6, IL8
		Miller (21): MA blood lymphocyte studies	16	488 SZ; 525 C	9	
		Tourjman (22): MA blood cytokine studies and AP	23	762 SZ	6	
		Miller (23): MA blood CRP studies	8	N/A	N/A	
		Upthegrove (24): MA blood cytokine studies in drug-naïve FEP	23	570 SZ; 683 C	9	
		Guo (25): MA blood cytokine studies	21	N/A	N/A	
		Goldsmith (26): MA blood cytokine studies acute–chronically ill	40	N/A	N/A	
		Fernandes (27): MA blood CRP studies	71	85,000 SZ + C	N/A	
		Wang and Miller (28): MA CSF cytokine studies	16	N/A	N/A	
		Capuzzi (29): MA blood cytokine studies on effect of AP in FEP drug-naive patients	8	505 SZ	6	
	Case–control study on autoantibody titers	Ezeoke (30): MA antibody titer studies	81	N/A	8	Increased prevalence of positive titers for 20 different autoantibodies
		Monroe (31): MA *T. gondii* titer studies	16	2,535 SZ; 1,707 C	9	Increase in *T. gondii* IgM antibodies in acute psychosis
Post-mortem studies	Case-control study on histological or molecular findings in *post-mortem* brain tissue	Bakhshi (14): SR of neuronal density studies	30	360 SZ; 390 C	4	Age-dependent increases in neuronal density
		Najjar and Pearlman (32): SR white matter pathology and neuroinflammation studies	15	350 SZ; 346 C	5	Approximately half of *post-mortem* studies reported increased microglial markers
		Trepanier (33): SR neuroinflammation markers	119	N/A	4[a]	Similar patterns of heightened innate immune gene expression in both brain and blood in schizophrenia
		Hess (34): 2 mega-analyses of microarray data from *post-mortem* prefrontal cortices and *ex vivo* blood tissues	19 (brain) 25 (blood)	315 (brain) 578 (blood)	N/A	
		Van Kesteren (35): MA histological/ molecular immunological parameter studies	41	783 SZ; 762 C	9[a]	
Neuroimaging	Case–control study on PET imaging for microglial activation	Data summarized in **Table 2**	11	176 SZ; 175 C	N/A	Increased uptake of tracer with certain tracers and kinetic models in certain patient populations; results vary
Clinical trials	RCT with adjunctive anti-inflammatory drugs	Sommer (36): MA RCT NSAID	5	264 SZ	N/A	Results vary, significant effects have been found for aspirin, celecoxib, estrogens, NAC, and minocycline
		Nitta (37): MA RCT NSAID	8	774 SZ	10	
		Sommer et al. (38): MA RCT anti-inflammatory agents	24	1,938 SZ	8	
		Oya (39): MA RCT minocycline	4	330 SZ	10	
		Solmi (40): MA RCT minocycline	6	413 SZ	11	
		Xiang (41): MA RCT minocycline	8	548 SZ	9	
		Zheng (42): MA RCT celecoxib	8	626 SZ	9	

SZ, schizophrenia patient group; C, control group; n, number of subjects; FEP, first-episode psychosis patients; MA, meta-analysis; SR, systematic review; N/A, not applicable/not available; ES, effect size; RCT, randomized controlled trial; RR, relative risk; CSF, cerebrospinal fluid; PET, positron emission tomography; NSAID, non-steroidal anti-inflammatory drug; NAC, N-acetylcysteine.

[a]*Included results from a paper which was later retracted.*

are now increasingly being used for the prospective *in vivo* study of human microglial activation in several CNS conditions. This means the evolution of early and late inflammatory changes can be followed over months and correlated with symptom severity in clinical longitudinal studies. The technique also allows to monitor the effects of treatment and to evaluate new anti-inflammatory or neuroprotective strategies. The oldest and most frequently used tracer is PK11195 radiolabeled with carbon-11, but its high lipophilicity and non-specific binding are thought to impede accurate quantification of tracer uptake. Since then, several newer second-generation tracers have emerged for use with either carbon-11 or fluor-18 labeling, boasting an improved pharmacokinetic profile and an up to 80-fold higher specific binding as demonstrated by animal blocking studies (54). Second-generation tracers radiolabeled with 18-F, which has a longer half-life, have made this technique available for nuclear imaging centers without cyclotron, although their use is complicated by the existence of a polymorphism (*rs6971*) in humans that occurs in 30% of the European population. Therefore, genotyping of study subjects at this locus is essential to allow interpretation of TSPO PET studies, by stratifying between genetic groups and exclusion of low-affinity subjects.

As a meta-analysis is lacking for this specific research domain, the results of the existing studies are summarized in **Table 2**. Interestingly, whereas some PET studies have revealed that activated microglia are present in frontal and temporal lobes and total gray matter in the following: participants at ultra-high risk of psychosis (55), patients within the first 5 years of disease onset (56) or during a psychotic state (57); other PET studies have shown no difference in microglial activation between healthy controls and patients in various clinical states (ultra-high risk, first-episode psychosis (FEP), after a recent diagnosis of schizophrenia or in chronic phases of the illness) (58–63); or even decreased uptake in first-episode medication-naïve patients (64). With four new negative papers published in the last year (61–63, 65), it seems this field is experiencing an episode of catharsis after the initial enthusiasm that accompanied the first positive exploratory results almost ten years earlier, and it is becoming increasingly unclear whether there is indeed enhanced TSPO uptake in schizophrenia.

One major drawback of this new technology is that it comes with considerable technical requirements. First, the kinetic properties of the TSPO tracers currently require complex dynamic scanning protocols (60–125 min PET scan, often with an arterial line) which are considered a challenging and relatively invasive procedure toward patients. Second, because of the low resolution yielded by PET, co-localization with additional magnetic resonance imaging (MRI) images is essential to assess regional specific uptake. Taken together with the restriction for patients not to use benzodiazepines prior to the scan, these technical requirements arguably compromise the generalizability of PET study samples due to significant non-response bias in patient groups with the highest symptomatic burden and limit the future clinical use of the procedure.

For studies to be conducted effectively, adequate statistical power is necessary, yet because of the high technical and financial burden of these studies, sample sizes are typically rather small. Even after stratifying for genotype, high inter-subject variability is seen in imaging with TSPO tracers. This variability may be due to technical variables such as (i) the degree of tracer plasma protein binding, which is estimated to introduce up to 30% of variability in primary outcome measure V_T (55), (ii) a relatively low signal-to-noise ratio (particularly with first-generation tracers), or (iii) other unidentified technical or clinical confounders. Intra-subject variability, measured as test–retest reliability, depends upon a number of factors including scanner noise, input function noise, tracer kinetics, and any fluctuating biological variability of the target within the inter scan period, with decreased reliability causing a loss of statistical power that is not accounted for in typical sample size calculations. For second-generation TSPO tracers, intra-class correlation coefficients have been reported around 0.76–0.92 for gray matter and 0.32–0.64 for white matter (64) (Ottoy et al., submitted).

Furthermore, a complicated kinetic modeling procedure is involved, in which the kinetic properties of the tracer together with the chosen mathematical model and outcome measure have a large impact on the results, as evidenced by the inconsistent results found across the TSPO PET imaging studies. The dominant methodology has been to adopt a regular two-tissue compartmental model (2TCM). However, a model which also accounts for endothelial and vascular TSPO binding (2TCM-1K) was recently shown to have improved performance in second-generation TSPO tracers, probably because in these tracers a higher ligand specific TSPO affinity leads to considerable endothelial binding in the blood–brain barrier. Additionally, a less invasive reference tissue model was developed to study TSPO binding using first-generation tracer PK11195 without arterial input function (61). A second important consideration is that the outcome measure is normally either BP (binding potential, i.e., a combined measure of the density of available receptors and the affinity of the radioligand to that receptor) or V_T (total volume of distribution, i.e., the ratio of the radioligand concentration in the region of interest to that in plasma at equilibrium). This means the increased uptake observed in a certain region of interest could reflect either increased non-specific tracer binding as well as a true biological signal (68). Strikingly in one of the studies, no difference was found between patients and controls when using the regular outcome measure V_T; yet when the authors accounted for inter-subject variability in the input function by using DVR (distribution volume ratio, i.e., the ratio of the V_T in the region of interest to V_T in the whole brain) as their outcome measure, large effect sizes (Cohen's $d > 1.7$) were found (55). This alternative region-based approach has been subject to the criticism that it does not clarify whether the higher distribution volume ratio values in schizophrenia subjects are a result of greater microglial activation in the region of interest or of lesser microglial activation in other regions included in the denominator of the outcome measure, such as the cerebellum or white matter (69). The authors of the original study thereupon defended their choice by explaining DVR is superior to V_T when plasma input function measures are unreliable (such as is the case with TSPO tracers, which generally have <5% free fraction in plasma) or subject to systematic group differences, for instance caused by elevated acute phase plasma proteins and cytokines in schizophrenia (70).

TABLE 2 | Positron emission tomography studies with TSPO tracer evaluating microglial activation in schizophrenia patients *versus* controls.

Reference	*n* SZ	*n* C	Tracer	Model; outcome measure	Clinical state	DOI (years)	Medication	Outcome
					Total (T) and positive symptom scale (P) score (PANSS mean ± SD unless otherwise specified)		% of patients on antipsychotics (AP); (mean CPZ) Benzodiazepines (BZD) excluded (duration)?	
Van Berckel et al. (56)	10	10	[11C]PK11195	2TCM; BP	Undefined Symptom scores unavailable	3.1 ± 1.7	AP 100% BZD?	SZ > C
Doorduin et al. (57)	7	8	[11C]PK11195	2TCM; BP	Psychosis T 73.6 ± 13.3 *P* 19.7 ± 3.0	5 ± 6	AP 100% BZD excluded (3 × $t_{1/2}$)	SZ > C
Banati and Hickie (66)	16	8	[11C]PK11195	2TCM; BP	Undefined Symptom scores unavailable	Range 0.3–30	Information unavailable	SZ > C
Takano et al. (58)	14	14	[11C]DAA1106	2TCM; BP	Chronic T 77.9 ± 20.1 *P* 19.1 ± 5.3	19 ± 12	AP 100% BZD excluded (<1 m)	SZ = C
Kenk et al. (59)	16	27	[18F]FEPPA	2TCM; V_T	Psychosis T 70.2 ± 9.7 *P* 19.3 ± 2.2	15 ± 9	AP 100%; (300 CPZ) BZD excluded (duration?)	SZ = C
Bloomfield et al. (55)	14	14	[11C]PBR28	2TCM-1K; DVR	Undefined T 63.7 ± 18.1 *P* 17.0 ± 6.1	Undefined	AP?% BZD excluded (duration?)	SZ > C
Coughlin et al. (60)	12	14	[11C]DPA713	Undefined; V_T	Undefined T unavailable *P* (SAPS) 3.8 ± 2.5	2.2 ± 1.4	AP?%; (474.5 CPZ) BZD excluded (6 m)	SZ = C
Van der Doef et al. (61)	19	17	[11C]PK11195	Reference tissue; BP	Undefined T 53 ± 10 *P* 12 ± 4	1.3 ± 1.1	AP 79% BZD excluded (4w)	SZ = C
Collste et al. (64)	16	16	[11C]PBR28	2TCM; V_T	FEP drug naïve T 77.4 ± 18.3 *P* 20.3 ± 4.9	0.7 ± 0.8	AP 0% BZD not excluded	SZ < C
Hafizi et al. (65)	19	20	[18F]FEPPA	2TCM; V_T	FEP unmedicated T 68.6 ± 13.0 *P* 19.2 ± 3.8	2.8 ± 3.3	AP 0% BZD?	SZ = C
Di Biase et al. (62)	33	27	[11C]PK11195	Reference tissue; BP	Recent-onset (*n* = 18) T 68.5[a] *P* (BPRS) 12.6 ± 4.6	1.5 ± 1.0	AP 78% BZD?	SZ = C
					Chronic (*n* = 15) T 86.5[a] *P* (BPRS) 19.5 ± 7.8	13.6 ± 8.8	AP 100% BZD?	SZ = C

SZ, schizophrenia patient group; C, control group; n, number of subjects; FEP, first-episode psychosis patients; DOI, duration of illness; 2TCM, two-tissue compartment model; BP, binding potential; V_T, total volume of distribution; DVR, distribution volume ratio; CPZ, chlorpromazine equivalent; SAPS, Scale for the Assessment of Positive Symptoms; ?, undefined.

SZ > C, increased uptake of tracer in schizophrenia patients compared to controls.

SZ = C, no difference in tracer uptake between schizophrenia patients and controls.

SZ < C, decreased uptake of tracer in schizophrenia patients compared to controls.

[a]*Mean Brief Psychiatric Rating Scale total scores were converted to corresponding Positive and Negative Syndrome Scale total scores using the equipercentile linking method (67).*

Also remarkably, when one of the pioneering author groups in this field replicated their study in a larger patient sample and using a reference region instead of arterial input function, they no longer found a difference between patients and controls as they did in the original study (56, 61).

Finally, the direct biological relationship between microglia and TSPO binding *in vivo* is still not fully understood. The exact cellular source of the upregulated TSPO binding in CNS pathology remains subject to discrepancy, caused by the extrapolation of *in vitro* data to the *in vivo* situation and differences in the models used (for instance lesions with or without blood–brain barrier damage) with literature reporting TSPO expression not only on microglial cells but also on astrocytes and some neuronal subtypes, as well as endothelial cells. In non-human primates, endotoxin-induced systemic inflammation caused marked increases in the signal of a second-generation TSPO tracer, confirmed *post-mortem* to be largely due to microglial binding (71). Although this animal model is not suitable to study the brain changes of schizophrenia patients—which are much more subtle than those in many "typical" inflammatory disorders and

autoimmune disorders—the source of TSPO radioligand binding has not yet been studied in more relevant models such as the (maternal) Poly I:C viral mimetic challenge. Given the subtlety of these changes as well as the different cell types involved, it is possible to imagine a scenario in which small increases and decreases in different cell types could cancel each other out causing no diagnosis-related differences regarding TSPO binding to be found despite biological changes being present.

Finally, even if increased TSPO binding truly reflects increased microglial activation, PET images do not provide the detailed morphological and functional data required to appreciate the function of microglia in pathophysiological states. Interpretation of such findings is therefore complex as they may reflect both a protective response and a neuroinflammatory process which causes psychotic symptoms and/or neurodegenerative effects.

Neuropathological Studies on Microglia

A recent paper of two mega-analyses of combined microarray data in brain and blood tissues re-emphasizes the raised expression of innate immune system genes in the brain but also in the blood of people with schizophrenia (34). However, anatomopathological investigation has been the gold standard for thorough characterization and investigation of human tissue. It offers the best possible resolution to evaluate changes at the cell level as well as interactions between microglial cells and other brain functional components such as neurons. Although data derived from *post-mortem* brain tissue of schizophrenia patients are scarce, an association between schizophrenia and microglial activation, particularly in white matter regions, has been observed (49). Yet a definitive statement cannot be made on whether neuroinflammation is present in schizophrenic *post-mortem* brain samples due to the significant number of negative studies and conflicting results that have been published by different groups with sometimes the use of the same microglial marker (59).

As the largest meta-analysis in this field, Trepanier et al. reviewed a total of 119 articles on neuroinflammation in *post-mortem* schizophrenic brains, 22 of which looked at a range of microglial markers. Of these, 11 studies reported an increase in microglial markers in *post-mortem* brains, whereas 8 studies found no effect and 3 studies found a decrease in microglial markers. HLA-DR is the strongest risk factor for schizophrenia and a component of the major histocompatibility class II involved in "non-self" recognition, antigen presentation and the most used marker of microglial activation in schizophrenia. The SR by Trepanier et al. reported differential expression of HLA-DR by immunohistochemistry in 11 out of 13 *post-mortem* studies (9 studies reporting increased and 2 decreased expression). The same review reported unchanged expression in 3 out of 3 studies using CD68, a marker of microglial lysosomes indicative of phagocytic microglia and involved in clearance of neuronal debris in neurodegeneration, and 2 out of 2 studies with Iba1 (ionized calcium-binding adapter molecule 1), a protein involved in membrane ruffling (72) and thus a marker associated with microglial motility, a function essential to brain surveillance (73).

More recently, Van Kesteren et al. reviewed 41 studies on immune involvement in *post-mortem* schizophrenic brains, with 11 studies investigating microglia on 181 patients and 159 controls. A significant increase in microglia was observed [standard mean deviation (SMD) = 0.69**] over all studies, yet again with significant heterogeneity. The authors also reported a significant increase in the overall expression of pro-inflammatory molecular components (14 studies on 330 patients and 323 controls; SMD = 0.37**).

It is worth noting that these two main meta-analyses included among their results a paper which demonstrated increased microglial staining and increases in IL1β and TNF expression in the frontal cortex, but was retracted afterward (74).

The most evident problem for this kind of study is the limited availability of patients' brain tissue. Since *post-mortem* research is by definition at the end of life, many clinical confounders related to the life and illness history of both patients and controls may complicate the interpretation of data. Known confounders include gender, age at death, use of medication, etc. The cause of death should also be considered, as the presence of systemic inflammation, hematological malignancies or neurological illness may confound the results, as can suicide, which is common in schizophrenia and has been linked to the presence of elevated pro-inflammatory cytokines regardless of diagnosis (75). It is therefore essential that all tissue samples are accompanied by detailed clinical information and that the control group has been mindfully selected. Heterogeneity may also be explained in part by the qualitative vs. quantitative analysis, the brain region and cortical layer in which the markers are measured (59). Furthermore, there may be differences in the diagnosis methods, inclusion and exclusion criteria, storage conditions, and demographics variables among the brain banks which provide the brain samples.

Besides the microglial cells, structural and functional abnormalities in the other two glial cell types have also been studied in relation to inflammation in schizophrenia. For instance, evidence related to reduced numbers and impaired cell maturation of oligodendrocytes has been linked to white matter abnormalities and disturbed inter- and intra-hemispheric connectivity. Astrocyte dysfunction, as suggested by some studies demonstrating abnormal expression of a variety of astrocyte-related genes as well as S100B protein, may contribute to certain aspects of disturbed neurotransmission in schizophrenia (76). However, these studies fall outside the scope of this paper and are not reviewed here.

SYSTEMIC INFLAMMATION AND ITS ASSOCIATION WITH PSYCHOSIS IN SCHIZOPHRENIA

Clinical research has focused to a great extent on peripheral immune system alterations in adult schizophrenia patients. Clinical observational studies in adult patients are relatively easy to organize and considered non-invasive, as blood sampling is often part of routine medical practice. The resulting large sample sizes and a lower selection bias imply study samples are generally more representative of clinical populations. Furthermore,

the identification of potential peripheral biomarkers will be useful to confirm diagnostic or prognostic questions in clinical practice.

To assess the role of immunity in illness symptomatology, it is important to carefully differentiate patient groups according to their clinical characteristics. This was done for the first time by Miller et al., who reviewed published data of levels of cytokines (or cytokine receptors or antagonists), and later also of blood monocytes or lymphocytes, *Toxoplasma gondii* and auto-antibody titers in patients with schizophrenia or related psychotic disorder and healthy control subjects in a series of meta-analyses. Their analyses included both cross-sectional studies and longitudinal studies in patients with an acute exacerbation of psychosis at baseline and following a period of antipsychotic treatment. All studies included in this paper met strict criteria for defining patients' clinical status [as acutely relapsed inpatients (AR), FEP, stable medicated outpatients or treatment-resistant psychosis], and the meta-analyses perform quite well on the AMSTAR scale. Yet together, the meta-analyses published by this group of authors make up 6 out of the 12 meta-analyses on blood inflammatory/immunological markers in schizophrenia (20, 21, 28, 30, 31).

Based on these meta-analyses, alterations in cytokines, chemokines, lymphocytes, and oxidative stress markers have been demonstrated in the blood of patients with schizophrenia during an acute psychotic event which normalize with antipsychotic treatment ("state" markers IL1β, IL6, TGFβ and increased CD4/CD8 ratio), as opposed to other "trait" markers that remain elevated throughout the stages of the illness (20, 77). In general, effect sizes for FEP are similar in direction and magnitude to those in AR, indicating prior exposure to antipsychotic medication does not change the "acute" profile. Interestingly, a similar finding was reported in patients with chronic schizophrenia infected by *T. gondii*, with positive IgM antibody titers (a marker of acute/recent infection, or also potentially persistent infection or reinfection, possibly with a different genotype) linked to acute psychotic exacerbations (31). Thus it seems that differential patterns of immunological activation exist in different "states" of the schizophrenia illness course, in which patients who are experiencing an acute psychotic episode can be distinguished from those who are in symptomatic remission.

Yet the correlation between peripheral and brain inflammation is far from certain. Data from CSF samples might be better at representing brain immunity changes than the peripheral blood samples; however, such CSF samples are much less readily available (28). In their meta-analysis, Wang et al. identified 16 studies of cytokines in CSF, but did not stratify according to patients' clinical state (28). Although the authors cautioned that their findings needed to be interpreted with caution due to the small numbers of studies and subjects, they reported that in schizophrenia many CSF alterations were also concordant with those reported in the peripheral blood. Communication between the systemic immune system and the brain and its consequence on microglia is a critical poorly understood component of the inflammatory response to systemic disease (78). Systemic infections activate neural and humoral pathways that communicate with the brain and initiate a coordinated set of metabolic and behavioral changes (79). However, these adaptive responses may become maladaptive when microglia have been "primed" by an ongoing pathology and respond to a systemic inflammatory challenge by switching their phenotype to an aggressive pro-inflammatory state (47), adversely affecting neuronal function and potentially leading to a psychotic decompensation through modulating effects of pro-inflammatory cytokines on neurotransmitter function (47).

Another recurring problem in the abovementioned clinical observational studies seems to be that although the effect sizes found in meta-analyses are moderate to large, for a majority of cytokines (or cytokine receptors or antagonists) assessed, there was significant heterogeneity in effect size estimates (20). Indeed it appears that due to the heterogeneity encountered, no individual assay seems apt to reliably differentiate between different patient groups in clinical practice, and large sample sizes are needed to detect these immunological disturbances which may reflect that they are either very low-grade and subtle differences, or they only occur in a subgroup of patients. The result is that both selection bias and methodological factors such as the choice of assay or analysis method and controlling for various confounding variables have a large impact on the results. In the meta-analysis on blood cytokine levels by Miller et al., 97% of studies controlled for age and gender as confounding factors, whereas the potential effects of race (41%), body mass index (35%), and smoking (24%) were often not considered (20). Furthermore, similarities have been found in the pattern of cytokine alterations during acute and chronic phases of illness in schizophrenia, bipolar disorder and major depressive disorder, pointing out the possibility of common underlying pathways that are not specific to schizophrenia (26).

CLINICAL TRIALS OF IMMUNOMODULATORY DRUGS AS ADD-ON TREATMENT FOR SCHIZOPHRENIA

Following the concept that neuroinflammation may play a central role in the symptomatology and prognosis of schizophrenia, novel therapeutic prospects have arisen which aim to modulate immune effects to influence schizophrenia disease course. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and celecoxib have been investigated for therapeutic intervention, as well as other compounds with anti-inflammatory properties which readily cross the blood–brain barrier such as davunetide (derived from activity-dependent neurotrophic protein (ANAP), a growth factor released by glial cells), fatty acids such as eicosapentaenoic acids and docosahexaenoic acids, estrogens, minocycline (a broad-spectrum tetracycline antibiotic with inhibitory effects on microglia), *N*-acetylcysteine (NAC), and even potent immunosuppressant drugs such as methotrexate. These drugs are being investigated as augmentation to antipsychotics (AP) in patients with a diagnosis of a schizophrenia spectrum disorders, and effects measured as change in symptom severity on the Positive and Negative Syndrome Scale (PANSS) or Brief Psychiatric Rating Scale (BPRS). The results of the most well-studied compounds (i.e., minocycline and NSAID) are summarized in **Table 3**.

TABLE 3 | Outcomes of meta-analyses of randomized controlled trials (RCTs) on minocycline and NSAID.

	N RCTs	Subjects	Treatment duration (weeks)	Total symptoms (PANSS total score or BPRS)	Positive symptoms (PANSS positive subscale or BPRS)	Negative symptoms (PANSS negative subscale or SANS)	General symptoms (PANSS general subscale)
Minocycline							
Sommer et al. (minocycline) (38)	4	182 drug–166 placebo	36 ± 18.8	SMD 0.22			
Oya et al. (minocycline) (39)	4	173 drug–157 placebo	25 ± 19.1	SMD 0.70*	SMD 0.26	SMD 0.86**	SMD 0.50*
Solmi et al. (minocycline) (40)	6	215 drug–198 placebo	19.7 ± 17.0	SMD 0.59*	SMD 0.22	SMD 0.76** (PANSS); SMD 0.60** (SANS)	SMD 0.44*
Xiang et al. (minocycline) (41)	8	286 drug–262 placebo	18.5 ± 13.4	SMD 0.64**	SMD 0.22*	SMD 0.69**	SMD 0.45*
NSAID							
Sommer et al. (NSAID) (36)	5	*N* = 264					
Nitta et al. (aspirin) (37)	2	133 drug–137 placebo	14 ± 2.8	Hedges *g* 0.29*			
Nitta et al. (celecoxib) (37)	6	255 drug–245 placebo	7.7 ± 2.1	Hedges *g* 0.21			
Sommer et al. (aspirin) (38)	2	133 drug–137 placebo	14 ± 2.8	Hedges *g* 0.30**			
Sommer et al. (celecoxib) (38)	5	236 drug–226 placebo	7.2 ± 2.4	Hedges *g* 0.15			
Zheng et al. (celecoxib) (42)	8	316 drug–310 placebo	8.3 ± 2.3	SMD 0.47**	SMD: 0.50**	SMD 0.32	SMD 0.35*

SMD, standardized mean difference; PANSS, Positive and Negative Syndrome Scale; SMD, standard mean deviation; BPRS, Brief Psychiatric Rating Scale.
**Sig at p = 0.05.*
***Sig at p = 0.01.*

Well-designed clinical trials offer the clinical relevance the healthcare field and community are waiting for. Since these trials are run with immunomodulatory drugs that are already available on the market, they come with the additional advantage of immediate usability and well-known clinical profiles in the case of positive outcome.

A first meta-analysis on the effect of NSAIDs as add-on to AP was published by Sommer et al. in 2012 and included 5 double-blind, randomized, placebo-controlled trials (4 studies on celecoxib and 1 on acetylsalicylic acid), reporting on 264 patients. The authors reported a mean effect size of 0.43, which was significant at $P = 0.02$ in favor of NSAIDs on total symptom severity (36). This paper was subsequently criticized by Nitta et al., who replicated the meta-analysis, adding three more unpublished studies to the analysis demonstrating an overall less convincing case (37). Nitta et al. conducted subanalyses based on treatment setting and disease phase, demonstrating a significant improvement in PANSS total scores with NSAIDs in studies of inpatients (Hedges' $g = 0.44$, $P = 0.029$) and first-episode patients (Hedges' $g = 0.39$, $P = 0.048$), but not in outpatients and chronic patients (37).

In 2014, Sommer et al. reviewed double-blind randomized placebo-controlled trials of a broad range of immunomodulatory compounds. Weak to moderate beneficial effects were reported with the use of aspirin (Hedges' $g = 0.3$, $P = 0.001$), NAC (Hedges' $g = 0.45$, $P = 0.009$), and estrogens (Hedges' $g = 0.9$, $P = 0.001$); while addition of celecoxib, EPA/DHA fatty acids, davunetide, and minocycline did not show efficacy (37, 38). That same year, a second meta-analysis by Oya et al. was published reviewing RCTs with minocycline (39). Compared to Sommer et al., this meta-analysis included two more original studies and excluded one study which was only published as congress proceeding. In this meta-analysis, minocycline was superior to placebo for decreasing PANSS total scores (39). Finally, during the course of 2017, two more meta-analyses on RCTs of minocycline in schizophrenia were published around the same time. Both Solmi et al. and Xhiang et al. demonstrated minocycline's superiority versus placebo for reducing PANSS total scores, negative symptom scores, and general psychopathology scores. While SMDs for the different outcome measures are quite similar across the last three meta-analyses, only in Xiang et al. the effect on positive symptom scores reached significance level—probably due to the higher number of RCTs and study subjects included in this meta-analysis (40, 41). A third meta-analysis published in 2017 evaluated celecoxib as add-on for schizophrenia once more, this time demonstrating a significant improvement of total and positive symptoms scores (42).

Clearly, the major limitation of these studies is that for each compound, the number of individual studies is relatively low. As a result, even though they have been well executed and gain the highest scores on the AMSTAR assessment, the outcomes within and between the different meta-analyses remain relatively heterogeneous. It seems therefore too early to make conclusions on the efficacy on symptom severity of schizophrenia of augmentation with anti-inflammatory agents. Stratifying results per symptom domain makes sense, as celecoxib seems to be more effective against positive symptoms, and minocycline against negative symptoms. Overall, we would argue that too little information is currently available about the clinical determinants of groups that may benefit from these treatments to optimize study cohort selection, as well as the nature of the immune alterations—causing the choice of study drug to be virtually unguided and ranging a complete spectrum from food supplements to potent immunosuppressant drugs.

THE MICROGLIAL PHENOTYPE

Besides the specific limitations of the studies mentioned above, one of the main problems underlying their limited clinical

usefulness is our very limited basic knowledge about one of its key players in human brain, the microglial cell. Our understanding about the physiology of microglia is derived mainly from studies of tissue macrophages (43). However, in contrast to the latter, microglia are derived from myeloid precursors migrating from the yolk sac to the CNS early in embryonic development (E8.5) (80), and throughout life reside behind the blood–brain barrier where they are difficult to study *in vivo*. They account for 0.5–16.5% of the total number of cells in the human brain depending of the region explored (81). Microglia are highly plastic cells (73) that can adapt their behavior and morphology to changes in their environment and adopt different profiles (43). This defines microglial activation as a spectrum, in which transient microglial activation can include adaptive and beneficial physiological and behavioral responses, whereas maladaptive immune responses can lead to neuronal dysfunction and tissue damage. Microglia are critically involved in the organization of the neuronal network (44). They have an important role in synaptic pruning (51, 82) during brain development as well as in adult life, optimizing synaptic communication (83). Microglia can develop a range of functional phenotypes beyond the classic M1 (classical activation; an activation state in which microglia would adopt a deleterious function) versus M2 (alternative activation; in which immune cells adopt a regulatory or tolerance-inducing profile) paradigm (43). However, it is the specific manner in which microglia are activated and the phenotype that they adopt that is important in determining the influence of microglia in neurodegeneration (78). Morphology does not indicate the function of microglia, which means that even if the presence of enhanced microglial activation in certain patient groups or disease states is proven unequivocally, we still do not know what this means in terms of microglial activity and whether to interpret this as beneficial or harmful. The activation may be pathological or part of an endogenous compensatory response to some other aspect of the disease process.

Neuropathological studies remain the gold standard to gain answers to these questions. Therefore, the use of a single microglial marker, often different between the *post-mortem* studies (Iba1, CD68, or HLA-DR), is not sufficient to identify the phenotype expressed by microglia. Furthermore, if their phenotype is related to a certain clinical state or illness phase, knowledge of the time course of the microglial activation is relevant. Adding to the complexity of microglia, some authors have argued that the microglial activation status is most probably a reflection of the history of life events [defined as innate immune memory (84)], including prenatal and *perimortem* influences, as well as an individual's genetic background (85), emphasizing the need for detailed clinical information in *post-mortem* or PET investigations of microglia.

NEUROPLASTICITY AND MICROGLIA

Neuroplasticity refers to the ability of the brain to develop and finetune its neural connections, including adjustments in response to changes in the environment, by alteration in the neurons or glial cells *via* cell division/apoptosis and synaptic/neurite remodeling. Microglia are key facilitators for neuronal plasticity during brain development (44, 45) and in adult life (86). Constant surveillance of the microenvironment by microglial processes (73) and their attraction to active rather than non-stimulated synapses imply that microglia might monitor the functional state of synapses leading to plastic changes in healthy adult brain (87). This could occur through remodeling of the extracellular spaces and elimination of synaptic elements, regulation of the neurotransmitters present in the synaptic cleft such as glutamate, or by direct contact with synaptic elements (87, 88) *via* the complement components (51).

Research, summarized in this paper, has identified alterations in the key actors and mechanisms involved in neuroplasticity. A quantitative meta-analytic summary of studies focused on neuron density provides support for the finding of altered neuron density in schizophrenia, with variation dependent on age (14). The vertical micro-circuits within the cortex, known as minicolumns, normally become thinner with age in controls (89), indicating a reduction in neuropil, but not in schizophrenia (90). The role that microglia may play in this process is strongly suggested by the finding that microglia are involved in synaptic pruning during healthy brain development (28). Given that neuropil expansion is a correlate of synapse number, an abnormality of microglia may be understood to be a basis for reduced synaptic and neuropil modulation.

The quantitative neuropathological changes in the cerebral cortex of schizophrenia, suggest that the "reduced neuropil hypothesis" (91) applies in early life in schizophrenia, but subsequently it is "reduced neuroplasticity" which leaves the cells and minicolumns relatively widely spaced in later life, compared to the typical pattern of healthy control neuropil thinning. This is consistent with neuroimaging data and offers a microanatomical explanation to account for many of the larger scale functional–anatomical changes observed in structural neuroimaging studies (14), which have shown that over time schizophrenia is associated with progressive decrease of whole brain volume and whole brain gray matter (92), frontal gray and white matter, parietal white matter, and temporal white matter (93).

Consequently, an altered aging trajectory is implicated as a factor in the pathogenesis of schizophrenia. This hypothesis is supported by the finding that the decrease in brain-derived neurotrophic factor (BDNF) which occurs in older age is amplified in schizophrenia. BDNF is a regulator of microglial-mediated synaptic plasticity (94), suggesting a greater reduction in neuroplasticity with advancing age in schizophrenia (95). The progressive anatomical abnormalities of brain structure recorded in longitudinal studies indicating greater severity following the first episode of illness may be the cumulative effect of reduced neuroplasticity over time. One of the consequences at the level of symptoms may be that a shift from positive symptoms to more negative symptoms, which has been reported during the disease course, is consistent with the implications of reduced plasticity—i.e., negative symptoms represent a relative loss of functions, that may become more dominant due to insufficient plasticity to keep up with ongoing cognitive demand, rather than the presence of additional cognitive phenomena described as positive symptoms.

CONCLUSION AND FUTURE DIRECTIONS: MULTIMODALITY AS KEY TO THE FUTURE OF NEUROINFLAMMATION RESEARCH IN SCHIZOPHRENIA

The evidence from the psychoneuroimmunology research field converges on a progressive illness model of schizophrenia in which primed microglia contribute to altered neuroplasticity, leading to structural and chemical abnormalities that accumulate as patients age. This model integrates both neurodevelopmental and neurodegenerative hypotheses in schizophrenia, as the combination of a pre- or perinatal immunological vulnerability serves as a background for ongoing abnormal peripheral and brain immune responses which throughout life constitute a basis for illness exacerbation and progressive defects. Yet although the psychoneuroimmunology field provides a compelling pathophysiological model, at this moment it has not attained a clinically significant level of relevance to comprehensively explain the etiology, diagnosis or potential treatment of the disorder. In addition, many of the findings appear non-specific to schizophrenia. To enhance the clinical relevance of this field, further links are needed to connect the immune-related hypotheses to other major research fields and hypotheses of schizophrenia psychopathology.

Such an interesting new link between immune alterations and neuroplasticity, in which aberrant microglial activation could cause reduced synaptic and neuropil modulation, has recently emerged, but still needs to be comprehensively investigated in the human brain. Furthermore, knowledge on the physiology and functional phenotypes of microglia, as well as their link with systemic inflammation, is crucially insufficient to interpret current findings. We would therefore argue that studies acting as a bridge between the preclinical and clinical studies are crucially missing. We therefore advocate multimodal research studies that explore neuroinflammatory mechanisms both in experimental models and schizophrenia patients; using *in vivo* multimodal imaging and analysis of immunological markers and micro-architectural changes in *post-mortem* brain tissue as translational means. In particular, the underutilized human brain approach has a powerful translational nature in complex neuropsychiatric conditions (9). Overcoming individual limitations, this combined approach evidently generates added value and has a high feasibility, as it is supported by literature, preliminary data, and active involvement of highly skilled research teams situated within the field that already possess the necessary know-how. The advent of a greater recognition of the role of the immune system and its effects on neuroplasticity in schizophrenia will allow identification of potential novel therapeutic avenues, thereby finally bringing the psychoneuroimmunology research field to its full potential.

AUTHOR CONTRIBUTIONS

All authors met ICMJE criteria and all those who fulfilled those criteria were listed as authors. All authors had access to the study data and made the final decision about where to present these data.

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OPINION
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Silencing of Viral Elements: An Available Cure for Schizophrenia?

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THE PROBLEM

Scientific evidence for various infectious agents as cause for psychosis in schizophrenia is not robust. Many pathogens that influence brain development might play a role in disease causation. It has been shown that influenza exposure before birth (1), exposure to herpes simplex during birth (2), or exposition to Toxoplasma gondii and other pathogens (3) increases risk for schizophrenia and/or compromises cognition. During psychosis, viral and also ancient retroviral elements become may activated in the brain (4, 5). The implication of this research for use in clinical practice is ambiguous, because a robust virus test is lacking. The current article explores the speculative, but testable hypothesis that HSV-1 a neurotropic virus infecting specific limbic brain regions is a necessary—but not sufficient—cause of psychosis in a significant proportion of schizophrenia patients. The test should qualify Koch's adapted postulates for latent pathogens (6): It should be present in the affected organ only in diseased cases, it should explain the pathogenetic changes of the individual and last, the pathogen should be transmissible and carry a corresponding disease in an animal model. We chose to focus on HSV-1, which specifically infects limbic brain regions and explains neuropathological and behavioral deficits in acute psychotic, and further detioration in chronically schizophrenic patients, but may also accounts for extremely high (up to 60%) concordance in monozygotic twins (7). HSV-1 is indeed abundantly present; up to 80% of adults harbor the virus and it is the only known virus, which has specific neurotropism for the limbic regions of the brain (8) involved in the inflammatory pathophysiology of schizophrenia (9). Transmitting the HSV in animals yields pathophysiological changes in the monoaminergic system mimicking aspects of the schizophrenia phenotype in humans (10). HSV is also omnipresent in evolutionary history of man (11). However, all these characteristics of the virus make it a candidate, may give a hint toward causation, or provide a little circumstantial evidence, but are by no means proof of causation.

THE HYPOTHESIS

Schizophrenia is a neurodevelopmental disease, with changes in the circuitry of the hippocampus shown postmortem, including a loss of lateralization in the dentate gyrus (12). The dentate gyrus is central to the cognitive adaptation of the human species across the life span, because this region is subject to adult neurogenesis, which secures life-long adaptation of memory to environmental influences, and neurogenesis is compromised in schizophrenia (13). The dentate gyrus is an primary target for HSV (14).

The virus hypothesis specified in this article is based on two remarkable phenomena, maybe combining to a third:

1. It is remarkable how pluripotent stem cells within the subgranular zone divide under influence of epigenetic mechanisms that (temporarily) silence the neuronal phenotype in favor of the

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stem cell phenotype, and how the suppression of neuronal phenotype is gradually relieved during differentiation into neurons (15). The master regulator of stemness and consecutive neuronal speciation is the transcription factor RE1 silencing transcription factor (REST) (16). REST is highly expressed in the milieu of the stem cell niche, suppresses hundreds of genes that are neuron specific, and thereby forces the cells in a dedifferentiated state, to enable replication. The niche, in which these stem cells replicate and form primitive neuronal progenitors, is the subgranular zone, and further differentiation into neurons occurs while they move toward the granular zone. This process of gradual speciation into neurons is guided by growth factors such as BDNF and nerve growth factor (NGF), which gradually suppress the transcription factor REST to enable neuronal genes to become more and more expressed on the way (17).

2. It is remarkable how in transneuronal tracing experiments the large DNA virus HSV, travels easily from the afferents of a sensory neuron deeply into the brain, without creating a severe encephalitis and death (18). Neuronal tracing experiments, with HSV, or with the related virus pseudo rabies virus (PRV), witness this peculiarity of nature. HSV travels anterogradely (along the normal direction of conduction of electrical stimuli along the axon), from the oral cavity sensory axon of the trigeminal nerve (Nervus V) via the efferent of this nerve to the next neuronal soma in the trigeminal nucleus (medulla oblongata), sending an efferent to the soma in the dopaminergic nuclei in the midbrain (substantia nigra), sending again an efferent into the limbic brain. An animal model shows (part) of this potential route of infection (19). The presence of HSV DNA as observed in the brain of healthy controls (20) is remarkable, because no apparent destruction is found on the supposed ways of anterograde transport of the virus (8). The suppression of replication of viral DNA in the infected neuron relies heavily on REST. Dominance of REST silences promoters of genes essential for replication of HSV and thereby prevents reactivation of the virus in neurons (21).
3. The remarkable hypothesis that might follow from the abovementioned is that stem cells in the dentate gyrus, which conserve stemness under influence of REST, contain very specific HSV gene elements that are forwarded into progenitors and thereby influence consecutive differentiation in a peculiar (schizophrenic) way: The necessary relief of REST to enable differentiation of the cell leads to partial derepression of the viral DNA element contained in the cell, which in combination with adverse life events (extreme social stress) is responsible for the neuroanatomical, neurophysiological, and behavioral phenotype of schizophrenia. The viral element present in stem cells, forwarded into progenitors, and partially expressed in differentiating cells, creates a certain variability on the differentiation process, that is not necessarily detrimental, but also might give rise to exceptional creativity, when kept in check. To be kept in check, the differentiation needs to be guided by growth factors that have a dual role: (1) to keep the virus in a sufficiently suppressed state to prevent activation of the (viral) replication machinery and (2) to support the developing neuron and its afferents with sufficient force to differentiate (cholinergic) transmitter connectivity that is important for new cognitions. NGF is produced within the hippocampus (22), supports cholinergic input to the hippocampus (23), and suppresses HSV (24).

THE TEST

Some aspects of the hypothesis have been already proven in experiments: As mentioned above, in neuronal tracing experiments have been shown how easy HSV (or PRV) reaches limbic regions of the brain and how this in line with the dopamine hypothesis of schizophrenia. The connectivity of substantia nigra with the dentate gyrus of the hippocampus supports the possibility of anterograde transport of the virus to the neurogenetic niche (25). In herpes simplex virus infected progenitors, or immortalized monoaminergic PC12 cells the virus is kept latent by NGF signaling (26), but NGF also forces the primitive cells into a neuronal differentiation program (27). The major weakness of the current virus hypothesis is that the exact nature of the viral elements that supposedly interfere with the differentiation program in neuronal progenitors is not yet defined in detail. Many viral elements of HSV are kept latent by epigenetic silencing of promoters of these elements by REST. REST recruits histone deacetylases and lysine-specific demethylases (LSD) for suppression of gene transcription by epigenetic modification (28). When suppression by REST is relieved, which is observed during differentiation of the stem cell progenitor toward neuronal speciation, the activation of viral genes would occur in a predictive manner as observed in the earliest phase in very delicate viral reactivation experiments: When immediate early genes of the virus become relieved from suppression by REST, the genes essential for DNA replication genes of the virus [thymidine kinase (TK) or ribonucleotide reductase] become transcribed (29, 30) An important candidate viral element present in human brain is the TK gene of HSV (31), The HSV-TK gene contains a promoter with a Sp1 responsive element (32), which is activated due to stress signals and plays an important role in reactivation of the virus (33) and is involved in schizophrenia (34). In our hypothesis, the HSV-TK gene element may become activated upon stress and thereby disrupt neuronal speciation in the hippocampus and cause schizophrenia. For this specific hypothesis, all postulates of Koch regarding this specific viral gene/promoter have to apply. The viral element should be present in the stem cell niche of the patient, should interfere with neuronal speciation and integration of the neuron in the hippocampus under stress, and a similar pathophysiology has to apply when the viral element is introduced in the hippocampal stem cell niche in an animal model. These requirements could be fulfilled as follows: distract DNA from postmortem dentate gyrus brain material of schizophrenia patients, assuming that the HSV-TK gene is still present as in postmortem cases of herpes encephalitis (31). Amplify the DNA sequence found in the autopsy material containing both the enhancer/promoter binding site and the gene of interest (in our case HSV-TK). If amplified DNA copies of this viral element are found (only) in patient brain tissue, this will satisfy the first postulate. Then introduce the viral element discovered in human brain tissue, by knock-in into human NSC (neuronal stem cells)

or PC12 cells that differentiate under control of NGF. Perform a detailed assessment of the phenotype of the differentiating cells with positive and negative control on the Sp1 stress response elements. If viral transduced neurons show a disrupted execution of the differentiation program and distrophical growth under Sp1 stress, the second postulate is fulfilled *in vitro*. Then, genetically introducing the viral element into mice hippocampi should provide us with the proof of the final postulate of Koch, the fact that the viral element transmits disease *in vivo*. In these experiments, the influence of the specific viral element discovered in human brain can be assessed regarding its influence on genesis, differentiation, migration and integration of neurons within the limbic network. To this aim, immunohistochemistry and fluorescent microscopy are very helpful, techniques that show us the normal neurogenetic mechanisms of cognition and what may go wrong (15).

THE CURE

Superficially, the cure for silencing of viral elements might seem easy: The only thing to do to keep the virus silent is suppress the transcription by silencing the promoter region of the viral element of interest epigenetically: keeping the activity of REST sufficiently high, as shown to be effective in viral knock-in REST gene experiments, would suffice (21). However, in patients, this pharmacological way to improve suppression by REST needs stepping out of the usual antipsychotic therapy of schizophrenia. We would need trials with drugs that support the epigenetically silencing REST complex, such as the LSD inhibitor Tranylcypromine (35). Is it a coincidence that LSD inhibitors are by another mechanism—by mono amino-oxidase inhibition—the most potent drugs to treat therapy resistant depression? Would suppression of viral elements by Tranylcypromine provide us with a cure for schizophrenia? Tranylcypromine might not only suppress expression of viral elements from HSV, but might also help to suppress other (retro)viral elements involved (4, 5). Suppressing the influence of stress on the expression of viral elements, might secure that neurogenesis is followed by proper differentiation of cell to neurons well integrated in the hippocampal circuitry and thereby prevent the accumulation of neurodevelopmental changes, found in postmortem hippocampi. It might basically cure schizophrenia by saving the patient from neurodevelopmental disarray. Medicinal treatment to *prevent* HSV replication might prove a far more effective treatment when compared with drugs that only *limit* HSV replication (36).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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OPINION
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How Will the Mild Encephalitis Hypothesis of Schizophrenia Influence Stigmatization?

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INTRODUCTION

People diagnosed with mental disorders, particularly those with schizophrenia, are severely stigmatized (1, 2). The image of people with mental disorders is strongly influenced by the mass media, which are then influenced by the prevailing medical opinion as well as by current research results. Therefore, researchers in psychiatry bear a certain responsibility for the stigmatization of their very own research objects.

Within the recent years, the mild encephalitis hypothesis receives more and more scientific interest. According to this hypothesis, a mild, but chronic, encephalitis underlies the symptoms of schizophrenia in a subgroup of patients. Infections, traumas, or autoimmune diseases can cause a mild encephalitis, which leads to psychiatric and/or neurological symptoms (3–5).

Since the mass media have recently started to report about the association of brain inflammation and schizophrenia, the mild encephalitis hypothesis is starting to influence the public's opinion about people diagnosed with schizophrenia, and thus will have a certain influence on the stigmatization. Whether it will increase or decrease stigmatization has not yet been investigated empirically. In the following, we discuss this question on grounds of theoretical concepts and empirical research on stigmatization of schizophrenia.

STIGMATIZATION OF MENTAL DISORDERS

Stigmatization is sociologically defined as the classification and stereotyping of people because of a negatively connoted attribute, together with segregation and loss of social status, discrimination in important contexts, and devaluation in a social hierarchy in a situation of exercise of power (6). Many stigmatized individuals internalize the negative evaluation, try to hide the negatively connoted attribute, and withdraw from society (self-stigmatization). Stigmatization often affects the social circle, particularly the families (courtesy stigma) (7).

Many biologically orientated researchers are convinced that biological explanations of psychiatric disorders will reduce stigma. This optimistic view is based on the attribution theory, assuming that the main reason for stigmatization is the attribution of guilt or responsibility for the onset and/or maintenance of the deviant behavior (8). Accordingly, biological, and particularly genetic, explanations should reduce blame against persons with mental disorders as soon as people understand that the strange or frightening behavior is not caused by evilness or weak will, but by a disease (9).

This conviction is contested by many social scientists. Because both the moral and the medical concepts assume an inborn predisposition for deviant behavior, a genetic explanation of deviant behavior does not diminish rejection (10). Genetic explanations assume mental disorders to be unchangeable, more serious, and hereditable (9, 11). People convinced of "genetic essentialism" believe that the genes are a person's essence and that the characteristics and behaviors of a person

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are based on his/her genetic makeup (11). Genetic explanations increase self-stigmatization (12) and courtesy stigma, particularly the stigmatization of genetic relatives of people with mental illness (9). Furthermore, this approach supports a paternalistic attitude towards mentally ill persons, questioning their autonomy and decisional capacity (13).

The attribution theory and the concept of genetic essentialism are not mutually exclusive; rather they grasp different aspects of stigmatization: the first one mainly the attribution of guilt and the second mainly the fear and the feeling of social distance (10).

EMPIRICAL RESEARCH ON STIGMATIZATION OF MENTAL DISORDERS

Empirical research supports the theory of genetic essentialism and widely disproves the attribution theory for major depression and schizophrenia. For example, a representative study with 1,241 participants (9) confirmed only one prediction of the attribution theory, namely, that people who are convinced of genetic explanations pleaded for lesser punishments for violent behavior of mentally disordered persons. However, there was support for predictions based on the concept of genetic essentialism. People who assume genetic causes of schizophrenia believe in a greater seriousness, tenacity, and pervasiveness of the deviance and hold more social distance against the siblings of mentally disordered persons.

A systematic review of population-based studies found that biogenetic beliefs about the cause of schizophrenia or depression were associated with greater social distance and thus stronger stigmatizing attitudes (1).

Based on the aforementioned and further studies on stigmatization, we have hypothesized that several factors influence whether a given biological model of a given psychiatric disorder will increase stigmatization: (1) disease-specific factors and (2) model-specific factors (10).

(1) Disease-specific factors: biological explanations increase the stigmatization of a given psychiatric disorder, as soon as people think that this disorder is associated with (a) high dangerousness/unpredictability, (b) high psychosocial disability, (c) poor treatment success, and (d) high responsibility for the onset and/or offset of the disease. Among these factors, the most important one is the perceived dangerousness/unpredictability, because this attribution leads people to seek social distance (2).
(2) Model-specific factors: there are different models of psychiatric disorders are, e.g., psychosocial models, the genetic model, the neurotransmitter disturbance model, or the mild encephalitis hypothesis. Model-specific factors can modulate the effects of disease-specific factors in various ways. Model-specific factors can influence the stigmatization, for example, the factor dangerousness/unpredictability either by changing the real dangerousness of people with this disorder or by changing the people's perception of the dangerousness. The first effect could take place if the model implied an effective treatment against psychosis and/or aggressiveness, the latter if the model convinced people that the disorder was not necessarily associated with dangerousness.

The differential effects of the model-specific factors might be contradictory. For example, genetic explanations of schizophrenia decrease the onset responsibility, but might squash hopes for successful treatments, at least in the laymen's perception.

Indeed, empirical research on the effects of different models on stigmatization has brought inconsistent results.

According to Rüsch et al. (12), the endorsement of genetic explanations was correlated with a stronger desire for social distance, whereas the endorsement of neurobiological explanations was not correlated with stigmatizing attitudes. In both cases, the attribution of responsibility was reduced.

According to Angermeyer et al. (14), the endorsement of a brain disease hypothesis is associated with increased anger and fear, which is associated with increased social distance. On the contrary, there was no significant association between the endorsement of hereditary factors and social distance, assumedly because the endorsement of hereditary factors increases on the one hand fear and on the other hand prosocial feelings.

In general, biological explanations of schizophrenia increase stigmatization, because schizophrenia has high degrees for three disease-specific factors (dangerousness/unpredictability, psychosocial disability, and poor treatment success). However, it remains an open question whether and in how far neurobiological explanations have a different effect on stigmatization as compared to genetic explanations. This situation is not only due to the inconsistent study results but also due to the rather crude biological explanations used in the studies.

ANTI-STIGMA MESSAGES

Accompanying research on stigmatization can contribute to a responsible psychiatric research that will not harm psychiatric patients by involuntarily increasing stigma. Empirical research on stigmatization of mental disorders is particularly necessary for communicating research results to the media and for designing anti-stigma campaigns which are not only well-intended but indeed beneficial for the concerned people. Since stigmatization is a multi-faceted phenomenon, interventions aiming at reducing stigma often have contradictory and unexpected effects.

According to a consensus paper on campaigns to reduce mental health-related stigma, the following message types should be used: (1) recovery-oriented, (2) "see the person," (3) social inclusion/human rights, and (4) high prevalence of mental disorders (15). Additionally, information on the continuous nature of psychopathological phenomena is recommended for anti-stigma messages (16).

INFLUENCE OF THE MILD ENCEPHALITIS HYPOTHESIS ON STIGMATIZATION

We expect that the mild encephalitis hypothesis will have different effects on the stigmatization of schizophrenia.

This hypothesis offers concrete hope for effective therapies with anti-inflammatory drugs for a subgroup of patients diagnosed with schizophrenia (17). Patients will probably accept these drugs better, so that their compliance will improve and the relapse rates might be reduced. With effective and potent drugs, many patients could be treated successfully, so that the dangerousness due to psychosis would vanish. Furthermore, their cognitive decline could be stopped, so that the level of cognitive functioning would be better. Diminished dangerousness and better cognitive functioning will positively affect on their social inclusion.

Because the mild encephalitis hypothesis contains no genetic determinism, but the concept of a genetic vulnerability, we expect that it will reduce the stigmatization of genetic relatives.

The mild encephalitis hypothesis might reduce the stigmatization further because it emphasizes the influence of infections and autoimmune disorders which can principally hit everyone, not only those with a special genetic makeup.

The mild encephalitis hypothesis might not influence the attribution of onset responsibility, because the patients are not responsible for any of the known causes of mild encephalitis. However, the attribution of offset responsibility might change significantly: if effective treatments without severe side effects were available, then the acceptance of the concept "liberty of illness" might diminish. People who refuse effective treatments will be considered as responsible for their enduring mental illness.

Finally, we expect that the stigmatization would be reduced significantly because the mild encephalitis hypothesis would support to shift the organizational authority over patients with schizophrenia from psychiatry to multi-disciplinary institutions combining psychiatry and neurology.

Therefore, we expect that the mild encephalitis hypothesis will contribute to a destigmatization of schizophrenia, of course particularly, if it will lead to effective drug therapies.

AUTHOR CONTRIBUTIONS

SM and RR have both contributed to the article with regard to development of ideas. SM wrote the first draft of the manuscript and developed the structure of the paper. Both authors read and approved the final manuscript.

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REVIEW
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Vitamin-D Deficiency As a Potential Environmental Risk Factor in Multiple Sclerosis, Schizophrenia, and Autism

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In this short review, we want to summarize the current findings on the role of vitamin-D in multiple sclerosis (MS), schizophrenia, and autism. Many studies have highlighted hypovitaminosis-D as a potential environmental risk factor for a variety of conditions such as MS, asthma, cardiovascular disease, and, more recently, psychiatric diseases. However, whether hypovitaminosis-D is a potential causative factor for the development or activity in these conditions or whether hypovitaminosis-D may be due to increased vitamin-D consumption by an activated immune system (reverse causation) is the focus of intense research. Here, we will discuss current evidence exploring the role of vitamin-D in MS, schizophrenia, and autism and its impact on adaptive and innate immunity, antimicrobial defense, the microbiome, neuroinflammation, behavior, and neurogenesis. More work is needed to gain insight into its role in the underlying pathophysiology of these conditions as it may offer attractive means of intervention and prevention.

Keywords: vitamin-D, multiple sclerosis, schizophrenia, autism, immunity, microbiome

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VITAMIN-D BIOLOGY

Vitamin-D is a member of the family of steroid hormones together with sex hormones, retinoid, and cortisol. Vitamin-D has pleiotropic functions and plays an important role not only in calcium homeostasis and bone metabolism but also in regulating immune responses and hormonal and metabolic processes. Furthermore, it influences neurotropic and neuroprotective processes in the brain and may also impact on neurotransmission and synaptic plasticity (1–4). Its receptor has been found expressed in most tissues and organs (5).

Vitamin-D is the only steroid hormone not synthesized from cholesterol, and this exclusive metabolic pathway distinguishes it from all other steroid hormones and suggests important functions (**Figure 1**). Life on earth began approximately 3.5 billion years ago, and vitamin-D became pivotal to the evolution of humankind. Through its role in calcium homeostasis and the endocrine system, vitamin-D played an important part in our movement from the ocean to land and in the subsequent development of the calcified skeleton of the terrestrial *Homo sapiens* (6–8). Human life started in surroundings abundant in ultraviolet B (UVB) rays, and to this day, people living in this environment have average vitamin-D levels around 115 nmol/L (9). Our subsequent settlement in the northern hemisphere was accompanied by skin color changes to improve light absorption. During the past 200 years, our lifestyle changed dramatically and occurs mainly indoors, culminating in sun

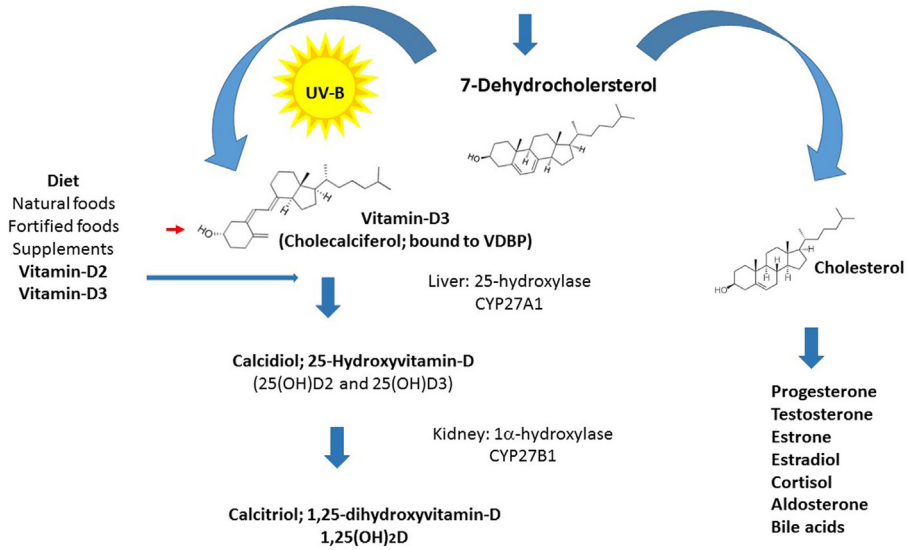

FIGURE 1 | **Vitamin-D synthesis: schematic diagram of vitamin-D synthesis**.

avoidance education, and the introduction of sun blockers over the last 50 years, leading to widespread hypovitaminosis-D (10).

Vitamin-D consists of two forms, vitamin-D_3 and vitamin-D_2, which are both biologically inert. The biosynthesis of the active form of vitamin-D (calcitriol) starts from its prime precursor 7-dehydrocholesterol and undergoes the key photochemical electrocyclization in the skin by irradiation with UVB light (at 290–315 nm), producing an intermediate that is spontaneously converted into vitamin-D_3 (calciferol or cholecalciferol). Cholecalciferol is then transported to the liver, where it is enzymatically hydroxylated in the side chain at position 25 (the number refers to the position in the molecule, which elicits its highly specific biological properties) to produce calcidiol (25-hydroxyvitamin-D_3). Vitamin-D_2 and vitamin-D_3 absorbed from the intestine are also metabolized in the liver. Calcidiol is subsequently converted to 1,25-dihydroxyvitamin-D [1,25$(OH)_2$D] also known as calcitriol in the kidney by the action of the 1α-hydroxylase enzyme (11–13). Enzyme levels are controlled by the parathyroid hormone, whose secretion is in turn triggered by low concentrations of calcium or phosphate (14, 15). The latter enzymatic hydroxylation reaction, producing calcitriol, has also been found to occur in lymphocytes and in the brain in microglia and probably in other locations (16). The half-life of calcidiol, which is dependent on vitamin-D-binding protein concentrations and genotype, is approximately 15 days and serves as a clinical measure of vitamin-D status, whereas the half-life of calcitriol is much shorter (5–15 h), therefore, its local production is advantageous (11, 17).

THE ROLE OF VITAMIN-D IN IMMUNITY AND IMMUNOMODULATION

Vitamin-D is known for its skeletal effects; however, in this review, we will focus on non-classical vitamin-D physiology and its involvement in immunity and inflammation (**Figure 2**). Two major observations link vitamin-D to immunity. First, most proliferating immune cells express the vitamin-D receptor (VDR) for active vitamin-D. The VDR is expressed in immune cells of the adaptive and innate immune system, such as T-cells, B-cells, monocytes, macrophages, dendritic cells (DCs), and neutrophils (18). Additionally, immune cells exhibit an active vitamin-D metabolism with the expression of the rate-limiting enzyme for vitamin-D synthesis, 1α-hydroxylase (CYP27B1) (19). Immune cells are, therefore, able to synthesize and secrete vitamin-D in both an autocrine and paracrine fashion, indicating that vitamin-D plays an important role in the immune system, where it affects antigen presentation, innate immunity, and T-cell activation, differentiation, and migration (19, 20).

THE IMPACT OF VITAMIN-D ON INNATE IMMUNE RESPONSES AND ANTIMICROBIAL RESPONSES

Genome-wide analyses and associated *ex vivo* and *in vitro* experiments have clearly demonstrated the importance of vitamin-D in orchestrating innate immune responses and maintaining optimal antibacterial responses in humans. Our innate immune system recognizes pathogen-associated molecular patterns (PAMPs) with the help of the so-called pattern recognition receptors including toll-like receptors (TLRs) to mount successful immune responses for the successful eradication of pathogens. A role for vitamin-D metabolism and signaling in innate immunity was provided by a genome-wide approach, which showed that the macrophage response to *Mycobacterium tuberculosis* involved an endogenous, intracrine vitamin-D system. Exposure to a TLR-2-interacting PAMP induced the expression of both CYP27B1 and VDR in macrophages (21). Furthermore, expression of

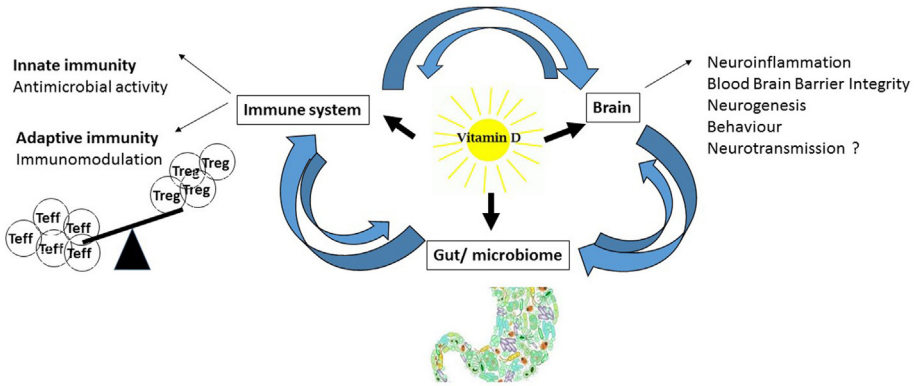

FIGURE 2 | **Effects of vitamin-D: schematic diagram of vitamin-D effects on the immune system, brain, and gut**.

antimicrobial proteins (cathelicidin, β-defensin-2, hepcidin antibacterial protein) can be induced by vitamin-D in macrophages upon pathogen encounter (22). The complex induction involved cooperation between VDR and NF-κB, which is the major transcription factor that regulates genes responsible for both the innate and adaptive immune responses but is also implicated in neuronal plasticity and memory (23). The innate immune response comprises a pronounced inflammatory component, and vitamin-D counteracted these events by promoting hyporesponsiveness to PAMPs *via* downregulation of TLRs on monocytes (24). In addition, other vitamin-D-mediated innate immune functions comprise the regulation of the nitric oxide pathway, iron metabolism, and autophagy, an intracellular degradation system thought to play an important role in neurodegeneration (19, 25).

THE IMPACT OF VITAMIN-D ON ANTIGEN PRESENTATION AND ADAPTIVE IMMUNE RESPONSES

Antigen-presenting cells are important players of our immune response and pivotal in priming and orchestrating adaptive immune responses. The function of monocytes, macrophages, and DCs can be modulated by vitamin-D as they exhibit an active intracrine vitamin-D system and express VDR and CYP27B1. Vitamin-D was also able to influence the differentiation of DCs (26). Furthermore, *in vitro* addition of vitamin-D to antigen-presenting cells inhibited the surface expression of antigens by major histocompatibility complex (MHC) class II and its costimulatory molecules, leading to reduced T-cell stimulatory capacity (27). Several studies highlighted a role for vitamin-D as inhibitor of T- and B-cell proliferation (28); however, it has become increasingly clear that the prominent effects of vitamin-D involve the modulation of the T-cell phenotype of $CD8^+$ cytotoxic T-cells and T-helper (Th) cells. Several T-cell subgroups have been characterized according to their distinct cytokine profiles. Interestingly, vitamin-D directly exerted its immunomodulatory effects on T lymphocytes by inhibiting the production of pro-inflammatory Th1 cytokines (IL-2, IFN-γ, and TNF-α, considered to be the key mediators in graft rejection and autoimmune diseases) and stimulated the production of anti-inflammatory Th2 cytokines (IL-4, IL-5, and IL-10), which have immunoregulatory functions (29). Vitamin-D also drove immunomodulation by suppressing inflammatory IL-17-expressing Th17 cells and promoted the production of regulatory T-cells (Treg) (30). Recent studies showed that Treg function correlated with serum concentration of vitamin-D in multiple sclerosis (MS) patients (31). Of interest is also the capacity of vitamin-D to influence T-cell homing (32).

The action of vitamin-D on cellular immune responses has been the focus of much research; however, not much is known on the effect of vitamin-D on B-cell homeostasis. However, it is known that the effect is not restricted to their IgG-producing capacity. Vitamin-D suppressed the differentiation of plasma cells and class-switched memory cells and regulated B-cell IL-10 production (33, 34).

VITAMIN-D AND THE MICROBIOME

The gut is the largest immune organ in the human body, and the gut microbiome plays an important role in health and disease. The gut microbiota is thought to communicate with the brain and regulate central nervous system (CNS) homeostasis through immune, vagal, and metabolic pathways. Alterations in its composition have been implicated in a wide range of neurological and psychiatric conditions including MS, schizophrenia, and autism, which have been reviewed in detail elsewhere (35, 36). The effect of vitamin-D on the microbiome, and *vice versa*, is less well researched, but a recent genome-wide association study (GWAS) showed that variation in the VDR influenced the composition of gut microbiota (37). This finding and the importance of the gut–brain axis in shaping behavior and brain development warrants further research as it will open up a new and exciting line of investigation.

VITAMIN-D AND THE BRAIN

An increasing body of evidence suggests that vitamin-D is an important player in mature brain function and brain ontogeny (38). The effects of gestational developmental vitamin-D deficiency in adult offspring led to persistent effects on brain anatomy, neurochemistry and function, where it impacted on neuronal differentiation, axonal connectivity, and dopamine ontogeny (39). Furthermore, vitamin-D influenced neural stem cells proliferation, survival, and neuron/oligodendrocyte differentiation supporting its remyelinating and neuroprotective effects (40). The effect of hypovitaminosis-D on synaptic imbalances warrants further study.

The VDR was found expressed in the human and rodent brain, and its widespread distribution suggests that vitamin-D may have autocrine/paracrine properties. The strongest immunohistochemical staining for the VDR and 1α-hydroxylase was found in hypothalamus and in the large neurons within the substantia nigra (16). Earlier studies using radiolabeled vitamin-D showed accumulation in nuclei of neurons, which suggested its role in regulating the production of several aminergic and peptidergic messengers and influencing the activity of certain sensory, motor, and endocrine–autonomic systems (41). The receptor was also found expressed in oligodendrocyte-like cells, human leukocyte antigen (HLA)-positive microglia, and glial fibrillary acidic protein-positive astrocytes (42).

Microglia cells are key players of the immune system in the CNS and play an important role in brain infections and brain development. Their activation interferes with neuronal survival by increasing oxidative stress and decreasing neurotropic support and has been linked to MS (43), schizophrenia (44, 45), and autism (46). As microglia cells play a pivotal role in neuroinflammation and neurodegeneration, downregulation of their proinflammatory cytokine production and release of free radicals by vitamin-D may be neuroprotective (47).

VITAMIN-D IN MS

Multiple sclerosis is an inflammatory, demyelinating disease of the CNS characterized by myelin loss, inflammatory lesions, and varying degrees of axonal pathology. It is a leading cause of disability in young adults, found to be more prevalent in woman, and affects 2.5 million people worldwide. The etiology of MS is still unknown, but autoimmune processes are thought to play an important role (48).

Susceptibility depends on genetic and environmental risk factors and their interactions (49). The study of environmental risk factors is of great interest as they can potentially be modulated, in contrast to the genetic susceptibility. This may offer exciting innovative strategies for disease prevention and intervention. Several risk factors are the current focus in MS research such as hypovitaminosis-D, viral infections (Epstein–Barr virus, human herpes virus-6, and human endogenous retroviruses), smoking, and the microbiome (43, 49–54). Interestingly, recent studies showed an association between vitamin-D and EBV status, which may highlight a role of vitamin-D in control of persistent EBV infection (51). In this review, we will focus on hypovitaminosis-D and summarize the latest findings in MS.

VITAMIN-D STUDIES IN MS PATIENTS

There is a substantial body of evidence on the role of vitamin-D in the development of MS. Two important prospective studies showed a protective effect of vitamin-D in MS. A nested case–control study in US military personnel reported that high serum concentrations of 25-hydroxycholecalciferol correlated with decreased MS risk (55). A more recent prospective study confirmed these findings and reported that levels of vitamin-D over 75 nmol/L were associated with a decreased MS risk (56).

Several observational studies have consistently shown an association of low serum levels of vitamin-D with increased MS risk and supported the findings from the prospective studies. Vitamin-D intake was found to moderately decrease the risk of MS in a large prospective study ($n = 187{,}563$) (57). In addition, the influence of vitamin-D on the disease course of MS is equally strong. Vitamin-D status correlated inversely with exacerbation risk in relapsing-remitting MS and suggested a beneficial effect on MS disease activity (58, 59). This effect was also found in patients on interferon-β treatment, where the lowest rate of new lesions was found in patients with vitamin-D levels over 100 nmol/L (60, 61). Of interest is also a potential role for vitamin-D in the conversion from clinically isolated syndrome, a first event suggestive of MS, to clinically definite MS. Low vitamin-D levels early in the disease course may predict higher risk of conversion to clinically definite MS (62).

Additional studies showed that genetic effects on vitamin-D pathways may also contribute to MS risk. Two recent Mendelian randomization studies evaluated whether genetically lowered vitamin-D levels influenced the risk of MS. The first study identified four single-nucleotide polymorphisms (SNPs), which were in or near genes strongly implicated in vitamin-D metabolism. Consecutive Mendelian randomization studies showed that genetically lowered 25(OH)D levels were strongly associated with increased susceptibility to MS (63). These findings were confirmed in a recent study, which reported strong evidence of a causal effect of low serum 25(OH)D on MS risk that is independent of established environmental risk factors and not subject to reverse causality (64). Interestingly, MS risk was found to be associated with the gene encoding the enzyme that activates vitamin-D (CYP27B1) and the genetic variant rs703842 in CYP27B1 in Caucasians (65, 66). Additional findings highlight that vitamin-D may also be able to regulate genes of the immune system that play a role in MS development. Molecular studies showed that MS associated loci were enriched for VDR-binding sites, including the promoter region HLA-DRB1 (67, 68).

Several vitamin-D supplementation trials are currently underway either as stand-alone or add-on therapy to disease modifying treatment. The largest study so far is the SOLAR study, which enrolled 229 interferon-β-treated MS patients with a 25-hydroxyvitamin-D plasma concentration below 150 nmol/L. This double-blind placebo-controlled study of high-dose oral cholecalciferol oil (14,000 IU/day) showed intriguing results. The primary endpoint "no evidence of disease activity"

was not improved by vitamin-D supplementation; however, the secondary endpoint showed a 32% reduction in the number of new combined unique active lesions in the cholecalciferol group. Furthermore, there was a trend toward absence of new T1 hypointense lesions in vitamin-D-supplemented patients, which became significant in those aged 18–30 years. Therefore, the results support the notion that vitamin-D supplementation is a safe and an effective add-on treatment in MS patients on β-interferon (69). The SOLAR study results support the findings of an earlier smaller Finish randomized trial in which patients receiving 20,000 IU vitamin-D_3 per week had better MRI outcomes than those receiving placebo (70). This study and observational small studies support the notion that similar benefit can be obtained from much lower levels of vitamin-D supplementation (equivalent to about 3,000–4,000 IU/day) and supraphysiological doses (14,000 IU/day) like in the SOLAR study are not needed.

VITAMIN-D IN SCHIZOPHRENIA

In the second part of our review, we will discuss the role of vitamin-D in schizophrenia. Schizophrenia is a debilitating psychotic disorder that develops most commonly during the late adolescent to early adulthood period across both genders, with females having a later age of onset. It is debilitating in the sense that the sufferer's ability to function normally in society is heavily impaired by a range of positive (hallucinations and delusions), negative (avolition, anhedonia, and alogia), and cognitive symptoms (71).

Schizophrenia lifetime prevalence is about 1% of the general population, meaning millions of people worldwide suffer from the disease (72). However, in some ways, this statistic fails to represent the true number of individuals affected by this mental illness, as friends and family members will also be heavily impacted.

Schizophrenia manifests as a mixture of cognitive, negative, and positive symptoms. Positive symptoms refer to psychosis, such as hallucinations and delusions, while negative and cognitive symptoms refer more to impairments in emotional, social, and intellectual functioning. Distinguishing between negative and cognitive symptoms is difficult, but they should be viewed as independent targets for intervention (73). However, both are linked closely and non-respondent to antipsychotics, making their treatment difficult (74).

An important observation, supporting the notion of a biological disease and, indeed, the connection between infection, immune responses, and psychosis, was made by Julius Wagner-Jauregg as early as 1883 (75). A relationship between fever and "madness" had been postulated over centuries; in clinical experimentation at the Vienna asylum, Wagner-Jauregg injected patients suffering from tertiary syphilis or dementia paralytica with potent immunostimulators such as tuberculin and malaria. Some patients made remarkable recoveries—far more than without treatment. Although nowadays these experiments would be ethically forbidden for good reasons, they marked a paradigm shift in psychiatry and Wagner-Jauregg received the Noble prize in 1927 for his work on "pyrotherapy" (fever therapy). The British and American clinicians W. L. Templeton and Leland Hinsie went on to try fever treatment therapy on schizophrenic patients with observed improvement in some patients, however, not permanent. This line of investigation was given up due to the danger of the malaria treatment and the transient nature of improvement. Looking back at these experiments with our knowledge of the twenty-first century immunology, one observes that these may have been the first findings signposting the role of the immune system in schizophrenia.

There appears to be a diffuse non-specific activation of the immune system in schizophrenia (76). Further evidence of this comes from genome-wide associations mapping to the MHC region in schizophrenia susceptibility and immune cells involved in adaptive immunity (CD19 and CD20 B-lymphocytes) (77, 78). Notably, in 2014, in what was at the time the largest genetic study of mental illness, the researcher identified 108 loci associated with schizophrenia. Interestingly, recent GWASs have now robustly identified immune-related SNPs linked to schizophrenia. Recent cross genomic studies, which addressed the common architecture between schizophrenia and those of other psychiatric and non-psychiatric traits, revealed links between schizophrenia and MS (79). A significant genetic overlap was found between schizophrenia and MS mainly within the MHC. This study demonstrated the involvement of the same HLA alleles in MS and schizophrenia, but with an opposite directionality of effect. Intriguingly, recent population-based studies found that several psychiatric comorbidities including schizophrenia, anxiety, depression, and bipolar disorder were more common in MS population than in a matched control cohort (79, 80), as were white matter changes and myelin-related dysfunction in schizophrenia (81).

This work suggests that a subgroup of patients with schizophrenia may demonstrate aspects of an autoimmune process. Interestingly, the elimination of autoantibodies against neuronal cell surface proteins by immunotherapy has led to symptomatic improvement in some cases of first-episode psychosis (82). One putative environmental risk factor for schizophrenia is infection. Early childhood infections of the brain increase the risk ~5-fold (83). Even during pregnancy, particularly the second trimester, maternal infections correlated with an increased risk to the offspring later in life (84). Injecting pregnant mice with synthetic double-stranded DNA poly I:C, to mimic viral infections/interferon responses, and lipopolysaccharide, a highly inflammatory component of bacterial cell walls, elicits morphological and behavioral changes characteristic of the brain in schizophrenia (85); however, the underlying mechanisms are not fully understood.

The autoimmune hypothesis is also strengthened by the finding of increased autoimmune disease in relatives of schizophrenic patients and the inverse relationship of schizophrenia with rheumatoid arthritis and connective tissue diseases (86). Notably, a very recent study singles out the gene C4, a component of the intricate complement system that works together with the immune response to regulate immune tolerance, autoimmunity, and anti-pathogen responses, as the strongest genetic risk factor for schizophrenia (87). These findings have

lent additional support to theories regarding immunological dysregulation as an underlying cause of schizophrenia. However, there is no evidence for a direct link between vitamin-D and C4 levels.

The hypothesis of an active immune/inflammatory component, which lends support to the "mild encephalitis hypothesis" (88) in at least a subgroup of schizophrenia patients, is of great interest and might prompt novel preventive or therapeutic strategies, such as immunomodulation and/or anti-inflammatory drugs. A recent meta-analysis of anti-inflammatory medications in the management of treatment-resistant schizophrenia showed therapeutic effects of fish oils, *N*-acetyl-cysteine, and estradiol (89).

VITAMIN-D STUDIES IN SCHIZOPHRENIA PATIENTS

Several lines of evidence support a role for vitamin-D deficiency in the risk for schizophrenia. Epidemiological data suggest that schizophrenia is more common in those born in winter and spring and its prevalence also rises with increasing latitude (90). These findings, added to the evidence that dark-skinned minority groups in cold countries have a greater risk of schizophrenia (91), have led to the hypothesis that low vitamin-D (especially during early life) may be implicated in the genesis of schizophrenia (92). A study based on Danish neonatal dried blood spots supported this hypothesis (93), compared with neonates in the fourth quintile (vitamin-D_3 concentrations between 40.5 and 50.9 nmol/L), those in each of the lower three quintiles had a significantly increased risk of schizophrenia (twofold elevated risk).

Patients with psychosis have lower levels of vitamin-D than matched controls, even at the first presentation with psychosis (94–96). A mini-meta-analysis confirmed that schizophrenia patients have lower vitamin-D levels than healthy controls with a medium effect size (97). A systematic review (based on seven studies) has confirmed that those with psychosis are significantly more likely to have low concentrations of vitamin-D (98). Moreover, looking at specific schizophrenia symptomatology, levels of vitamin-D have been shown in some studies to inversely correlate with depression and negative symptoms, in patients with psychosis, controlling for other contributors (99).

Low vitamin-D may have also detrimental effects on brain development. The Dutch Hunger Winter and Chinese Famine studies have suggested a role for hypovitaminosis-D in the development of schizophrenia. However, findings regarding vitamin-D deficiency and its link to psychosis have the potential to be confounded by factors such as other nutrient deficiencies and ethnicity/skin tone. As a result, investigations around this association with schizophrenia are mainly supported by the more clear evidence of vitamin-D as a protective neuro-immunomodulator, which strongly suggests that hypovitaminosis-D during development would have profound effects on offspring outcome (100).

Concerning the impact of low vitamin-D on the adolescent and adult brain, a study based on a UK birth cohort ($n = 3,182$) found an association between low vitamin-D among children with a mean age of nine years and an increased risk of later psychotic-like symptoms during adolescence (mean age 14 years) (101). Diet also appears to be important—a large population-based study of Swedish women ($n = 33,623$) reported a significantly greater risk of psychotic-like experiences in those with low vitamin-D intake (102). Thus, the evidence suggests that low vitamin-D not only disrupts early brain development but may also compromise later periods of brain growth and maturation.

A prospective Finnish birth cohort study looked at one way of addressing this risk and found that vitamin-D supplementation in males during the first year of life resulted in a reduced risk of them later developing schizophrenia (103).

It is therefore plausible that vitamin-D may reduce inflammation and enhance resilience to neurobiological or pathogen-induced insults, which might increase risk of schizophrenia. Indeed, two cross-sectional studies have reported that vitamin-D is inversely associated with levels of C-reactive protein, a marker of inflammation, in psychosis (96, 104).

Despite all this circumstantial evidence, a recent Mendelian causation study (105), looking at SNPs associated with serum vitamin-D and schizophrenia in 34,241 schizophrenia cases and 45,604 controls, found no evidence for causal effect of vitamin-D on the risk for schizophrenia. Moreover, currently, there is no evidence from randomized controlled trials of vitamin-D supplementation in the relevant populations, although a trial is underway. Those randomized controlled trials are needed to confirm the effect of vitamin-D supplementation on inflammation in patients with schizophrenia.

VITAMIN-D IN AUTISM SPECTRUM DISORDER (ASD)

Autism spectrum disorders are a heterogeneous group of complex neurodevelopmental disorders that undermine optimal brain development. A recent surveillance study identified 1 in 68 children (1 in 42 boys and 1 in 189 girls) as having ASD. Recent data collated for ASD suggest that the cost is at least £32 billion a year (106).

While ASD is currently diagnosed on the basis of abnormalities in social communication and repetitive behaviors, it is increasingly being recognized as a whole-body disorder. The core behavioral characteristics such as altered communication and social skills, cognitive and learning deficits, and stereotypic behaviors are being intrinsically linked to complex biological processes.

Increasing evidence suggests that altered immune responses in ASD may be related to the severity of behavioral impairment and other developmental outcomes (107). Abnormal cytokine profiles have been described with elevated levels of pro-inflammatory cytokines (e.g., IL-6, IL-8, IL1β, IFN-γ, and eotaxin) (108). Several *postmortem* and neuroimaging studies have found chronic neuroinflammatory processes such as microglial activation in the CNS (109, 110). It has been suggested that this chronic immune activation could be a response to an early autoimmune

attack on the brain by mother-to-fetus transfer of autoantibodies and/or maternal infection (111–114). Research into antibody-mediated CNS disorders may help identify a subgroup of patients with antibody-mediated illness, which may be relevant to autism and schizophrenia (115). Findings from animal models in ASD point toward inflammatory processes; and anti-inflammatory/immune-modulating drugs in ASD have been trialed (116). It is now well established that individuals with autism have much higher than expected rates of a range of comorbidities, which support dysregulation of immune mechanisms, inflammation, and a potentially altered gut–brain axis and resemble findings in other inflammatory and autoimmune diseases (117). Autoimmune and gastrointestinal problems are often present in ASD, and nutritional approaches have become widely used in managing ASD (118).

Autism spectrum disorder is an extremely heterogeneous disorder. It is suggested that the origin of ASD influences the phenotype—thus, e.g., ASD caused by maternal infection during pregnancy may trigger a different set of symptoms than more genetically driven forms of ASD or combinations of environmental (pollution, neurotoxins, etc.) and genetic factors (118). In addition, gender seems to play an important role as girls with ASD often present with milder social and communicative symptoms, relatively intact symbolic play skills and fewer obsessional interests (119). Ecological studies observed the correlation between the number of ASD cases and a number of environmental factors such as latitude, season of birth, mother's skin type, and the climate, implicating a possible role for vitamin-D in ASD (120).

VITAMIN-D STUDIES IN ASD

Several studies found lower vitamin-D levels in children with autism compared to their siblings, parents, and non-family controls (17, 121). Low vitamin-D levels were already present at birth in children later diagnosed with ASD but not in their healthy siblings (122). Subsequent research demonstrated that the vitamin-D status of mothers corresponded with their offspring's vitamin-D status at birth. Low levels of vitamin-D during pregnancy impacted negatively on the cognitive status, early development, and ASD diagnosis (123).

Two studies have addressed the impact of vitamin-D supplementation on ASD. One found improved core symptoms in children supplemented with pharmacological doses of vitamin-D (124). Furthermore, a preliminary study found that vitamin-D supplementation during pregnancy and early childhood decreased the occurrence of ASD in siblings (125). However, optimal vitamin-D dosage and levels are not yet determined, and proper randomized trials are needed.

Several studies looked at vitamin-D-specific gene variants and the risk of ASD. Recently, paternal and child genetic abnormalities in vitamin-D metabolism in ASD were reported (126). Notably, paternal VDR *Taq*I homozygous variant genotype and VDR *Bsm*I and offspring's GC AA-genotype/A-allele were associated with ASD, whereas offspring's CYP2R1 AA-genotype was significantly associated with decreased risk of ASD.

Further support for a role of vitamin-D comes from a recent study, which reported association between polymorphisms in the VDR gene and vitamin-D levels in ASD children (127). Vitamin-D levels were influenced by *Fok*I polymorphisms and haplotype GTTT (*Bsm*I/*Taq*I/*Fok*I). Interestingly, this polymorphism resulted in compensatory higher vitamin-D levels due to lower VDR activity in ASD akin to the findings reported in MS (127, 128).

Of particular interest is the observed strong gender bias in ASD (four males:one female), which may be suggestive of abnormalities in steroid metabolism, which comprises the hormones cortisol, testosterone, estrogens, progesterone, and vitamin-D. Indeed, ASD children have significantly higher levels of a number of C21 and C19 steroid hormones, especially androgens (129, 130). Altered steroid hormone levels have also been identified in children with Smith–Lemli–Opitz syndrome (SLOS), which carries a comorbid ASD risk of 50–86% (131). Mutations in the DHCR7 gene that codes for the enzyme 3β–hydroxysterol-Δ(7)-reductase, the catalyst for the final step in cholesterol biosynthesis (132), lead to hypo-cholesterolemia and often higher levels of 7-dehydrocholesterol in SLOS patients. Dysregulation of the steroid metabolome, in synergy with genetic predisposition and other environmental risk factors (e.g., methylation, maternal infection, neurotoxins and other chemicals, premature birth, paternal age), may act as a potential risk factor for the development of ASD, schizophrenia, and other mental disorder with hypovitaminosis-D being one of the possible hallmarks (133).

Autism spectrum disorder research and diagnosis would benefit from greater analysis of metabolic markers and genetic polymorphisms, to aid patient stratification and identify therapeutically relevant biomarkers to inform diagnosis, prevention, and treatment strategies. Collaborations between geneticists, immunologists, steroid chemists, endocrinologists, nutritionists, psychiatrists, and psychologists will be needed to decipher the complex pathophysiology of ASD.

CONCLUSION

Multiple sclerosis, schizophrenia, and autism are multifactorial disorders caused by the effects of multiple genes in combination with environmental factors. As environmental risk factors are modifiable—in contrast to genetic susceptibility—they offer potential strategies for intervention and prevention. Great efforts are being made in identifying risk factors in these conditions and vitamin-D is one of the culprits, with most evidence in MS, where hypovitaminosis-D seems to contribute to disease activity and vitamin-D supplementation studies have shown some promise.

There is also some evidence, albeit less clear, that hypovitaminosis-D may act as risk factors for schizophrenia and autism, and further research and longitudinal studies are needed. Inflammatory responses appear to play a significant role in the etiology of both schizophrenia and autism. Whether and how vitamin-D contributes to the pathophysiology of these

conditions is unknown. Further insight into the role of vitamin-D, in schizophrenia and autism, especially as it relates to the immune system, inflammation, and neuroprotection, will help shed light on the underlying pathophysiology of these conditions and may aid the design of better treatment strategies for the twenty-first century.

AUTHOR CONTRIBUTIONS

U-CM, AK, and EK helped in drafting the manuscript, and U-CM, AK, EK, and FG helped in reviewing the manuscript.

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Vitamin D Deficiency in Adult Patients with Schizophreniform and Autism Spectrum Syndromes: A One-Year Cohort Study at a German Tertiary Care Hospital

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Introduction: Vitamin D has many immunomodulatory, anti-inflammatory, and neuroprotective functions, and previous studies have demonstrated an association between vitamin D deficiency and neuropsychiatric disease. The aim of our study was to analyze the prevalence of vitamin D deficiency in a 1-year cohort of adult inpatients with schizophreniform and autism spectrum syndromes in a naturalistic inpatient setting in Germany.

Participants and methods: Our study was comprised of 60 adult schizophreniform and 23 adult high-functioning autism spectrum patients who were hospitalized between January and December of 2015. We compared our findings with a historical German reference cohort of 3,917 adults using Pearson's two-sided chi-squared test. The laboratory measurements of 25-hydroxyvitamin D2/3 [25(OH)vitamin D] were obtained using a chemiluminescence immunoassay.

Results: In the schizophreniform group, we found decreased (<20 ng/ml) 25(OH)vitamin D levels in 48/60 (80.0%) of the patients. In the autism spectrum group, decreased levels were detected in 18/23 (78.3%) of the patients. 25(OH)vitamin D deficiencies were found in 57.3% of the historical control group. Particularly, severe deficiencies (<10 ng/ml) occurred much more frequently in the schizophreniform (38.3%) and autism spectrum groups (52.2%), when compared to the control group (16.3%). The recommended 25(OH)vitamin D values of >30 ng/ml were observed in only 5% of the schizophreniform patients, 8.7% of the autism spectrum patients, and 21.9% of the healthy controls.

Discussion: We found very high rates of 25(OH)vitamin D deficiencies in both patient groups and have discussed whether our findings might be related to alterations in the immunological mechanisms. Irrespective of the possible pathophysiological links between vitamin D deficiency and schizophrenia or autism spectrum disorders, a more frequent measurement of vitamin D levels seems to be justified in these patient groups. Further prospective, controlled, blinded, and randomized research should be conducted to analyze the effectiveness of vitamin D supplementation on the improvement of psychiatric symptoms.

Keywords: schizophrenia, autism spectrum disorder, vitamin D, inflammation, mild encephalitis

INTRODUCTION

The role of vitamin D levels in skeletal health is well known; for example, vitamin D deficiency can cause or exacerbate osteoporosis, lead to muscle weakness, and increase the risk of bone fractures (1). Moreover, vitamin D has many immunomodulatory, anti-inflammatory, and neuroprotective functions (2). Previous studies have demonstrated an association between vitamin D deficiency and metabolic (e.g., atherosclerosis), neoplastic (e.g., colon cancer), and immune disorders (e.g., Type 1 diabetes mellitus) (1, 3). In neuropsychiatric research, the associations between vitamin D deficiency and multiple sclerosis (MS), Parkinson's disease, schizophreniform disorder, autism spectrum disorders, and Alzheimer's disease have recently been described (4–6).

Vitamin D Deficiency in Neuropsychiatry

The association between MS and vitamin D deficiency is an established issue in the respective neuropsychiatric research, and it may be an independent risk factor for the development of MS. Moreover, vitamin D seems to modulate the course of the disease, in that higher vitamin D levels are correlated with reduced MS activity (2, 7). Earlier studies have also shown clear indications of an association between vitamin D deficiency and schizophreniform disorder. In the largest study of psychoses to date, vitamin D deficiency was identified in 86% of the cases, and in 49%, a severe deficiency of <10 ng/ml was reported (8). A recent meta-analysis found vitamin D deficiencies in 65.3% of these patients (9). In another meta-analysis, statistically significantly lower vitamin D levels were found in autistic patients compared to healthy controls. However, most of these studies were performed in children (10). A developmental vitamin D deficiency might lead to alterations in the structural and functional (e.g., alterations in the dopaminergic functions) brain features. Furthermore, an adult deficiency may be associated with diverse immunological alterations, as well as neurochemical changes (6, 10).

Vitamin D Metabolism

Vitamin D is produced in the skin through the conversion of provitamin D3 to previtamin D3; however, the nutritional intake of vitamin D2/3 is small. Vitamin D can be stored in fat tissues or modified in the liver by the 25-hydroxylase enzyme to 25-hydroxyvitamin D2/3, which is then transformed to the active metabolite 1,25-dihydroxyvitamin D2/3, mainly in the kidneys. The synthesis of vitamin D3 is enhanced by increased levels of parathyroid hormone, which in turn, increase the calcium concentrations. Reduced phosphate can also lead to the production of vitamin D3 (3). The serum level of 25-hydroxyvitamin D2/3 [in this paper abbreviated as "25(OH)vitamin D"] is an established marker of the current vitamin D status and was therefore measured in our study.

Vitamin D Levels in Germany

The German Nutrition Society[1] and the American Institute of Medicine[2] have defined a vitamin D deficiency as levels <20 ng/ml. These references are based on calculations with respect to the distribution of the "vitamin D requirement curve" in the general population. Serum levels of ≥20 ng/ml are necessary for bone health in 97.5% of the individuals in the population (11); however, preferred vitamin D values are generally above 30 ng/ml (1, 3, 9, 12). A German reference cohort of adults is available from the Robert Koch Institute and was reported in a statement from the German Nutrition Society. In this cohort of 3,917 adult subjects ranging from 18 to 79 years, an average vitamin D level of 18 ng/ml was found, and overall vitamin D deficiency (levels <20 ng/ml) was found in 57.3% of subjects. The specific breakdown was as follows: 2% were below 5 ng/ml, 14.3% were 5–10 ng/ml, 41% were 10–20 ng/ml, and concentrations between 20 and 30 ng/ml were found in 20.8% of the subjects. Recommended vitamin D levels of >30 ng/ml were identified in 21.9% of these subjects (13).

Rationale for the Study

In our clinic, we offer a broad diagnostic workup for patients with schizophreniform syndromes, including a broad range of laboratory measurements, cerebrospinal fluid (CSF) analyses, electroencephalography (EEG), and cerebral magnetic resonance imaging (cMRI) (14–16). In doing so, we have found non-specific immunological CSF alterations in 54.4% and overall abnormalities, including EEG and MRI findings, in 75.6% of the patients (14). Moreover, among our patients with autism spectrum disorders, we routinely perform laboratory analyses, EEGs, and cMRIs (17, 18). In our earlier structural imaging studies, we found no differences between those patients with high-functioning autism and the healthy controls (19), although neurochemical alterations in the glutamatergic prefrontal system were detected with MR spectroscopy (20, 21). From January to December of 2015, we measured the 25(OH)vitamin D levels of the schizophreniform and autistic patients in our specialized ward for these diseases, with the aim of analyzing the prevalence of 25(OH)vitamin D deficiency in this 1-year cohort of adult inpatients in Germany. We hypothesized that there would be increased rates of 25(OH) vitamin D deficiency in both patient groups, when compared with the large German reference cohort. Moreover, we conducted an exploratory analysis of the possible correlations between the 25(OH)vitamin D levels and psychopathological scores.

PARTICIPANTS AND METHODS

Study Sample

For this research, we included patients with schizophreniform and high-functioning autism spectrum syndromes, who were admitted to our specialized ward from January to December of 2015. The study was part of a larger project analyzing immunological markers, which received approval from the local ethics committee (Faculty of Medicine, Freiburg University, EK-Fr 609/14). Those patients who had been transferred from our sheltered ward to our special unit for schizophreniform and autism spectrum disorders were excluded, since they did not receive this standard diagnostic procedure. Similarly, those patients who were already being treated with vitamin D were excluded ($N = 2$). The diagnostic procedure was conducted by experienced in-house senior consultant psychiatrists, following

[1] www.dge.de

[2] www.nationalacademies.org/hmd/

the criteria of the International Classification of Diseases, tenth revision. This approach led to the inclusion of 60 schizophreniform and 23 high-functioning autism spectrum patients in our study. The schizophreniform syndrome group was comprised of 41 patients with schizophrenia, 7 with schizoaffective disorders, 6 with organic schizophreniform disorders, 2 with substance-induced psychosis, and 4 patients each with acute polymorphic psychotic disorder, organic hallucinations, delusional disorder, or schizotypal disorder. The autism spectrum group included 9 patients with Asperger's syndrome and 14 with atypical autism. The autistic patients were admitted to our clinic for diagnostic purposes and participation in a specific psychotherapy program to improve social interaction (The Freiburg Asperger Specific Therapy Manual for Adult Patients or the FASTER program) (22), as well as for treatment of comorbidities (e.g., depression). All of the patients were Caucasian and lived in Germany. The historical German control cohort was collected from the Robert Koch Institute in 1998 within the scope of the German health survey, which analyzed 3,917 adult subjects between 18 and 79 years old (13).

Laboratory Measurements

The laboratory measurements of the patients' 25(OH)vitamin D levels were determined using a chemiluminescence immunoassay at our Institute for Clinical Chemistry and Laboratory Medicine.[3] Vitamin D deficiency was defined as serum 25(OH)vitamin D levels <20 ng/ml, relative vitamin D insufficiency was 20–30 ng/ml, and the preferred vitamin D range was >30–60 ng/ml (1, 9, 11).[4] The control group was also analyzed using a chemiluminescence immunoassay (13).

Psychometric Data

The psychopathological scores for attention and memory, formal thought disorder, fear and compulsion, delusions, affectivity, energy and psychomotor domain, circadian rhythm, and suicidal tendency were acquired in a standardized way according to the AMDP system (Arbeitsgemeinschaft für Methodik und Dokumentation in der Psychiatrie[5]). Together with the sociodemographic data, all of the data were obtained using our clinic's electronic documentation system.

Statistical Analyses

The statistical analyses were performed using the Statistical Package for the Social Sciences software, version 22 (SPSS 22[6]) and R software, version 3.2.2.[7] The main results were presented descriptively. The group comparisons (i.e., schizophreniform/autism patient groups vs. historical controls) for the rate of decreased 25(OH)vitamin D levels were calculated using Pearson's two-sided chi-squared test and Yates' continuity correction (in *R*). The correlation analyses between the 25(OH)vitamin D levels and psychometric scores were conducted separately for each group using the Pearson correlation coefficient (in SPSS 22). For the statistical analyses used to develop further hypotheses, a p-value <0.05 served as the criterion of significance.

RESULTS

Sociodemographic Data

The average age of the entire cohort was 32.98 ± 11.05 years old, ranging from 18 to 71 years old, and male patients were predominantly included in both patient groups. The sociodemographic details are presented in **Table 1**. Fifty-five of the 60 schizophreniform patients and 15 of the 23 autism spectrum patients were medicated. Fifty patients of the schizophreniform group received neuroleptics; 29 schizophreniform patients received neuroleptics as a monotherapy, in 21 patients in combination (e.g., with anticonvulsants or lithium in patients with schizoaffective disorders). The autistic patients were treated with antidepressants and/or neuroleptics.

Laboratory Findings

For the schizophrenia spectrum group, we found decreased 25(OH)vitamin D levels in 80% of the patients, and relative insufficiency in 15%; therefore, the preferred 25(OH)vitamin D range was observed in only 5% of the cases. In the autism spectrum group, we found decreased levels in 78.3% of the patients, relative insufficiency in 13%, and preferred values in 8.7% [see **Table 1** for the absolute values and exact distribution of the 25(OH)vitamin D levels]. In the control group, 25(OH)vitamin D deficiencies were detected in 57.3% of the patients, relative insufficiency in 20.8%, and a preferred 25(OH)vitamin D range in 21.9%. In particular, severe deficiencies (<10 ng/ml) were much more common in the schizophrenia (38.3%) and autism spectrum groups (52.2%), when compared to the controls (16.3%). From January to August of 2015, all of the patient measurements fell below the recommended threshold of 30 ng/ml (**Figure 1**). The details of patients within the recommended vitamin D levels are discussed in **Figure 1**. The 25(OH)vitamin D levels in the schizophrenia group ($N = 41$) were lower than among those patients with other psychotic syndromes ($N = 19$), although this finding was not statistically significant (13.64 ± 8.22 vs. 17.85 ± 12.39; $p = 0.124$).

Statistical Analyses

25(OH)vitamin D deficiencies (<20 ng/ml) were significantly more common in the schizophrenia group, when compared with the historical controls ($Chi^2 = 11.559$, df = 1, $p = 0.001$), and a trend in the same direction was detected in the autism spectrum group ($Chi^2 = 3.2964$, df = 1, $p = 0.069$). A severe 25(OH)vitamin D deficiency (<10 ng/ml) was significantly more frequent in the schizophrenia ($Chi^2 = 19.131$, df = 1, $p \leq 0.001$) and autism spectrum patients ($Chi^2 = 18.826$, df = 1, $p \leq 0.001$). Vitamin D deficiency/severe deficiency in the control group did not significantly differ between the male and female groups, and the same was true for the schizophreniform and the autism spectrum patient groups. The correlation analyses for the schizophreniform group showed no significant correlations between their 25(OH)vitamin D levels and any psychopathological score; there were also no significant correlations when we analyzed unmedicated ($N = 5$)

[3] www.uniklinik-freiburg.de/ikcul.html
[4] www.dge.de/wissenschaft/referenzwerte/vitamin-d/
[5] www.amdp.de
[6] www-01.ibm.com/software/analytics/spss
[7] www.r-project.org

TABLE 1 | Vitamin D findings in the schizophreniform and autism spectrum syndrome groups.

	Schizophreniform syndromes ($n = 60$)	Autism spectrum syndromes ($n = 23$)	Entire patient cohort ($n = 83$)	German control group ($n = 3\,917$)[a]
Demographic information				
Age – *mean* ± *SD*	33.5 ± 11.3	31.7 ± 10.6	33.0 ± 11.1	n.a.[b]
Age – *range*	18–71 years	19–57 years	18–71 years	18–79 years
Gender – *ratio*	35 males:25 females	16 males:7 females	51 males:32 females	1,706 males:2,211 females
Laboratory findings				
Vitamin D levels (in ng/ml) – *mean* ± *SD*	15.0 ± 9.8	14.5 ± 9.8	14.9 ± 9.8	18 ± 12.6[c]
Vitamin D levels from 0 to 5 ng/ml	6 (10%)	2 (8.7%)	8 (9.6%)	2%
Vitamin D levels from 5 to 10 ng/ml	17 (28.3%)	10 (43.5%)	27 (32.5%)	14.3%
Severe vitamin D deficiency (levels <10 ng/ml)	23 (38.3%)	12 (52.2%)	35 (42.2%)	16.3%
Vitamin D levels from 10 to 20 ng/ml	25 (41.7%)	6 (26.1%)	31 (37.3%)	41%
Overall vitamin D deficiency (levels <20 ng/ml)	48 (80%)	18 (78.3%)	66 (79.5%)	57.3%
Relative insufficiency of vitamin D levels from 20 to 30 ng/ml	9 (15.0%)	3 (13.0%)	12 (14.5%)	20.8%
Recommended vitamin D levels from 30 to 60 ng/ml	3 (5.0%)	2 (8.7%)	5 (6.0%)	21.9%[d]

[a]*Reported in Linseisen et al. (13).*
[b]*Information not available.*
[c]*SD for the historical control group was post hoc and calculated by us.*
[d]*Reported are values >30 ng/ml.*
Abbreviation: SD, standard deviation.

and medicated ($N = 55$) groups separately. However, for the autism spectrum group, we found a correlation between 25(OH) vitamin D levels and circadian rhythm ($r = 0.458$, $p = 0.028$; $N = 23$), as well as between 25(OH)vitamin D and energy levels ($r = 0.421$, $p = 0.046$; $N = 23$). These notable correlations were still significant in the medicated patient group ($N = 15$) but were not found in the unmedicated patients ($N = 8$).

DISCUSSION

The main findings of our study were decreased 25(OH)vitamin D levels in 80% of the schizophreniform patients and 78% of the patients with autism spectrum disorders. In particular, severe deficiencies (<10 ng/ml) were much more common in both patient groups, when compared to the healthy controls.

Limitations

In this study, we have described a 1-year cohort of electively hospitalized adult patients in our special unit for schizophreniform and autism spectrum syndromes at a tertiary care university hospital in Freiburg, Germany. We compared our findings to a historical German control group of 3,917 adult subjects between 18 and 79 years old and used the same method to measure 25(OH)vitamin D in another laboratory (13). However, this control group was not matched for gender, age, body mass index, sunlight exposure, or seasonality. The (severe) vitamin D deficiency in the control group did not significantly differ between males and females, and the same was true in the schizophreniform (35 males:25 females) and autism spectrum (16 males:7 females) patient groups. Therefore, we do not believe that gender distribution had a decisive influence on our findings. However, we were unable to correct for the other influencing factors (age effects, body mass index, sunlight exposure, and seasonality), because this information was unavailable for the control group. Therefore, social withdrawal in the patient cohort *per se* might be responsible for the detected vitamin D deficiency. Moreover, the representative control cohort could have included subjects with unrecognized psychiatric diseases, because information about the prevalence of psychiatric comorbidity in the reference cohort was not available. The information about the control group is only available in German; we therefore have included the main information in **Table 1**. We analyzed a 1-year cohort to determine the possible effects of seasonality as a function of sun radiation, but our results are not comparable with those of previous studies of populations living at other latitudes and longitudes, due to the different levels of sun exposure. Since vitamin D levels depend on sun exposure and the intensity of radiation, it is mandatory to perform research in different regions. Moreover, *in vitro* studies have suggested an interaction between vitamin D levels and antipsychotic drugs (23), in that antipsychotic exposure is associated with lower vitamin D levels. Thus, our results may have been influenced by the effects of the medications taken by our patients. Clearly, the definition of the reference ranges for vitamin D is controversial; however, there is concurrence that vitamin D levels of less than 20 ng/ml are too low (1, 9, 11). We used these established cutoff values following the German Nutrition Society (see text footnote 1) and the American Institute of Medicine (see text footnote 2). As described in the Section "Introduction," these references are based on calculations with respect to the distribution of the "vitamin D requirement curve" in the general population. Therefore, we cannot speak from normal values in the common sense (like a 95th percentile), because many of the healthy controls also suffered from vitamin D deficiency. Further research should explore this aspect.

Finally, the measurement of 25(OH)vitamin D using a chemiluminescence immunoassay is well established, and the preanalytical stability has been previously described as solid and reliable (24). In addition, our serum samples were analyzed directly after blood collection; therefore, the methodological aspects are unlikely to be responsible for the alterations we found.

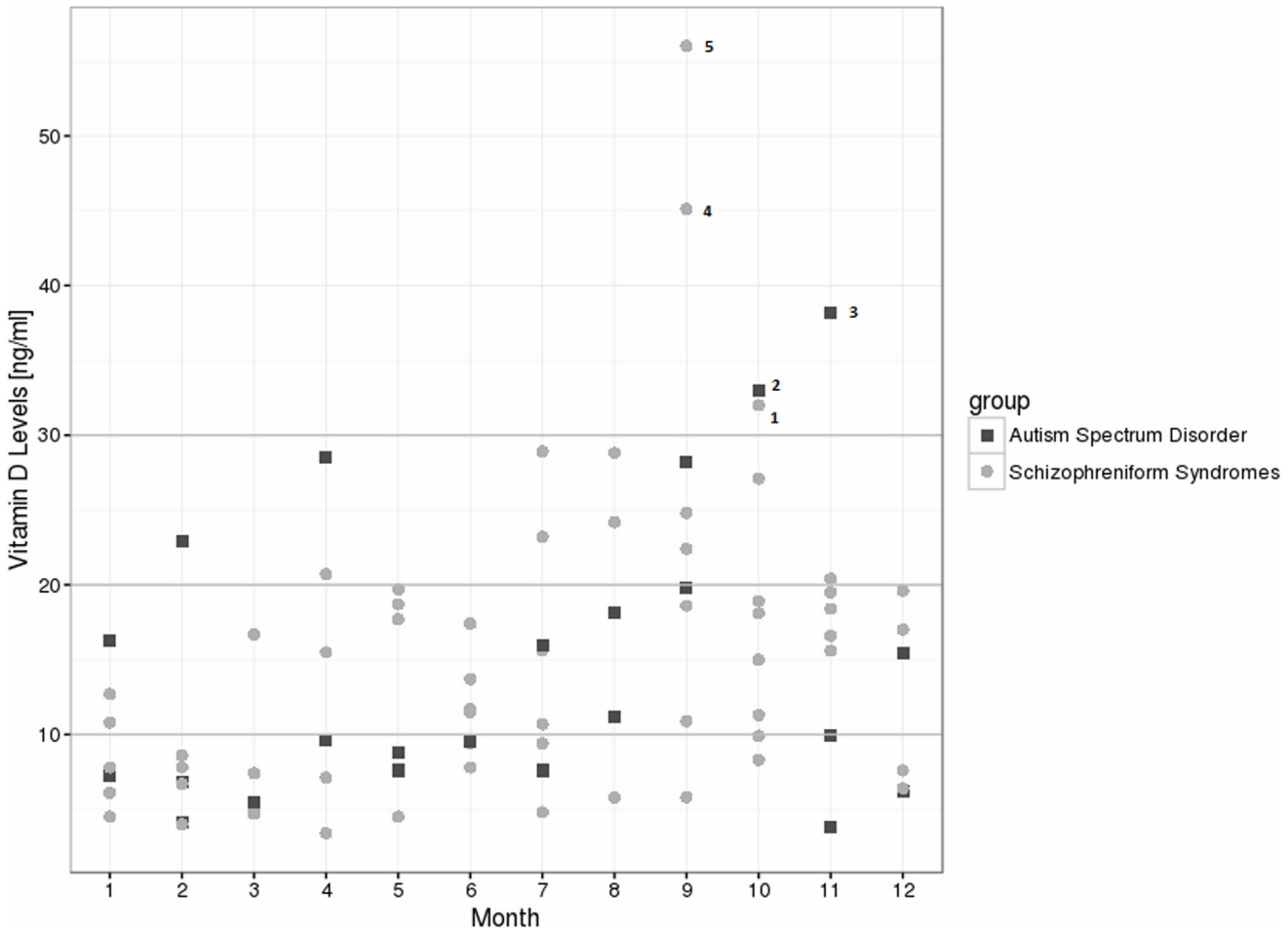

FIGURE 1 | Vitamin D level distribution over 1 year among the schizophreniform and autism spectrum disorder groups. Five patients reached recommended levels >30 ng/ml: *patient 1*: 43 years, female, schizophreniform syndrome (organic schizophreniform disorder), treated with olanzapine, body mass index (BMI) of 26.7 kg/m^2; *patient 2*: 25 years, male, autism spectrum disorder (Asperger syndrome), no psychiatric medication, BMI of 19.4 kg/m^2; *patient 3*: 41 years, male, autism spectrum disorder (atypical autism), treated with olanzapine and clomipramine, BMI of 23.7 kg/m^2; *patient 4*: 37 years, male, schizophreniform syndrome (schizophrenia), treated with olanzapine and escitalopram, BMI of 25.6 kg/m^2; *patient 5*: 21 years, male, schizophreniform syndrome (schizoaffective disorder), treated with quetiapine and venlafaxine, BMI of 26.4 kg/m^2.

Comparison with Previous Studies

A systematic review of all available studies with schizophreniform cohorts through 2013, which consisted of a total of eight studies, found an overall prevalence rate of vitamin D deficiency of 65.3% (9), which is lower than the rate in our German cohort. The odd ratios indicated that children with vitamin D deficiency were 2.16 times more likely to develop schizophrenia (9). The largest uncontrolled study to date analyzing the prevalence rates in 324 psychotic patients comes from the United Kingdom (8), and the authors found lower vitamin D levels in 86% of the participants and mean vitamin D levels of 12.4 ng/ml ± 7.3 (in comparison with 14.97 ng/ml ± 9.83 in our schizophreniform patient group). Well in line with our findings, the authors described severe vitamin D deficiency (<10 ng/ml) in 49% of the subjects. We also found strong suppressed 25(OH)vitamin D levels (<10 ng/ml) significantly more often, when compared to the historical control group. The differences in the mean vitamin D levels could be explained by the different levels of sun exposure, seasonal effects (i.e., different distribution of the measurements over the year), and the inclusion of different ethnicities (i.e., a broader spectrum in the study by Lally and colleagues) (8). With respect to the subtype of the psychotic disorder, other earlier studies found that patients with schizophrenia displayed lower vitamin D levels when compared to those with non-schizophreniform psychoses (25). In line with that, we also detected a trend in this direction. Moreover, lowered vitamin D levels have previously been associated with negative symptoms (4, 26). However, in this study, we were unable to detect such correlations for the schizophreniform patients.

In the first meta-analysis of vitamin D levels in autistic patients, which included 11 studies, every single case–control study reported significantly reduced vitamin D levels in those patients with autism. Corresponding to this, the authors described a statistically significant reduction in the vitamin

D levels in the autism group, when compared to the controls. However, no adult studies were included in the meta-analysis (10). Research that combined adolescents and adults ($N = 40$), and adults with autism spectrum disorders ($N = 10$) showed similar findings (10, 27, 28). When looking at dimensional associations in the correlation analyses of the autism spectrum disorder group, we found a significant dimensional association between the 25(OH)vitamin D and energy levels, as well as with the circadian rhythms, in that higher 25(OH)vitamin D levels were associated with a higher severity of symptoms. However, this observation does not support the hypothesis that vitamin D deficiency is a marker of symptom severity. Based on the small sample size, these findings clearly need further investigation.

Pathophysiological Interpretation: Inflammation and Schizophrenia

One previous study found that acute psychotic patients had significantly lower vitamin D levels, when compared to psychotic patients in remission and healthy controls (29). One might therefore speculate that vitamin D levels can modulate disease activity in this subgroup of patients. Nevertheless, until now, we have not been able to determine whether vitamin D deficiency is the cause, or rather a sequela of the psychiatric disorder. Many findings support the idea that vitamin D-associated autoimmune processes may play a role in the primary prevention and pathogenesis of schizophreniform disorders. The most elaborate insight into the role of vitamin D in neuropsychiatric disorders comes from MS research. The role of vitamin D in immunomodulation has been supported by the discovery that vitamin D receptors are expressed in most immune cells, including those in the brain (30, 31). Since the 25-hydroxylase enzyme, the rate-limiting enzyme for vitamin D synthesis, is also presented in these immune cells, they are able to secrete vitamin D in both autocrine and paracrine ways. Earlier studies have shown that vitamin D is indirectly able to reduce the T cell stimulatory capacity. In addition, vitamin D has direct effects on immunoregulatory T lymphocytes by stimulating the production of Type 2 helper T cell cytokines and reducing the production of Type 1 helper T cell cytokines. Furthermore, vitamin D inhibits B cell differentiation and immunoglobulin secretion, as well as T cell and B cell proliferation (31). *Via* such different effects, vitamin D contributes to immunoregulation and may decrease inflammation and produce immunoprotective effects (10, 31). Therefore, vitamin D may also lead to reduced C-reactive protein levels (8). In accordance with these observations, vitamin D deficiency may result in the withdrawal of these anti-inflammatory effects and may therefore support mild inflammatory processes (32). In non-psychiatric patients, vitamin D was found to be present in the CSF; however, the CSF concentrations were lower than those found in the corresponding sera (33). Moreover, it is still unknown how vitamin D reaches the brain (34). We previously demonstrated a blood–brain barrier dysfunction in a subgroup of more than 20% of the schizophreniform patients (14), and one could speculate that this could lead to altered intrathecal vitamin D levels. Vitamin D also seems to play a role in control of infections, for instance, by influencing the risk for tuberculosis or Epstein–Barr virus infection (35, 36). Therefore, vitamin D might not only have direct effects on immunological processes but could also influence infectiological mechanisms, which might be the link to an infectious hypothesis of schizophrenia. Further research should analyze the association between vitamin D levels and other immunological serum and CSF markers. Furthermore, in terms of the neurochemical function, alterations in the γ-aminobutyric acid, glutamate, and dopamine metabolism were found to be associated with vitamin D metabolism (6, 34), which might be another way in which vitamin D contributes to the CNS information processing in schizophrenia or autism. However, the precise pathophysiology of the immunological processes in relation to vitamin D metabolism and different disorders remains unclear, and more research is necessary in this field.

The Role of the Vitamin D Measurement in Basic Neuropsychiatric Diagnostics

Based on the current evidence, we are far from stating that vitamin D deficiency does play a critical role in the pathophysiology of schizophreniform disorders or autism. However, there is reasonable evidence that this might be the case, in particular, with respect to the established data on MS. More importantly, irrespective of such possible pathophysiological aspects, it is well established that low vitamin D levels do play a causal role in other systemic aspects of physical health (osteoporosis, muscle weakness, atherosclerosis, neoplastic disorders, diabetes mellitus, etc.) (1, 3). Due to our findings of 25(OH)vitamin D deficiency in about 80% of these patients, we believe that more frequent measurements of vitamin D levels in inpatients with schizophreniform and autism spectrum syndromes are justified. In order to do an overall assessment of the vitamin D status, the measurement of the serum 25-hydroxy vitamin D level is recommended, because it reflects total vitamin D from sunlight exposure, dietary intake, and conversion out of the stores in the liver and fatty tissue (1).

Treatment of Patients with Vitamin D Deficiency

Interventional studies in which vitamin D has been administered in schizophreniform disorder patients have shown mixed results (37, 38). For example, in a Finnish birth cohort of over 9,000 individuals, vitamin D supplementation in the first year of life reduced the risk of schizophrenia by 77%. Interestingly, this finding was detected in males, but not in females (39). In a small study of a schizophreniform immigrant population, daily vitamin D supplementation (of 1,000 IU/day) did not lead to changes in their psychiatric symptoms (40). The mixed results of earlier studies in schizophreniform patient cohorts might be a consequence of non-comparable doses of vitamin D. Furthermore, small early interventional studies in infantile autism spectrum groups have provided encouraging results; however, studies in adult autism, especially blinded, randomized placebo-controlled trials are absent (41).

CONCLUSION

We found a very high prevalence of 25(OH)vitamin D deficiency in adult patients with schizophreniform and autism spectrum

syndromes admitted for inpatient treatment in a tertiary referral center for psychiatry and psychotherapy. It is unproven, but possible, that these findings may be associated with immunological alterations in a subgroup of these patients. However, irrespective of such speculative pathomechanisms, we suggest more frequent measurements of vitamin D levels in patients with these disorders, in light of the high prevalence of vitamin D deficiency. The individualized supplementation of patients with schizophreniform and autism spectrum disorders with vitamin D deficiencies may be considered, with regard to the possible consequences of further vitamin deficiency (osteoporosis, enhanced cardiovascular risk, etc.). Further prospective, controlled, randomized research should be conducted to assess the efficacy of vitamin D supplementation in patients with and without suppressed vitamin D levels and schizophreniform/autistic syndromes. If vitamin D treatment should turn out to be effective, one might also discuss supplementation with moderate doses without any measurement due to the high prevalence of vitamin D deficiency, because the measurement is very costly as compared to the vitamin itself.

AUTHOR CONTRIBUTIONS

LTvE and DE initiated the study and conducted the data analyses. DE wrote the paper. AB supervised the laboratory measurements. BF supported the statistical analyses. All of the authors were crucially involved in the theoretical discussion and performance of this study. Furthermore, all of the authors read and approved the final version of this manuscript.

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frontiers in Psychiatry

ORIGINAL RESEARCH
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Alterations in Cerebrospinal Fluid in Patients with Bipolar Syndromes

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Bipolar disorder (BD) is a severe and lifelong condition. Primary endogenic polygenetic forms are common. Secondary organic forms have received increasing interest recently due to the detection of immunological encephalopathies that mimic various psychiatric syndromes, including BD. However, only limited data about routine findings of cerebrospinal fluid (CSF) analyses in BD are available. Therefore, we investigated the frequency of alterations in the CSF in patients with BD and the association with autoantibodies, cerebral magnetic resonance imaging, and electroencephalography findings. CSF samples of patients with BD collected from January 1998 until December 2015 were analyzed retrospectively. Patients with preexisting causes for alterations in the CSF (e.g., patients with obvious past or current neurological disorders) were excluded. In total, 63 patients with BD fulfilled the inclusion criteria for the study. In 1.6% of the patients with BD, an increased white blood cell count was found in the CSF. Increased albumin quotients were found in 12.9% of the patients, oligoclonal bands (OCBs) in 1.6%, and increased immunoglobulin (Ig) G indices in 3.2% (OCBs were not measured in case of increased IgG indices). No significant differences in CSF findings were found between patients with manic and depressive episodes. The main findings of this open uncontrolled study are that alterations in the CSF may be found in a small, but potentially relevant, subgroup of patients with BD. These findings are discussed in light of the new concepts of mild encephalitis and immunological encephalopathy. The detection of patients with possibly secondary organic bipolar syndromes could open up new causal treatment options with immunomodulatory medication.

Keywords: cerebrospinal fluid, bipolar disorder, blood–brain-barrier dysfunction, immunological encephalopathy, mild encephalitis

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INTRODUCTION

Bipolar Disorder

Bipolar disorder (BD) is a severe, lifelong condition with prevalence rates of 1–4% (1, 2). The underlying pathophysiological mechanisms are not completely understood. Primary polygenetic forms might be common (3). However, secondary forms due to inflammatory processes have received increased interest in the last few decades since autoantibody-associated immunological encephalopathies that

mimic various psychiatric syndromes were detected (4). In line with this, we earlier published several case reports about patients with autoantibody-associated immunological encephalopathies who presented with isolated depressive or schizophreniform syndromes (5–8). Other authors have also described bipolar syndromes associated with underlying immunological encephalopathies [e.g., caused by thyroid autoantibodies (9–11)]. The idea of immunological dysfunction leading to mild encephalitis (12) is supported by epidemiological observations (i.e., increased rate of other autoimmune diseases in BD) and laboratory findings [e.g., circulating immune markers, inflammatory central nervous system (CNS) alterations (13)].

Cerebrospinal Fluid Analyses and Earlier Findings in Bipolar Disorder

Cerebrospinal fluid (CSF) analysis is the most precise method for detecting CNS inflammation. In routine CSF analysis, the total white blood cell (WBC) count, total protein concentration, albumin quotient, intrathecal immunoglobulin synthesis (IgG/A/M), IgG index, oligoclonal bands (OCBs), and lactate concentration are measured (14). Reports of routine CSF findings in patients with BD are scarce and have limited sample size. Pazzaglia et al. described increased total protein concentration in 55% of male patients with BD compared to only 10.5% in female patients with BD (15). This gender-specific finding was confirmed in another study (16). However, increased total protein concentrations are an unspecific and imprecise marker for blood–brain barrier (BBB) function, which is assessed more precisely by the albumin quotient (14). BBB dysfunction might be due to antipsychotic treatment in patients with BD (17). Elevated lactate concentrations are typically found in patients with purulent meningitis but also in patients with ischemic stroke and mitochondriopathies (14). Interestingly, Regenold et al. (18) also found increased lactate concentrations in 5 out of 15 patients with BD. The researchers interpreted this finding as a consequence of mitochondrial dysfunction (18).

Rationale of the Study

There is relevant evidence that inflammatory processes might lead to secondary forms of BD. However, only limited data about CSF basic diagnostic findings in BD are available. The current retrospective study was performed within this context. The aim of this project was to investigate the frequency of alterations in the CSF in BD. It was hypothesized that alterations would be found in the WBC count and BBB function, as well as that intrathecal humoral immune response would be found in the CSF of bipolar patient subgroups as markers of immunological dysbalance. In addition, CSF findings for patients with manic and depressive episodes were compared in an exploratory manner to detect possible differences.

PARTICIPANTS AND METHODS

The study was approved by the local ethics committee (Faculty of Medicine, Freiburg University, EK-Fr 609/14). All measurements were part of the routine clinical workup. All patients gave written informed consent for lumbar punctures and cerebral magnetic resonance imaging (cMRI) diagnostics.

CSF Samples

The CSF samples collected from January 1998 until December 2015 were analyzed retrospectively. The information was extracted from our CSF database, which was established in previous projects (19, 20). Routine CSF results were available for 75 patients with BD. Some of the patients were included in a previous research project (21). Comorbid somatic, neurological, and psychiatric diseases were obtained from medical reports. Patients with obvious past or current brain disorders, such as infectious encephalitis or meningitis, demyelinating diseases, stroke, brain tumors, epilepsy, delirium, dementia, traumatic brain disease, or earlier brain surgery were excluded as were hemolytic CSF samples. Headache due to migraine or tension headache and mild cognitive impairment were not exclusion criteria. Finally, 63 patients with BD were included in the descriptive and statistical analyses (**Tables 1** and **2**).

Procedure of Basic Diagnostic Analyses

Laboratory investigations were performed in the CSF laboratory of the Department of Neurology of the University Medical Center Freiburg.[1] The immunological assessment methods were described in previous papers (19, 20). Briefly, CSF WBC count and cytological differentiation were performed with manual microscopy (Leica DMRB, Germany) using a Fuchs–Rosenthal counting chamber (Hecht-Assistant, Germany). The basic quantitative protein diagnostics included total CSF protein, albumin, and immunoglobulin G, M, and A concentrations in the CSF and serum (ProSpect System, Siemens, Erlangen, Germany). Paired CSF and serum samples collected on the same date were analyzed simultaneously. For detection of BBB dysfunction, age-related albumin quotients were calculated (22). Intrathecal immunoglobulin synthesis was considered significant if the intrathecal immunoglobulin fraction exceeded 10% of the Reibergram (22), the IgG index was >0.7 mg/l, and/

[1] www.uniklinik-freiburg.de/neurologie/klinik/diagnostische-einrichtungen/liquor-labor.html.

TABLE 1 | The bipolar disorder patient cohort.

Available bipolar disorder CSF cohort from 1998 until 2015	75
Reasons for exclusion	
Infectious encephalitis	2 (1× viral encephalitis, 1× tick-borne encephalitis)
Demyelinating diseases	2 (2× multiple sclerosis)
Delirium	1 (most likely medication-induced)
Dementia	2 (1× Alzheimer's disease, 1× multifactorial dementia)
Brain tumor/brain surgery	1 (medulloblastoma)
Movement disorders	2 (1× Parkinson's disease, 1× unclear spastic tetraparesis)
Traumatic brain injury	1 (severe cerebral contusion)
Previous stroke	1 (repeated ischemic stroke)
Bipolar disorder cohort for analyses	63

or if the OCBs – measured using isoelectric focusing followed by immunofixation (Hydragel Isofocusing, Sebia, France) – were present exclusively or predominantly in the CSF (23). In cases of an increased IgG index, no OCBs were measured. To avoid false-positive findings, we also described the albumin quotient and the immunoglobulin fraction of the Reibergram in these cases. Clear-cut alterations in the CSF were defined as increased WBC count, increased age-dependent albumin quotient, or intrathecal immunoglobulin synthesis. Antineuronal antibodies against onconeural intracellular or synaptic antigens were analyzed using an immunoblot employing recombinant neuronal antigens as the substrate (ravo Diagnostika, Freiburg, Germany). CSF antibodies against neuronal cell surface antigens were measured using a non-specific cell-based [transfected human embryonic kidney (HEK) cells] indirect immunofluorescence assay (Euroimmun, Luebeck, Germany). The cMRI images were evaluated by experienced senior neuroradiologists, and the electroencephalograms (EEGs) were analyzed by in-house physicians.

Statistical Analysis

All laboratory and instrument-based diagnostic findings, as well as clinical information, were entered into a Statistical Package for the Social Sciences (SPSS) database. The main findings of alterations were presented descriptively in absolute values and percentage figures. For group comparisons (for the CSF findings) between manic and depressive patients, an independent-sample *t*-test for continuous variables was performed. For group comparisons (for alterations in cMRI and EEG) between patients with and without alterations in the CSF, a Pearson's two-sided chi-square test was performed. A p value of less than 0.05 was considered statistically significant for the statistical analyses.

TABLE 2 | Available datasets.

Diagnostic measurements	Number of samples
CSF basic diagnostics (WBC count, protein concentration, albumin quotient, and intrathecal immunoglobulin synthesis)	63
Intracellular synaptic and onconeural antigens (GAD, amphiphysin, Yo, Hu, Ri, Cv2/CRMP5, Ma1, Ma2, and SOX1)	29
Antibodies against neuronal cell surface antigens (NMDAR, AMPA-1/2-R, GABA-B-R, and VGKC-complex: LGI1 and CASPR2)	23
Electroencephalography data sets	61
Magnetic resonance imaging data sets	50[a]

[a]*For eight patients, only cranial computer tomography was available.*
WBC, white blood cell; GAD, glutamic acid decarboxylase; Yo/Hu/Ri, abbreviations of first patients' name; Cv2/CRMP5, anti-collapsin response-mediator protein; Ma1/Ma2, 37, and 40 kDa neuronal proteins; SOX1, sry-like high-mobility group box 1; NMDAR, N-methyl-D-aspartat-receptor; AMPA-1/2-R, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; GABA-B-R, γ-aminobutyric acid receptor; VGKC-complex, voltage-gated potassium channels complex; LGI1, leucine-rich glioma-inactivated 1; CASPR2, contactin-associated protein-like 2.

RESULTS

Demographic Data

The mean age of all patients was 48.35 ± 15.22 years. The gender ratio in the study was 25 men to 38 women. At the time of the lumbar puncture, the patient group comprised 18 patients with manic episodes, 35 with depressive episodes, and 7 with mixed episodes. In three patients, lumbar puncture was performed after recovery or remission of an acute manic or depressive episode; in all other patients, lumbar puncture was performed while the patients were symptomatic.

Routine CSF Diagnostics

In the BD group, 1.6% showed an increased WBC count. A false-positive pleocytosis due to hemolytic CSF was excluded. In 40% of the samples, an increased total protein concentration and, in 12.9%, an increased albumin quotient was found. OCBs were detected in 1.6% and increased immunoglobulin (Ig) G indices in 3.2% (OCBs were not measured in case of increased IgG indices). The CSF findings are summarized in **Table 3**. The characteristics of the patients with CSF alterations are presented in **Table 4**. In Patient 3, the increased IgG index was in line with a normal albumin quotient and a relevant IgG fraction in the Reibergram (~50%). In Patient 4 (**Table 4**), the increased IgG index was found in combination with a normal albumin quotient and a borderline IgG fraction in the Reibergram (~10%). In summary, the basic CSF diagnostic findings showed alterations in 28 of the 63 patients with BD (44.4%). Apart from the increased total protein concentration, clear-cut alterations were found in 12 of 63 patients (19%). None of the patients with clear-cut CSF pathologies had combined alterations in WBC count, albumin quotient, and intrathecal immunoglobulin synthesis. No autoantibodies against intracellular antigens or neuronal cell surface antigens

TABLE 3 | CSF basic diagnostics in bipolar patients.

CSF measurement	Mean ± SD	Number of cases	Frequency of alterations	Threshold
White blood cell count (n = 62)	1.35 ± 0.87	↔: 61; ↑: 1 (cell count: 6/µl)	1.6%	<5/µl
Total protein concentration (n = 60)	436.40 ± 218.93	↔: 36; ↑: 24	40%	<450 mg/l
Albumin quotient (n = 62)	5.97 ± 3.00	↔: 54; ↑: 8	12.9%	<40 years: 6.5×10^{-3}; 40–60 years: 8.0×10^{-3}; >60 years: 9.3×10^{-3} (14)
Immunoglobulin-G-index (n = 63)	0.50 ± 0.13	↔: 61; ↑: 2	3.2%	Immunoglobulin-G-index ≤0.7 mg/l (14)
Oligoclonal bands (n = 61[a])	–	No: 58; Yes: 3 – OCB restricted to CSF: 1 – OCB mirror pattern: 2	4.9%; OCB restricted to CSF: 1.6%; OCB mirror pattern: 3.3%	No oligoclonal bands

CSF, cerebrospinal fluid; OCBs, oligoclonal bands; ↔, value within normal range; ↑, value above normal upper limit of normal.
[a]*In cases of increased IgG-Index, no oligoclonal bands were measured.*

TABLE 4 | Patients with increased WBC count, CSF-specific OCBs, or elevated IgG index.

Nr.	Patient	CSF	cMRI	EEG	Neuropsych.	Other findings	Comment
1.	56 y, female, bipolar disorder, depressive episode	Increased WBC count (6 cells/µl), protein concentration slightly increased (510 mg/l)	Over years, unchanged left cerebellar cavernoma with associated developmental venous anomaly without hemorrhage; mild supratentorial atrophy and unspecific white matter lesions	Normal	Slight slowing in word fluency, retentiveness, and attention	No infectious causes (borreliosis, lues, etc.); autoantibody screening not performed	–
2.	21 y, female, bipolar disorder, depressive episode	CSF-specific oligoclonal bands	Alterations of the right lateral ventricle with mild gliosis and reduced white matter volume	Intermittent generalized slowing	Deficits in all domains of TAP	Increased thyreoglobulin antibodies; autoantibody screening negative (including rheumatological screening), the "MRZ" reaction was negative	No typical relapses suggestive for multiple sclerosis episodes were reported; the Swanton criteria for dissemination in time and space were not fulfilled
3.	61 y, female, bipolar disorder, depression	Increased IgG-index (1.3); IgG fraction in the Reibergram: ~50%	Mild increased CSF spaces, microangiopathic white matter lesions, the cMRI was affected by many artifacts	Normal	n.p.	Comorbid hypothyroidism (thyroid autoantibodies not measured) and psoriasis vulgaris, autoantibody screening not performed	No typical relapses suggestive for multiple sclerosis episodes were reported
4.	37 y, male, bipolar disorder, manic episode	Increased IgG-index (0.81); IgG fraction in the Reibergram: ~10%	Normal	Dysrhytmic EEG with β-overlap	n.p.	Earlier lues infection; autoantibody screening not performed	Elevated IgG index possibly due to clinically inapparent CNS infection after peripheral syphilis infection

CSF, cerebrospinal fluid; cMRI, cerebral magnetic resonance imaging; EEG, electroencephalography; TAP, test for attentional performance; n.p., not performed; MRZ-reaction, polyspecific, intrathecal humoral immune response against measles, rubella, and varicella zoster virus; y, years; IgG, immunoglobulin G.

TABLE 5 | cMRI and EEG pathologies in patients with bipolar syndromes.

Localization of cMRI alterations[a]	Frequency absolute[b] (%)
White matter lesions/cerebral microangiopathy	22/50 (44)
Cortical atrophy	2/50 (4)
Postischemic changes	1/50 (2)
Other alterations	3/50 (6)
Cerebellar cavernoma	1/50 (2)
Mild asymmetrical hippocampi	1/50 (2)
Mild gliosis	1/50 (2)
Anatomic variations	2/50 (4)
Arteriovenous malformation	1/50 (2)
Aplasia of the left transverse sinus	1/50 (2)
Overall cMRI alterations	30/50 (60)
EEG pathologies	**Frequency absolute[b] (%)**
Continuous generalized slow activity	2/61 (3)
Continuous regional slow activity	0/61 (0)
Intermittent generalized slow activity	9/61 (15)
Intermittent regional slow activity	3/61 (5)
Epileptic activity	0/61 (0)
Overall EEG pathologies	14/61 (23)

[a]*For eight patients, only cranial computer tomography was available.*
[b]*Only the predominant cMRI lesion or EEG alteration is listed for each patient.*
cMRI, cerebral magnetic resonance imaging; EEG, electroencephalography.

were found. Seven of the eight patients (87.5%) with increased albumin quotients were male. The EEG was abnormal in one of these eight patients (12.5%), and the cMRI showed pathologies in four out of six patients (66.7%).

cMRI and EEG

In 60% of the patients, cMRI alterations were detected. In most cases, unspecific white matter lesions (44%) were detected. EEG abnormalities were found in 23% of the patients, the majority of which were intermittent generalized slow activities (IRDAs in 15%; **Table 5**).

Subgroup Analyses

A comparison of the CSF findings in patients with manic ($n = 18$) and depressive symptoms ($n = 35$) showed no statistically significant differences in the WBC count (1.24 ± 0.44 vs. 1.40 ± 1.06; $p = 0.543$, $n = 52$), total protein concentration (382.50 ± 172.99 vs. 465.65 ± 255.47; $p = 0.245$, $n = 50$), albumin quotient (5.48 ± 2.48 vs. 6.36 ± 3.47; $p = 0.350$, $n = 52$), and IgG index (0.49 ± 0.09 vs. 0.50 ± 0.16; $p = 0.838$, $n = 53$). In addition, patients with ($n = 12$) and without ($n = 51$) clear-cut alterations in the CSF were compared. Alterations in the CSF were detected significantly more often in male patients (8 of 12 were male; chi-square = 4.5, $p = 0.034$). The cMRI pathologies (chi-square = 0.01, $p = 0.904$) and the rate of EEG alterations (chi-square = 0.33, $p = 0.564$) was not statistically different between the two groups.

DISCUSSION

As the main findings of this open, retrospective, uncontrolled study, we observed pathological alterations in the CSF in 19% of patients with BD. The CSF findings did not differ between manic and depressive patients in a statistically significant way. Male patients showed a higher rate of clear-cut alterations in the CSF.

Limitations

The first main limitation of this retrospective study is the missing control group. Therefore, the current study's findings could not be compared to those of healthy controls. However, due to the potential side effects of lumbar punctures (e.g., headache, vomiting, nerve damage, and infection), at the current stage of research, it was not ethically justifiable to puncture healthy controls. Therefore, we compared our findings with well-established reference values based on extensive work by Reiber et al.[2] Furthermore, we compared our results to those for other psychiatric patient cohorts, which were analyzed with the same methods in the same laboratory (19, 20). In cases of increased IgG indices, OCBs were not measured earlier in our laboratory. However, the findings of increased IgG indices are susceptible [e.g., due to increased albumin quotients; (22)]. To avoid the production of unreliable results, we also looked at the albumin quotient and the IgG fraction in the Reibergrams. Further studies should always analyze OCBs. The second main limitation of this study is due to its open and retrospective nature. At the University Clinic of Freiburg, for many years, CSF analyses had been offered and generally was performed only in selected patients with clinical signs or constellations suggestive of organic features. This of course leads to a probable selection bias of the sample. Therefore, the findings cannot be generalized to the overall group of bipolar patients presenting to a psychiatric clinic. However, to avoid an overinclusion of patients with suggestive organic features in this cohort, all patients with comorbid disorders associated with alterations in the CSF were excluded (see **Table 1**). Thus, only patients without preexisting organic causes for secondary organic bipolar syndromes were analyzed, and thus, from a clinical perspective, a cohort of patients with apparently endogenic primary BD was created. Due to the retrospective approach, we were not able to analyze the role of infrequent possible extracranial-influencing factors, such as spinal canal stenosis. Because of the retrospective character and the limited number of patients, other potentially relevant factors like medication have not been adequately addressed in this study. This also led to an incomplete dataset (e.g., autoantibodies were not measured in all patients). Thus, bigger prospective studies should be performed with predefined measurement protocols, including analyses of medicated and unmedicated BD patients. In spite of these shortcomings, this study is clinically important because it is, to the authors' knowledge, the first study that focused primarily on complete routine CSF findings in combination with cMRI and EEG findings. Previous studies mostly focused on the role of BBB dysfunction (15, 16).

Results in the Context of Previous Studies

The results are in line with those of previous studies that described increased total protein concentrations in some patients with BD (15, 16). We also found CSF alterations predominantly in male patients (16). In comparison to patients with psychosis, the rate of alterations in the CSF was lower. In patients with psychosis, increased cell counts were found in 3.4% (vs. 1.6% in BD), an elevated albumin quotient in 21.8% (vs. 12.9% in BD), and augmented intrathecal immunoglobulin synthesis in 7.2% [vs. potentially 4.8% in BD; (19)]. These differences support the idea that bipolar syndromes occur only in a small subgroup due to secondary inflammatory processes. This observation relates well to the clinical presentation of intermittent full remission without causal (e.g., immunomodulatory and anti-infectious) therapy. One might speculate that secondary forms due to inflammatory or neurodegenerative processes lead to chronic courses of the disease if the forms are not treated causally. However, our findings of clear-cut alterations in the CSF in 19% of patients are compatible with mild encephalitis (12) and immunological encephalopathies (4) in defined subgroups of patients with BD. These patients often show unspecific alterations in the CSF. Patients with Hashimoto's encephalopathy (HE) showed mild lymphocytic pleocytosis in 25% of cases, increased protein levels in 85%, and OCBs in 8.3% (24). Patients with anti-*N*-methyl-D-aspartic acid receptor (NMDAR) encephalitis showed initially abnormal findings in 80% of the cases. WBC counts were often mildly increased, and in 60% of patients, CSF-specific OCBs were detected (25). EEG alterations were found in 95% of patients with HE and nearly all patients with anti-NMDA encephalitis (4). In the cMRI of patients with HE, often normal or non-specific white matter lesions were detected (in 74%), and about 20% of the patients had diffuse increased signaling on T2-weight images (24). In 50% of patients with anti-NMDA-R encephalitis, the cMRI was unremarkable, and in the other half of the patients, T2 or FLAIR signal hyperintensity was found in the hippocampi, and in the cerebellar or cerebral cortex, frontobasal and insular regions, basal ganglia, and brainstem (25).

Alterations in the CSF in the Current Study and in General

Increased WBC counts, intrathecal immunoglobulin synthesis, and increased albumin quotients are unspecific but noticeable CSF findings (22). Increased WBC counts and intrathecal immunoglobulin synthesis are associated with acute and chronic inflammatory processes. In contrast, increased albumin quotients are unspecific findings. They can be caused by a broad spectrum of inflammatory CNS processes but also by systemic disorders, such as alcohol or diabetes-induced polyneuropathy, as well as by spinal canal stenosis (14).

WBC Count

Slightly increased WBC counts (5–30 cells/μl) can be due to stimulus pleocytosis (e.g., epileptic seizure) and are often found in immunological encephalopathies (4, 14). WBC counts above 30/μl are increased significantly and are distinct signs of infectious neuroinflammation [i.e., neuroborreliosis, neurosyphilis, meningitis, brain abscess, etc. (14)]. One patient in our cohort (Patient 1, **Table 4**) showed an isolated mild increase in WBC count (6 cells/μl) without other specific alterations in the CSF and no evidence of infectious agents. cMRI showed an over-the-years unchanged left cerebellar cavernoma with an associated developmental venous anomaly without hemorrhage. Moreover, cMRI depicted mild supratentorial atrophy and unspecific white matter lesions. No epileptic seizures were reported. One might

[2] www.horeiber.de/liquor.html.

speculate that the combination of mild pleocytosis in combination with cerebral atrophy, white matter lesions, and clinically evident cognitive deficits might be associated with mild chronic immunological encephalopathy. Alternatively, the findings could be unspecific, especially because no increased intrathecal immunoglobulin synthesis was detected.

Intrathecal Immunoglobulin Synthesis

One female patient (Patient 2, **Table 4**), with a normal WBC count, intact BBB function, and normal IgG index, showed weak CSF-specific OCBs; serological analyses showed increased thyroglobulin antibodies. The cMRI showed alterations around the right lateral ventricle with mild gliosis and reduced white matter volume. The EEG showed generalized slowing; the test for attentional performance (TAP) showed deficits in all domains. These findings are compatible with multiple sclerosis (MS) or HE. However, a relapsing disease course suggestive for MS was not reported by the patient. Additionally, the cMRI Swanton criteria for dissemination in time and space were not fulfilled and the MRZ reaction, a polyspecific, intrathecal humoral immune response against neurotropic agents (measles, rubella, and varicella zoster virus), which is possibly the most specific available CSF marker for MS (26), was also negative. Therefore, we were not able to diagnose MS or an early stage of this disease although previous studies showed an association between BD and MS (27, 28). The OCBs could also be an "immunological scar" after a previous CNS infection; however, a history of previous CNS infections was negative. HE could also be a possible reason. HE can be diagnosed by exclusion if the patient (1) suffers from encephalopathy with psychiatric symptoms (hallucinations, etc.), seizures, myoclonus, etc.; (2) has subclinical or mildly overt thyroid disease; (3) shows maximal non-specific cMRI alterations; (4) shows the presence of serum thyroid antibodies; and (5) shows the absence of autoantibodies against intracellular and cell-surface antigens (29). However, no steroid treatment was performed in this patient, which might have supported this differential diagnosis (8). Patient 3, with an increased IgG index, normal albumin quotient, and significantly increased IgG fraction in the Reibergram (~50%) also showed atrophic alterations in the context of hypothyroidism. Clarification for Hashimoto's thyroiditis was not performed, and perhaps, again, HE could be one possible reason. This patient also suffered from psoriasis vulgaris; therefore, an autoinflammatory predisposition seems to be plausible. Such associations with autoimmune diseases have been described previously (30). One might speculate that the bipolar symptoms and the thyroid and skin disease are different signs of immune dysregulation in this patient. In line with this, a previous case report described a patient with BD and psoriasis. During the depressive episodes, the skin lesions worsened, and during the manic episodes, the psoriasis improved without specific treatment (31). The increased IgG index in Patient 4 is associated with a normal albumin quotient and a borderline IgG fraction in the Reibergram (~10%). Alterations in this patient are most likely due to previous clinically inapparent syphilis infection of the CNS following the previous syphilis infection in the primary stage that was successfully treated with antibiotics. Unfortunately, a diagnostic workup for neurosyphilis (including calculation of the antibody index) and measuring of OCBs – which would have provided more clarity – were not available for this patient. Patients with depressive (in 5%) and manic syndromes (in 3%) have earlier been described as rare but possible clinical presentations of neurosyphilis (32, 33).

Albumin Quotient

The age-dependent serum and CSF albumin quotient is the reference standard for measuring BBB function (22). In a previous study we detected increased albumin quotients in 21.8% of patients with psychosis (19). In the present bipolar cohort the rate of patients with increased albumin quotients was 12.9% and, therefore, lower than in patients with psychosis but higher than in patients with other neurological disorders, like optic neuritis, where BBB dysfunction was only found in 3.8% (19, 34). Increased albumin quotients are unspecific but can also be found in most types of immunological encephalopathies. Some authors suggest that intermittent BBB dysfunction is required for the development of autoantibody-associated immunological encephalopathy. Following this line of thought, common autoantibodies in the serum [e.g., anti-NMDAR-antibodies (35, 36)] can reach the brain only in patients with BBB dysfunction (35).

The Role of CSF Analyses in Patients with Bipolar Disorder

Currently, CSF analyses are not generally recommended in patients with bipolar syndromes according to present guidelines (e.g., Nice guidelines[3]; German S3-Praxisleitlinie[4]). To avoid overlooking clinically potentially important immunological and infectious encephalopathies in a previous paper, the authors suggested to consider CSF analyses for patients with (1) laboratory- (e.g., hyponatremia and thyroid autoantibodies) and instrument-based alterations (e.g., EEG and cMRI alterations), (2) (sub)acute beginning of the symptoms, (3) an association between the beginning of symptoms and a feverish condition or vegetative derailment, (4) atypical clinical presentation, and (5) additional neurological symptoms [e.g., epileptic seizures, myoclonic jerks, other movement disorders (4)].

CONCLUSION

This open uncontrolled study revealed evidence for rare, but relevant, alterations in the CSF in patients with bipolar syndromes. Alterations in the CSF in patients with BD are less frequent than in patients with psychosis. In daily clinical practice, findings in MRI and EEG basic diagnostics, or an atypical clinical presentation (e.g., with neurological symptoms), should also lead the clinician to consider an underlying encephalopathic pathomechanism. Future studies should analyze CSF findings in a prospective, controlled setting and correct for frequent influential factors (e.g., alcohol use, medication, diabetes, age, and gender). The

[3] http://www.nice.org.uk.
[4] www.dgppn.de.

detection of patient subgroups with infectious or immunological encephalitis could open up new causal treatment options.

AUTHOR CONTRIBUTIONS

LTvE and DE initiated the study and conducted the data analyses. DE wrote the paper. DE and BH performed the data collection. All of the authors were crucially involved in the theoretical discussion and performance of this study. Furthermore, all of the authors read and approved the final version of this manuscript.

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T Cell Deficits and Overexpression of Hepatocyte Growth Factor in Anti-inflammatory Circulating Monocytes of Middle-Aged Patients with Bipolar Disorder Characterized by a High Prevalence of the Metabolic Syndrome

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Background: We previously reported T cell deficits and pro-inflammatory gene activation in circulating monocytes of two cohorts of bipolar disorder (BD) patients, a cohort of postpartum psychosis patients and in bipolar offspring. Pro-inflammatory gene activation occurred in two clusters of mutually correlating genes, cluster 1 for inflammation-related cytokines/factors, cluster 2 for motility, chemotaxis, and metabolic factors.

Aim: To verify these cellular immune abnormalities in yet another cohort [the bipolar stress study (BiSS) cohort] of relative old (52 years, median) BD patients and to relate immune abnormalities to hair cortisol levels, measured in this cohort and representing long-term systemic cortisol levels, and to the presence of the metabolic syndrome (MetS), which was prevalent in 29% of the BiSS patients.

Methods: Monocyte immune gene activation (quantitative polymerase chain reaction) and T cell deficits (fluorescence-activated cell sorting analysis) were determined in 97 well-controlled, largely euthymic BiSS BD patients. Monocyte genes included the cluster 1 and 2 genes, the genes for the glucocorticoid receptor (GR) α and GRβ, and the gene for hepatocyte growth factor [HGF, a marker of monocyte-derived circulating angiogenic cells (CACs)]. CACs serve vessel repair. Abnormal numbers are found in patients with MetS and vascular damage.

Results: As compared to healthy controls: (1) the pro-inflammatory cluster 1 genes were downregulated, and the GRα and the HGF gene were upregulated in the monocytes of the BiSS patients and (2) T cell deficits were shown (reduced numbers of lymphocytes in particular of T cells). Within the reduced T cell population, a shift had taken place in the T-helper populations: T-helper 17 and T-helper 2 increased and T regulatory cells

decreased. Correlations between hair cortisol, the MetS, monocyte gene activation, and T cell deficits were not found.

Conclusion: T cell deficits most likely are a trait phenomenon of BD, since they have also been found in the other cohorts of BD patients and in bipolar offspring. Monocytes of this cohort showed an anti-inflammatory set point, suggesting that pro- and anti-inflammation are state characteristics of BD. The monocyte gene profile indicated an increased CAC activity; the question arises whether this is due to putative vessel damage in these relatively old patients with a high prevalence of the MetS.

Keywords: bipolar disorder, T cell deficits, angiogenic cells, gene expression, hypothalamic–pituitary–adrenal axis

INTRODUCTION

The last 10 years evidence has accumulated that dysfunctions of T cells, and monocytes/macrophages are important factors in the development of bipolar disorder (BD). The majority of studies focused on cytokine levels in serum, and a recent meta-analysis confirmed the elevation of pro-inflammatory, anti-inflammatory, and regulatory cytokines in BD (1).

Cellular studies have accumulated too. Our team has studied in the past 10 years the percentages of circulating subsets of T cells and the monocyte inflammatory gene expression in three patient cohorts of BD patients. We have studied (1) a cohort of 56 BD patients from the Dutch site of the Stanley Foundation Bipolar Network (D-SFBN) cohort, mean age 42 years (2, 3), (2) a cohort of 90 BD patients, aged 43 years (mean) from the MOODINFLAME-Groningen-Leuven site (4), and (3) a cohort of 140 children of a bipolar parent in follow-up, from 16 through 29 years, the so-called Dutch bipolar offspring (DBO) study (5). In these studies, we detected that the monocytes of subjects were characterized by the abnormal expression of two coherent clusters of inflammation-related genes. One cluster of monocyte genes represented pro-inflammatory cytokines and key regulating compounds for the production of these cytokines (cluster 1 with genes, such as IL1B, IL6, TNF, PDE4B, and ATF3). The other cluster represents monocyte genes playing a role in chemotaxis, motility, nuclear signaling, and metabolism (cluster 2 with genes, such as CCL2, CCL7, NAB2, PTPN7, and DHRS3). The two clusters were found upregulated in monocytes particularly during a manic/depressive episode (4, 6), in longstanding disease with an early onset (6), and in 16-year-old DBO subject irrespective of the presence of mood symptoms (5).

The pro-inflammatory state of the monocytes was also found in yet another cohort of patients (a fourth cohort), namely, patients with postpartum psychosis, using not only gene expression but also microRNA profiling (7, 8). Postpartum psychosis is a pathologic condition related to BD. In postpartum disorder, monocytes not only showed increased levels of cluster 1 and 2 genes (7) but also decreased levels of miR-146a (8). MiR-146a is a well-established inflammation-regulating microRNA, and its decrease underscored the pro-inflammatory state of the patient monocytes. In addition, *in silico* studies showed monocyte miR-146a to target genes of the pro-inflammatory monocyte signature, such as PTGS2 and IRAK2.

Indeed, microRNA studies form an entire new field to investigate a new layer of epigenetic regulation of cellular function. While the study of Weigelt et al. (8) and those of others (9) target circulating leukocytes, most studies in psychiatry have focused on abnormal microRNA expression in the brain investigating microRNAs playing a role in brain development [reviewed in Ref. (10)].

Apart from the pro-inflammatory state of monocytes, we also detected in these four cohort studies that the T cell system was set at another equilibrium. In all cohorts, there was a deficit in the number of T cells, while within the T cell population there were abnormal fluctuations over time in the circulating levels of anti-inflammatory T regulatory (Treg) cells and T-helper 2 (Th2) cells and of pro-inflammatory T-helper 1 (Th1) and T-helper 17 (Th17) cells (7, 11, 12).

These observations enforced our idea that the immune system of individuals at high risk to develop BD (the DBO subjects) and of BD patients was abnormal and dynamically activated and deactivated over time showing episodes of inflammatory activation, particularly in early stages of the disease process in adolescence and the absence of mood symptoms and during active disease.

Recently, we collected circulating leukocytes of another cohort of BD patients. These patients belonged to the so-called Bipolar Stress Study (BiSS) cohort and had a mean age of 52 years (significantly older than the Stanley Foundation, the MOODINFLAME, and the DBO cohort). The BiSS study is a 2-year longitudinal study, designed to identify risk factors that have an impact on the clinical course and the treatment of outpatients with BD (13). The cohort has been studied in depth clinically (14–17) and is special in that the subjects had also been studied for the presence of the metabolic syndrome (MetS) (18). BiSS subjects showed a significantly higher prevalence of MetS when compared to subjects with MDD and non-psychiatric controls of the same age (28.4 versus 20.2 and 16.5%, respectively, $p < 0.001$), also when adjusted for sociodemographic and lifestyle factors. It is known that patients with severe mental illness do have a higher risk for cardiovascular disease (19).

Also special is that BiSS subjects had been studied for their hypothalamic–pituitary–adrenal (HPA) axis activity over a longer period of time *via* measuring their hair cortisol level (16). The hair cortisol test is relatively novel and reflects the mean HPA-axis activity over a period of time, in our case over the last 3 months before the leukocyte collection. Staufenbiel et al. (17)

reported an increase of hair cortisol levels in the BD patients of the BiSS cohort, but only in those with negative life events within 3 months before the cortisol determination. Hair cortisol was found normal across the entire BiSS cohort.

The MetS, the HPA axis, and the immune system have strong mutual influences (20–22). The collection of circulating leukocytes in the BiSS cohort at the end of the 2-year follow-up period enabled us to study the monocyte gene expression and T cell subset distribution in the BiSS subjects in relation to the mean HPA-axis activity in the 3-month period before blood collection and in relation to the presence/absence of the MetS.

We therefore not only determined the earlier reported cluster 1 and 2 gene sets in the monocytes of the BiSS subject but also the gene expression levels of the genes for the glucocorticoid receptor α (GRα, activating) and GRβ (blocking receptor). We hypothesized that there would a relationship between the expression of the GR receptor in monocytes, the cluster 1 and 2 gene expression, and the hair cortisol levels.

Not only did we add the genes for the GR but also additionally determined the expression of the gene for hepatocyte growth factor (HGF). In a recent study on type 2 diabetic (T2D) and MetS patients (23), we found HGF gene expression raised in the circulating monocytes of the T2D patients. HGF is an important vascular repair factor (24) and monocyte-derived circulating angiogenic cells (CACs), capable of repairing endothelial damage, are characterized by the expression of HGF (25). We hypothesized that the HGF expression would be raised in particularly BiSS subjects with the MetS.

In addition to monocyte gene expression studies, fluorescence-activated cell sorting (FACS) analysis was performed on the collected leukocytes of the BiSS cohort to determine the percentages of monocytes, lymphocytes, B cells, NK cells, T cells, as well as the distribution of the T cells into the cytotoxic T cells and the helper T cells. The latter population was further quantified for its subpopulations, i.e., the regulatory T cells, the Th1 cells, the Th2 cells, and the Th17 cells. We hypothesized that we would detect again the T cell deficits and an abnormal distribution of T-helper subsets within the circulating T cell population of the BiSS subjects, similar as was found before.

Thus, our prime objective of this study was to determine the monocyte gene activation patterns and leukocyte distribution abnormalities in this new BiSS cohort of BD patients, and to compare outcomes to the outcomes of the previous studied BD cohorts, which had been investigated with the same techniques. Our secondary objective was to study correlations between the immune outcomes and the hair cortisol level and the presence/absence of the MetS.

MATERIALS AND METHODS

Patients

The study design and patient population have been described elsewhere in detail (13–15, 17). For this study, only patients with hair cortisol measurements were selected. The study was conducted as part of the MOODINFLAME (EU-FP7-HEALTH-F2-2008-222963) and the PSYCHAID project (EU-FP7-PEOPLE-2009-IAPP-MarieCurie-286334) on the samples cross-sectional collected at the end of the 2-year follow-up study. The study protocol was approved by the Medical Ethics Committee of the Clinic for Mood disorders, Parnassia Group (presently PsyQ), The Hague, The Netherlands, and all patients gave informed consent after a full description of the study.

To summarize, adult patients ($n = 97$) with BD I, II, and not otherwise specified were included. As this was a naturalistic study design, we included all outpatients with BD. Diagnosis of BD and psychiatric comorbidities were based on DSM-IV criteria and were assessed with the MINI International Neuropsychiatric Interview Plus (26). Sociodemographic data and disease-related data were collected in interviews by trained psychologists. Disease-related data included disease characteristics (age of onset, disease duration, mood classification, comorbidities including the MetS) and medication use (lithium, antipsychotics, anti-depressives, and benzodiazepines). Mood classification was administrated with the self-rated Quick Inventory of Depressive Symptomatology and the observer based Young Mania Rating Scale (27, 28). MetS was defined according to the National Cholesterol Education program—Adult Treatment panel III (29).

Exclusion criteria were age below 18 years and the diagnosis schizoaffective disorder. As the BiSS study was a naturalistic study, we included all patients with BD and only noted comorbidities, such as thyroid disease, diabetes, and endocrine disease in general. Patients with glucocorticoid usage in the last 6 months or without sufficient hair growth at the posterior vertex were also excluded (this was interfering with the hair cortisol determination). Methods and detailed data on the hair cortisol levels and GR polymorphisms are available in previous studies on this cohort (15–17). The reference upper limit of the normal hair cortisol level was defined by Manenschijn et al. (30) as 52 pg/mg hair in healthy non-obese individuals. The reference lower limit has not yet been determined.

Healthy Controls (HCs)

The HC group was established by combining HCs of the studies in the MOODINFLAME and PSYCHAID consortia (Departments of Psychiatry, University Hospitals of Rotterdam, Groningen, Münster, Leuven, and München). These HCs had been selected at the same time as the patients, and material was processed in the same way by the same technicians together with the materials of the patients. Exclusion criteria for HC in the MOODINFLAME and PSYCHAID studies were psychiatric and immune disease or endocrine disease in both the subject and first-degree family members and medication use (apart from oral contraceptives). Of a total number of 519 HC with peripheral blood mononuclear cell (PBMC) data available, only HC with complete information on gender, age, and BMI was included for comparison ($n = 272$). From these 272 controls, we selected 47 controls for the monocyte gene expression evaluation, since these controls had been tested in the same assays as the BD cases (to avoid inter-assay variation). For the leukocyte subset determination, we finally selected 72 controls matched for age and gender (see Results).

Blood Collection and Preparation

Blood was drawn using sodium heparin tubes for immune cell preparation, and the heparinized blood was subsequently

centrifuged to prepare PBMC suspensions as described previously in detail (7, 31). PBMCs were frozen in 10% dimethyl sulfoxide and stored in liquid nitrogen.

Determination of Monocyte Gene Expression

$CD14^+$ monocytes were isolated from the frozen PBMCs using a magnetic cell sorting system (Miltenyi Biotec, Germany) resulting in a purity of monocytes >95%. RNA was isolated from the purified monocytes, and 1 μg of RNA was reverse transcribed using the cDNA high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). The cDNA was used in quantitative polymerase chain reaction (qPCR) to determine gene expression using the comparative threshold (CT) cycle method (32). qPCR was performed using a TaqMan Universal PCR mastermix and TaqMan probes (Applied Biosystems). Gene expression was normalized to expression of housekeeping gene ABL1, and the resulting ΔCT values were used for further analyses.

Genes of interest were selected based on previous findings of abnormally expressed genes in patients with BD (4, 5, 11), MDD (31), type 2 diabetes (23), and systemic autoimmune diseases [the IFN-induced inflammatory genes (33)], and known to be involved in major inflammatory or immune activation pathways in monocytes and macrophages. We selected the most consistently over expressed genes. A list of included genes is given in Table S1 in Supplementary Material.

Monocyte Gene Expression Analysis

It has been published extensively before that the determined genes are expressed in three mutually coherent clusters, i.e., cluster 1, cluster 2, and the IFN cluster (7, 11, 23, 31, 33). For this study, principle component analysis was used to determine which genes could be allocated to these clusters. Furthermore, the analysis showed the order of correlation of each gene in the found clusters, enabling the use of a limited set of the highest correlating genes (key genes) for further comparisons. ΔΔCT values (ΔCT values of patients relative to the mean ΔCT value of controls within the same assay) were used to compare gene expression of BD patients with HC for the key genes of each cluster. The results were expressed as fold change (FC) relative to HC. The researcher was blind to the identity of the sample during the FACS measurement and analysis of the data.

Determination of the Percentage and Subset Distribution of Circulating Leukocytes

Lymphocytes, monocytes ($CD14^+$), B cells ($CD19^+$), NK cells ($CD56^+$), total T cells ($CD3^+$), T-helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$), and regulatory T cells ($CD25^+FoxP3^+$) were determined using FACS and described as percentage relative to total mononuclear cells. Furthermore, Th1 (IFN-γ^+), Th2 ($IL4^+$), and Th17 ($IL17A^+$) subsets were assessed and described relative to the total number of leukocytes. All used procedures including the gating strategy are extensively described in Snijders et al. (12). The researcher was blind to the identity of the sample during the FACS measurement and analysis of the data.

Statistics

Statistical analysis was performed using SPSS software, version 23.0 (IBM Corp, Armonk, NY, USA). Normally distributed continuous variables were expressed as mean with 95% confidence intervals and compared using two-sided *T*-tests. Log transformation was applied on Th1, Th2, and Th17 cells and hair cortisol levels to achieve normality. Non-normal data, mainly the various clinical variables, were described with median (range) and compared with Mann–Whitney *U* tests.

Principle component analysis was used on the 36 genes (excluding HGF and both GR isotypes) with oblique rotation (direct oblimin). The Kaiser–Meyer–Olkin measure verified the sampling adequacy, KMO = 0.89. An initial analysis was run to determine the number of factors to be extracted. A combination of three factors with the highest eigenvalue and clear inflections shown in the scree plot explained 63.31% of the variance. The maximum number of convergence was 25.

For monocyte gene expression one-sample *T*-test was performed on normally distributed ΔΔCT values, and data are presented as FC of the patient gene expression relative to the HC value set to 1 for each given gene.

RESULTS

Clinical Characteristics of the Patients, including the Prevalence of the MetS and Hair Cortisol

The large majority of the BD patients were BD I cases (76%), had longstanding disease (mean duration 23 years), were on lithium (76%), were well-controlled with low self-rated QIDS scores (median 5), and showed a high prevalence of the MetS (29%). Further detailed characteristics of BD patients are given in **Table 1**.

Hair cortisol levels of the BiSS cohort have been published in detail before (16, 17). Using the cut-off determined by Staufenbiel et al. (17), the large majority of BD patients showed normal hair cortisol levels ($n = 84$, median = 25.6, range = 10.6–51.9), the remaining patients had elevated cortisol levels ($n = 13$, median = 77.2, range = 56.7–370.8).

Monocyte Gene Expression

A total of 39 genes were included in qPCR, shown with description in Table S1 in Supplementary Material. Monocyte gene expression could successfully be determined in 97 BD patients. Monocyte gene expression of patients was compared to monocyte gene expression of 47 HC of whom blood was tested in the same assays (to avoid inter-assay variation for monocyte gene expression). Of these 47 healthy subject data on gender, age and BMI were available: median age was 48 years (range 24–72), percentage females 57, and a mean BMI of 25.6 (SD 3.6). BMI and gender were not statistically significantly different between patients and HC. The age of the HC (48 years, mean) was statistically different from the age of the patients (52 years, mean, $p < 0.01$). However, age was not found to correlate to monocyte gene expression (data not shown). Nevertheless, and to be sure, corrections were carried out in statistics for age, gender, and BMI.

The results of the principle component analysis are shown in Table S2 in Supplementary Material. As expected the three distinct clusters could be found as in previous studies (7, 11, 23, 31, 33). The first cluster was by and large corresponding to the previously described cluster 1 (pro-inflammatory genes) and the second by and large to the previously described cluster 2 (genes involved in chemotaxis, cell adhesion nuclear signaling, and motility) using the previous cohorts of BD patients. The principle component analysis also showed a third cluster, i.e., a cluster of interferon-induced genes, on which we have extensively published in systemic autoimmune diseases, such as Sjögren disease, SLE, and systemic sclerosis (33). In these latter diseases, this gene set is over expressed in monocytes due to high levels of type II IFNs in the circulation.

Some of the lower ranking genes of cluster 1 (like HSPA1A/B) and of cluster 2 (like CD9) in the previous studies did not belong to the present clusters in this study anymore (Table S2 in Supplementary Material). Since there is not an exact overlap between the clusters in the various studies, we only took the three top genes of the principle component analysis into consideration for further analysis and calculations. These top genes belong in each of the previous study also to the most discriminative genes for the cluster.

The fold expression of the top three genes per cluster as found with principle component analysis is shown relative to HCs in **Table 2** (the outcomes of the entire list of measured genes can be found in Table S3 in Supplementary Material). Gene cluster 1, as measured by the top key genes IL1B, CCL20, and IL6, was significantly downregulated in BD patients, Table S3 in Supplementary Material shows that indeed the vast majority of cluster 1 genes (13 out of 15) is significantly downregulated. We take this observation as indicating that the monocytes of the BiSS BD patients are set at an anti-inflammatory set point.

Of the cluster 2 key genes, only the top key gene NAB2 showed a significantly reduced expression as compared to the expression level in the monocytes of HCs; Table S3 in Supplementary Material shows that indeed only a minority of cluster 2 genes was significantly downregulated (5 of 15 genes).

The IFN genes were normally expressed in the monocytes of the BD patients.

The expression of the GRα and HGF gene was significantly upregulated in the monocytes of BD patients as compared to those of HC ($p = 0.039$ and $p = 0.004$, respectively) (**Table 2**).

Table 3 shows that the expression level of HGF in monocytes correlated significantly positive with the expression of the activating GRα and with the IFN cluster, but negative with the expression levels of the cluster 1 and 2 genes: the higher the HGF expression the higher the GRα expression and the IFN expression, but the lower the expression of IL1, CCL20 and IL6, and NAB2, CCL2, and CCL7.

Table 3 also shows that the gene expression of the blocking GRβ expression in the monocytes of the patients correlated positive to the cluster 1 gene expression, an observation reported before (7, 31).

TABLE 1 | Characteristics of bipolar patients and healthy controls.

	All bipolar disorder patients (*n* = 97)
Age (median, range), years	52 (20–83)
Female (*n*, %)	61 (63)
BMI (mean, SD)	26.2 (4.5)
DSM-IV classification (*n*, %)	
Bipolar I	74 (76)
Bipolar II	22 (23)
Non-specified	1 (1)
QIDS score (median, range)	5 (0–22)
Mood classification (*n*, %)	
Euthymic[a]	50 (52)
Mild depression[b]	23 (24)
Moderate depression[c]	14 (14)
Severe depression[d]	5 (5)
Very severe depression[e]	1 (1)
Manic[f]	0
Not determined	4
Age of onset (median, range), years	21.5 (20–59)
Disease duration (median, range), years	23 (0–61)
Metabolic syndrome (*n*, %)	28 (29)
Endocrine disease (*n*, %)	10 (10)
Thyroid disease[g]	7 (7)
Diabetes mellitus type 2	3 (3)
Smoking (*n*, %)	32 (33)
Medication (*n*, %)	
Lithium	74 (76)
Antipsychotics	25 (26)
Antidepressants	30 (31)
Benzodiazepines	29 (30)

[a]*Self-rated Quick Inventory of Depressive Symptomatology (QIDS-SR) score ≤5, YMRS <13.*
[b]*QIDS-SR score 6–10.*
[c]*QIDS-SR score ≥11.*
[d]*QIDS-SR score ≥16.*
[e]*QIDS-SR score ≥21.*
[f]*Young Mania Rating Scale score ≥13.*
[g]*Stable disease (normal TSH + fT4).*

TABLE 2 | Monocytes in bipolar disorder patients compared to healthy control (HC).

	FC[a]	95% CI	*p* Value
Cluster 1			
IL1B	**0.72**	0.59–0.89	**0.002**
CCL20	**0.58**	0.43–0.78	**0.001**
IL6	**0.76**	0.61–0.96	**0.021**
Cluster 2			
NAB2	**0.80**	0.67–0.96	**0.017**
CCL7	0.93	0.65–1.32	0.669
CCL2	1.05	0.82–1.34	0.703
Interferon-related cluster			
IFI44	1.01	0.90–1.13	0.860
IFIT3	1.04	0.90–1.19	0.631
Glucocorticoid receptor (GR) α	**1.03**	1.00–1.07	**0.042**
GRβ	0.97	0.87–1.09	0.626
Hepatocyte growth factor	**1.10**	1.03–1.18	**0.005**

[a]*Fold change (FC) relative to HC.*
The most important genes for each respective cluster are shown as found by principle component analysis (Table S2 in Supplementary Material).
A full list of compared genes is included in Table S3 in Supplementary Material.
Values marked in bold indicate numbers that are significant on the 95% confidence limit.

TABLE 3 | Correlations of hair cortisol, glucocorticoid receptor (GR) α and GRβ receptor gene expression, hepatocyte growth factor (HGF) gene expression, and cluster 1-, 2- and interferon-related cluster scores.

	HGF	Hair cortisol	GRα	IL1B	CCL20	IL6	CCL7	NAB2	CCL2	GRβ	IFI44	IFIT3
HGF		0.21	0.34	−0.42	−0.38	−0.50	−0.43	−0.53	−0.35	−0.18	0.18	0.41
Hair cortisol	0.21		0.12	−0.12	−0.06	−0.05	0.00	−0.14	−0.02	0.13	0.14	0.19
GRα	0.34	0.12		−0.39	−0.24	−0.30	−0.10	−0.20	−0.15	−0.04	0.12	0.25
GRβ	−0.18	0.13	−0.04	0.35	0.38	0.40	0.34	0.38	0.33		0.11	−0.07

Correlations significant at the p = 0.05 level are colored.

The disease characteristics as given in **Table 1**, including the mood state, the medication, and the presence of the MetS, did not correlate to the various gene expression levels in the monocytes of the BD patients (data not shown).

In the correlations between hair cortisol and other parameters, we used an analogous approach. The hair cortisol levels did not significantly correlate to any of the above described immune parameters (**Table 3**). We did, however, confirm the correlation of the hair cortisol with negative life events in the previous months to bloodletting as reported earlier by Staufenbiel et al. (17).

Leukocyte Subset Determinations

For leukocyte subset determination, inter-assay differences performed on PBMC in the various cohorts of MOODINFLAME and PSYCHAID and obtained at different sites and collection times appeared to be negligible, therefore a total number of 519 HC with PBMC data were available for evaluation against the BiSS cases; however, only HC with complete information on gender, age, and BMI ($n = 272$) were included for comparison with the BiSS cases. Out of these 272 HC, a selection was made to match for age (maximum 3-year difference), gender, and BMI (maximum three BMI points difference) with the BiSS patient group; this resulted in 72 pairs (BD cases–HC pair) for leukocyte subset determination. The mean age of these pairs was 52 years (range 20–67), percentage females 63, and mean BMI 25.3 (SD 3.2) not statistically different from the entire group of BiSS BD patients (**Table 1**).

Table 4 shows that there was a reduction of the percentage of lymphocytes in the BD patients. The percentage of monocytes was raised. Since many of the patients were on lithium, we carried out a separate comparison between lithium-treated and non-lithium-treated BD patients. This comparison did not show an effect of lithium treatment (see **Table 4**). Correlations with the usage of other drugs were also not found (data not shown).

With regard to the subpopulations of lymphocytes, **Table 4** shows that it was particularly the $CD3^+$ (pan) T cells, which were decreased and within this population both the $CD3^+CD8^+$ cytotoxic T cells and the $CD3^+CD4^+$ T-helper cells. Within the population of $CD3^+CD4^+$ T-helper cells a shift had taken place, there was a significant increase in both the percentages of Th2 and Th17 cells, while the Treg cells had decreased. These abnormalities were also present in patients not on lithium, except for the Th2 increase; this was only seen in the lithium treated BD patients.

B cell, NK cell, and Th1 cell levels in the BD patients were not different from the HCs.

In the correlations between hair cortisol and the leukocyte subsets, we again used an analogous approach. Correlations between hair cortisol and any of the leukocyte subsets could not be detected. This also applied for correlations between the leukocyte subsets and clinical characteristics of the patients, including the mood state and the presence of the MetS (data not shown).

DISCUSSION

This study shows that the pro-inflammatory gene cluster 1 expression was downregulated in the monocytes of the BD patients of the here studied BiSS cohort, indicating a reduced inflammatory state of the circulating monocytes as compared to that of HCs.

This reduced inflammatory state of the circulating monocytes of the BiSS patients is in contrast to the pro-inflammatory state observed in previously studied cohorts of BD patients while in all cohorts the same collection and laboratory determination techniques were used. In the BD patients of the D-SFBN cohort, an increased inflammatory gene expression was found that correlated to the activity of disease, particularly to manic symptoms (2, 3, 6). The patients in the D-SFBN cohort were indeed special in that a relative high percentage had active severe disease (38%). In the MOODINFLAME Leuven–Groningen cohort, the raised pro-inflammatory gene expression in monocytes was again only found in patients with active mania or depression, these patients constituted around 10% of the cohort (4). Our data thus support a concept that in BD patients over expression of monocyte inflammatory genes can only be found during active episodes of the disease, in particular during mania. The BiSS cohort only had six patients with severe depression.

Our observations on monocytes are by and large in accord with serum cytokine/inflammatory factor studies that also found that BD patients with an active episode and particularly those who have or develop manic symptoms have an increased inflammatory activity, especially high CRP, compared with individuals who do not develop manic symptoms (34).

However, before to conclude that the pro-inflammatory state in BD is only linked to (manic) mood derailment in BD, it is relevant that in the studies on the DBO children over expression of monocyte inflammatory genes was also found in adolescents at 16 years of age, but in this case irrespective of present or later mood symptoms (5). Five years later, in early adulthood (at 21 years of age), the pattern of monocyte gene expression had changed in the DBO children to a reduced expression of cluster 1 genes, but still increased cluster 2 gene upregulation. At 28 years,

TABLE 4 | Peripheral blood mononuclear cells (PBMCs) in bipolar disorder (BD) patients compared to matched healthy controls (HCs).

	All HC ($n = 72$)		All BD patients ($n = 72$)			HC no lithium[b] ($n = 16$)		BD no lithium ($n = 16$)			HC lithium[d] ($n = 56$)		BD lithium ($n = 56$)		
	Mean	**SD**	**Mean**	**SD**	p^a	**Mean**	**SD**	**Mean**	**SD**	p^c	**Mean**	**SD**	**Mean**	**SD**	p
Lymphocytes	76.85	0.88	72.54	0.94	**<0.001**	79.81	1.33	76.28	1.72	0.088	76.00	1.06	71.47	1.08	**0.003**
$CD19^+$ B cells	7.32	0.28	7.56	0.30	0.56	6.65	0.50	7.38	0.60	0.32	7.52	0.32	7.61	0.36	0.84
$CD56^+$ NK cells	9.11	0.58	10.21	0.64	0.19	10.75	1.30	10.64	1.59	0.95	8.62	0.65	10.10	0.71	0.12
$CD14^+$ monocytes	17.63	0.74	21.01	0.70	**0.001**	14.23	1.19	18.62	1.54	0.020	18.54	0.85	21.72	0.80	**0.007**
$CD3^+$ T cells	57.66	0.99	53.31	1.17	**0.005**	59.98	1.80	56.53	2.67	0.25	56.99	1.19	52.38	1.32	**0.009**
$CD3^+CD8^+$ cytotoxic T cells	16.31	0.69	14.65	0.67	0.083	18.50	1.72	16.16	1.81	0.32	15.68	0.76	14.22	0.72	0.11
$CD3^+CD4^+$ T-helper cells	38.42	0.90	36.18	1.11	0.11	38.74	2.33	37.53	2.37	0.69	38.33	0.98	35.79	1.29	0.15
$CD3^+CD4^+CD25^{hi}FoxP3^+$ Treg	1.56	0.07	1.46	0.05	0.21	1.51	0.18	1.60	0.13	0.65	1.58	0.07	1.41	0.06	0.076
$CD3^+CD4^+IFN\text{-}\gamma^+$ T-helper 1 cells	4.36	0.26	4.43	0.29	0.73	4.78	0.61	5.27	0.89	0.80	4.23	0.29	4.19	0.29	0.80
$CD3^+CD4^+IL4^+$ T-helper 2 cells	0.43	0.03	0.53	0.04	**0.037**	0.53	0.10	0.41	0.07	0.26	0.40	0.03	0.56	0.04	**0.002**
$CD3^+CD4^+IL17A^+$ T-helper 17 cells	0.25	0.02	0.33	0.03	**0.003**	0.25	0.03	0.30	0.03	0.18	0.25	0.02	0.34	0.04	**0.009**

Percentages of indicated subpopulations are given per PBMC (mononuclear white cells). The p values in comparison of the lithium and not-lithium using BD patients versus their respective controls are given below.

Values marked in bold indicate numbers that are significant on the 95% confidence limit.

p^a: all BD patients versus all matched HCs.

HC no lithium[b]: matched HCs corresponding to BD patients with no lithium usage.

p^c: BD patients without lithium usage versus corresponding matched HC.

HC lithium[d]: matched HCs corresponding to BD patients with lithium usage.

monocyte gene expression had virtually normalized. Neither increases nor decreases in monocyte inflammatory gene expression were linked to mood symptomatology in the DBO children in the various stages of investigation.

Collectively, these findings on the inflammatory state of monocytes point in the direction of dynamic changes of inflammatory gene expression in monocytes over time in subjects at risk to develop BD and in BD patients. Apparently the inflammatory set point of monocytes is dependent on the (environmental or hormonal) state of the organism, an observation that is supported by twin studies in BD, showing that common endogenous or environmental factors play a prime role in the pro-inflammatory set point of the monocytes (35). In sum, only episodes of pro-inflammatory activation occur in circulating monocytes of individuals at risk to develop BD and in BD patients, and these episodes are probably environmentally and endogenously precipitated and only in part linked to the presence and activity of (manic) mood symptoms.

The present study also shows that the gene expression of the vascular repair factor HGF was significantly raised in the monocytes of BD patients of the BiSS cohort as compared to HCs. We previously found a similar gene expression profile, i.e., a reduced pro-inflammatory cluster 1 and raised HGF expression, in the circulating monocytes of 64 T2D patients (23, 36). These T2D patients were on average 62 years old.

We took the observation in the studies of Baldóon Rojas et al. of a high HGF expression and a low expression of inflammatory signs in the circulating monocytes of T2D patients as indicating that many of the circulating monocytes in the T2D cases were differentiating into CACs and involved in the repair of endothelium damaged by the diabetic process. CACs are bone marrow-derived monocytes involved in vascular regeneration and angiogenesis, are anti-inflammatory, and exert their repair function by secreting vascular growth factors, such as HGF, which is considered as a marker of CACs (25). Healthy angiogenesis is crucial for vascular regeneration (24).

We therefore like to hypothesize that the elevated gene expression of HGF in the anti-inflammatory monocytes of the patients of the BiSS cohort probably reflects that a high percentage of the monocytes had developed into vessel-repair-supporting CACs to counteract a putative endothelial damage in the BD patients of the BiSS cohort. Indeed, the MetS and dyslipidemia were prevalent in the BD patients of the BiSS cohort (18), yet there were no correlations between the HGF expression and the MetS. Therefore, formal studies in older BD patients focusing on monocyte-derived CAC development in relation to parameters of vascular damage need to be performed to verify or refute the here proposed idea of the development of anti-inflammatory CACs as overruling mechanism preventing a pro-inflammatory state of monocytes in BD patients. Also endothelial damage in atherosclerosis is considered a pro-inflammatory environment (37), and it is therefore at first sight puzzling that the CACs are anti-inflammatory. However, there is literature that it is particularly in situations of chronic mild hyperglycemia (38) and in situations of unstable plaque formation (39) that CACs and circulating monocytes become pro-inflammatory and dysfunctional. Therefore, special care should be taken in follow-up studies whether such conditions exist in bipolar patients next to a putative heightened endothelial damage.

The GRα was also higher expressed in the monocytes of the BD patients of the BiSS cohort as compared to those of HCs, and the expression of the GRα expression correlated to the expression of HGF. Since the GRα is the activating receptor signaling down-regulation of pro-inflammatory molecules during glucocorticoid

exposure, an upregulation of the GRα suggests a higher sensitivity for glucocorticoids to downregulate the inflammatory state of monocytes.

These data suggest that in principle a raised cortisol level might be instrumental in downregulating the inflammatory state of the circulating monocytes in BD patients. Such a raised cortisol level was reported in BiSS BD patients who experienced recent negative life events (17). However, although we were able to verify the previous correlation between high hair cortisol levels and negative life events, we were unable to find in this study a correlation between both high hair cortisol levels/negative life events on the one hand and a reduced inflammatory state of the circulating monocytes of the BiSS cohort on the other hand.

We therefore assume that not negative life events and a higher HPA-axis activity, but the earlier mentioned putative endothelial damage and consequent transition of the circulating monocytes into anti-inflammatory vessel repair cells CACs plays a overruling and prime role in the here found characteristic profile (high HGF, reduced pro-inflammatory compounds) of the circulating monocytes in the BD patients of the BiSS cohort. But as stated before, this needs formal verification, also with regard to an enhanced monocyte sensitivity to dexamethasone in functional *in vitro* exposure tests.

With regard to the T cell findings in the BiSS cohort the following remarks. Snijders et al. (12) took the monocyte data on the DBO adolescents of Mesman et al. (5) further and showed that the DBO 16-year-old adolescents had deficits in the number of circulating lymphocytes, particularly of total $CD3^+$ T cells and within this population particularly in the Treg cell subset. Interestingly, she found that the lower the level of the anti-inflammatory Treg cells in the DBO adolescents, the higher the monocyte inflammatory gene activation. These data corroborate earlier findings that Treg cells are capable of downregulating monocyte inflammatory activation and that in particular a defective Treg cell system might form the underlying condition allowing endogenous or environmental factors to induce an early raised pro-inflammatory state of monocytes in the 16-year-old DBO subjects at risk to develop BD.

During further aging of the DBO children, the aberrant T cell state showed a dynamic course over time. During young adulthood (mean age 21 years), the partial T cell deficit, i.e., the reduction in the total number of $CD3^+$ T cell was still detectable, but not of the Treg cell subset anymore, but of the pro-inflammatory Th1 and Th17 cell subsets, suggesting a phase of relative anti-inflammation at young adulthood, but in the presence of deficits of total T cells. Also the upregulation of cluster 1 inflammatory genes in monocytes had disappeared and even a downregulation of such genes as compared to HC monocytes was found (see before), enforcing the idea that young adulthood at 21 years of age was a phase of relative anti-inflammation.

In late adulthood (mean age 28 years), the various subsets of T cells (Th1, Th2, Th17, and Treg cells) and the abnormal monocyte gene expression had largely normalized as compared to the controls, only the deficit of lymphocytes and total $CD3^+$ T cells could still be detected.

Collectively, these DBO data show that T cell deficits deregulate the inflammatory response in adolescence and young adulthood in individuals at risk for BD, and we assume that the immune disequilibria represent vulnerability factors for later BD development by influencing morphological brain and hippocampus development during adolescence and early adulthood, since T cells and non-inflammatory microglia are essential support cells for proper brain development (see **Figure 1**).

Although the T cell and monocyte inflammatory disequilibria were more or less normalized in adulthood in the DBO subjects, a partial T cell defect (a deficit in lymphocyte and total T cell numbers) still existed. This report on the BiSS BD patients with a mean age of 52 years still finds the apparently over time consistent partial T cell deficit in showing reduced levels of lymphocytes and total $CD3^+$ T cells also in the relatively old BiSS cohort, while the restoration within the $CD4^+$ T-helper cells subset had further continued into an overshoot, showing significantly raised levels of Th2 and Th17 subpopulations, while Treg cells were reduced.

A composite of the dynamics over time of T cells and monocytes as found in the various cohorts studied by us is shown in **Figure 1**. In this figure, it is also hypothesized that particularly in adolescence and early adulthood changes in brain structure and physiology are induced by the T cell deficits and inflammatory influences. In later phases of life, partial corrections of the immune aberrancies occur, and only during an active episode signs of a peripheral monocyte inflammatory state can be found.

Limitations of This Study

Data on the leukocyte subpopulations could only be given as percentages of the total number of leukocytes or lymphocytes. Absolute counts of the same leukocyte populations were not performed. Future studies should focus on more exact and extensive measurements of the actual circulating numbers of discriminating leukocyte populations.

Vasculature related factors have not been measured in the BiSS cohort, such as endothelin and the carotid intima thickness, which would have given a better impression of the state of the endothelium. Future studies should involve patients with a better metabolic characterization and characterization of the state of their vasculature.

Another limitation is that the exclusion criteria for patients and controls were not exactly the same. Patients of the BiSS cohort were not excluded if known with an endocrine or immune disease. However, endocrine diseases were recorded in the BiSS group (seven patients had thyroid disease and three diabetes), and of note the presence of endocrine disease did not correlate with immune outcomes (data not shown). Yet future studies should try and match as much as possible for the various conditions having an effect on the immune state of both patients and controls (age, gender, BMI, comorbidities, smoking, stress, etc.).

CONCLUSION

Despite the limitations, we are confident that this study shows that lymphopenia, and in particular a reduced percentage of T cells, is a characteristic of relatively old BD patients. Since lymphopenia and a reduced percentage of T cells are phenomena also found in children at risk for BD and younger BD patients, a deficit of T cells might be a trait phenomenon of BD.

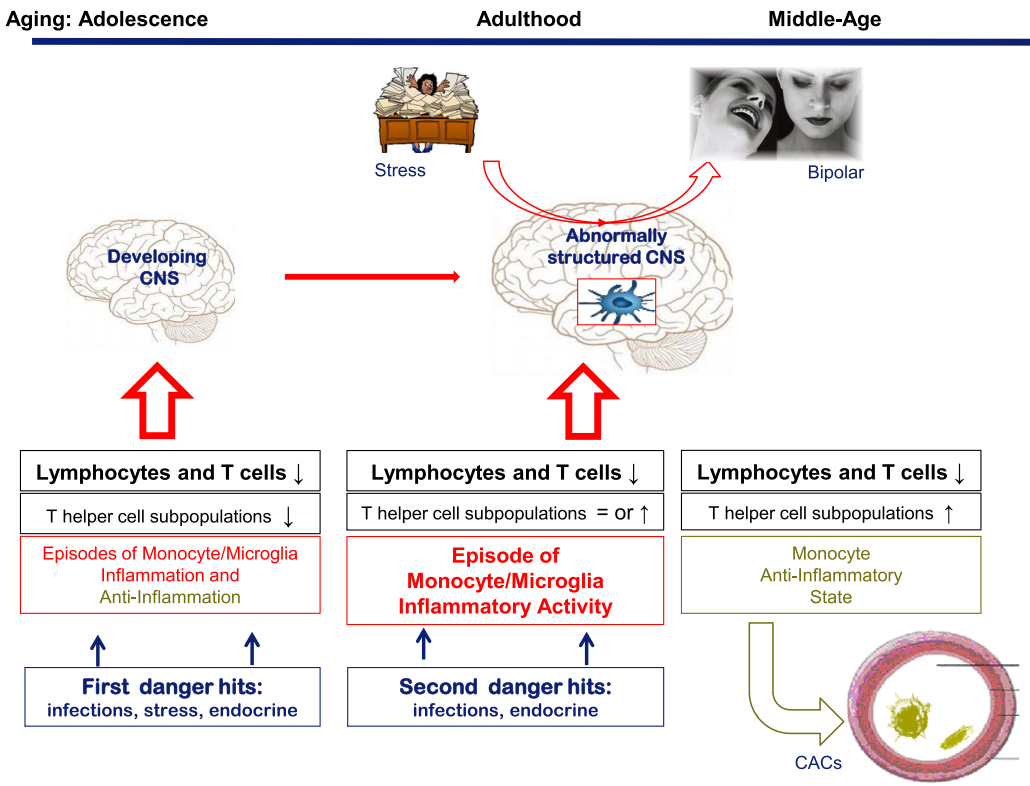

FIGURE 1 | A hypothetical cartoon on the natural history of the lymphocyte and monocyte changes in bipolar subjects over time and their consequences for brain function and support function of blood vessels. In the cartoon, it is assumed that in healthy conditions both T cells and monocytes/microglia are essential for proper brain development and function. When there is a deficit in T cells or changing inflammatory states as in bipolar disorder (BD) and the pre-stages of BD, we assume that brain development and function are abnormal leading to a vulnerability for mood derailment.

The monocyte gene expression profile of the relatively old BD patients showed a downregulation of inflammatory genes and an upregulation of HGF, suggesting CAC activity of the cells. This profile is different from the pro-inflammatory profile found in 16-year-old offspring of a bipolar parent and in BD patients with active disease. Therefore, the changes in monocyte profiles suggest dynamic changes over time in the inflammatory state of monocytes of individuals at risk for manic-depressive episodes. We therefore assume that the monocyte inflammatory set point is a state rather that a trait phenomenon of BD.

AUTHOR CONTRIBUTIONS

All the authors collected part of the study cohort, evaluated the data, particularly the hair cortisol and metabolic parameters, and assisted in writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fpsyt.2017.00034/full#supplementary-material.

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High Kynurenine (a Tryptophan Metabolite) Predicts Remission in Patients with Major Depression to Add-on Treatment with Celecoxib

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Background: Signs of an inflammatory process have been described in major depression.

Methods: In a double-blind, randomized study of celecoxib or placebo add-on to reboxetine in 40 depressed patients, celecoxib treatment has beneficial effects. In order to evaluate the tryptophan/kynurenine metabolism and to identify predictors for remission, tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA), and quinolinic acid (QUIN) were estimated in the serum of 32 patients before and after treatment and in a group of 20 healthy controls.

Results: KYN levels were significantly lower in patients ($p = 0.008$), and the QUIN/KYN ratios were significantly higher ($p = 0.028$). At baseline, the higher KYN/TRP ratio was predictive for remission during celecoxib add-on treatment ($p = 0.04$) as well as for remission in the overall patient group ($p = 0.01$). In the placebo group, remitters showed a higher KYNA/QUIN ratio ($p = 0.032$). In the overall group, remitters showed lower KYNA/KYN ($p = 0.035$) and QUIN/KYN ($p = 0.011$) ratios. The lower the formation of downstream metabolites, especially QUIN, the better the treatment outcome.

Conclusion: The high KYN/TRP ratio predicted remission after treatment with celecoxib in this small sample of depressed patients. Eventually, the KYN/TRP ratio might be a marker for those patients, which benefit from an additional anti-inflammatory treatment.

Keywords: depression, cyclooxygenase-2, celecoxib, kynurenines, remission

INTRODUCTION

Activation of the inflammatory response system in major depression (MD) is well documented (1–5). Recent meta-analyses clearly showed elevated interleukin-6 (IL-6) levels in patients with MD (6–9).

However, the findings of these meta-analyses differed regarding levels of the inflammatory markers C-reactive protein (CRP), IL-1, IL-1RA, and TNF-α, with more hints toward increased CRP levels and no association for TNF-α and IL-1 in depression (8). In general, the inflammatory response

system appears to be activated, but the levels of the different markers vary across studies.

Prostaglandin E2 (PGE2) is an important mediator of inflammation (10). Increased PGE2 in the saliva, serum, and cerebrospinal fluid of depressed patients has been described previously (11–14). The enzyme cyclooxygenase-2 (COX-2) is involved in the function of PGE2 in the inflammatory pathway. The COX-2 inhibitor celecoxib, an add-on to different antidepressants, has demonstrated beneficial effects in the treatment of depression (15, 16). Although not all patients who received celecoxib add-on remitted, celecoxib showed significant advantages over the placebo add-on. However, side effects, including cardiovascular effects, have been observed during the use of COX-2 inhibitors, particularly in long-term treatment. With these specific side effects of celecoxib, screening and monitoring for cardiovascular risk factors and events is important, when treating MD with COX-2 inhibitors. Also, a recent meta-analysis with a total of 150 patients has shown that the adjunctive celecoxib group had better remission and response rates than the placebo group (17).

Taken this together, it would be desirable to predict remission to the therapy with celecoxib. Predictive markers of the immune system for antidepressant therapy response have been described before. Decreased IL-6 levels were predictive for response to antidepressant pharmacotherapy (18, 19). A very recent study identified increased cytotoxic T cells and decreased natural killer cells as possible predictors for treatment response in MD (20).

Additionally, a meta-analysis showed that persistently elevated TNFα was associated with prospectively determined treatment resistance for depressed patients (21). Products of the tryptophan/kynurenine metabolism, however, have not yet been studied under the aspect of antidepressant therapy, although they are induced by an enhanced inflammatory response and proposed to be involved in the pathophysiology of depression (22, 23). Enzymes of the tryptophan–kynurenine metabolism are regulated by pro-inflammatory cytokines and prostaglandin E2 as a coactivator, in particular the indoleamine 2,3-dioxygenase (IDO), which metabolizes tryptophan to kynurenine. Moreover, metabolites of tryptophan metabolism are plausible biomarkers for depression since the biological ranges are fairly narrow, the detection rate in blood is good, and they discriminate satisfactorily between depressed patients and controls (24). The precise degradation of tryptophan leads to different neurotransmitters that are excitotoxic (25) or *N*-methyl-D-aspartate receptor antagonists (26). The hypothesis of the current study is that the measurement of the way tryptophan is metabolized could help to identify remitters already before the onset of treatment. Therefore, in this 6-week study, we evaluated key tryptophan metabolites to investigate whether they predict the outcome of treatment with celecoxib as an add-on to an antidepressant.

MATERIALS AND METHODS

Patients and Controls

In total, 60 subjects participated in this study. Of these, 40 participants were patients (20 males and 20 females) aged between 23 and 63 years. All patients were diagnosed with MD according to DSM IV (DSM IV: 296.2 × single depressive episode or 296.3 × recurrent depressive episode) and needed to have a 17-item Hamilton Depression Scale (HAMD-17) score of at least 15 (range for included depressed patients was from 15 to 38). Patients suffering from psychotic depression or also other inflammatory diseases (e.g., multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease) were excluded. Current intake of NSAID for any reason (including pain) was an exclusion criterion. Also, a history of substance or alcohol abuse/dependence and severe physical illnesses were exclusion criteria. Each patient was included after written informed consent. The study was examined and approved by the ethics committee of the medical faculty of the University of Munich in accordance with the Declaration of Helsinki 1975, revised Hong Kong 1989. The depressed patients were study participants of a double-blind randomized, placebo-controlled, and prospective parallel group trial of celecoxib add-on to reboxetine. After a wash-out period (or without, in case patients were not medicated) of 3–7 days (according to the prior drug used; no patient had prior fluoxetine treatment), the patients were randomized to either celecoxib or placebo. The treatment period lasted 42 days (6 weeks). Patients were permitted to take benzodiazepines if needed for bridging the gap until reboxetine showed its effects. The results of the clinical parameters were reported in detail elsewhere (16). Briefly, 20 of the 40 patients (12 males, 8 females) were allocated to treatment with celecoxib as an add-on to reboxetine and 20 (8 males, 12 females) to placebo add-on to reboxetine. The dose of reboxetine was flexible and ranged from 4 to 10 mg/day, according to the clinical needs. Celecoxib was administered at a dose of 400 mg/day.

The baseline mean scores on the HAMD-17 were 25.4 (SD 4.0) in the celecoxib group and 24.6 (SD 5.9) in the reboxetine plus placebo group. After exclusion of drop-outs and patients whose blood samples were no longer available from the baseline sample collection, a total of 32 subjects (18 in the celecoxib group and 14 in the placebo group; age 44.6 ± 11.6 years; age range: 25–65 years; 16 females, 16 males) were included in the study. Remitters were defined as patients whose scores on the HAMD-17 had decreased to 7 or less by the end of the study. Six of 18 patients in the celecoxib group and 3 of 14 in the placebo group were remitters. The relatively high drop-out rate (in particular in the reboxetine plus placebo group) might partly be explained by the limited antidepressant effects of reboxetine and partly by the side effects of reboxetine (16).

A total of 20 healthy, age-matched controls (age 40.0 ± 10.4 years; age range: 24–60 years; 5 females, 15 males) were recruited to allow comparison of the tryptophan metabolism parameters. A non-structured clinical interview was used to exclude participants with a personal or familial history of psychiatric illness, diagnosed autoimmune disease, or substance or alcohol abuse. These interviews were performed by an experienced psychiatrist. The healthy controls were free of chronic or acute physical illness associated with altered states of immunity and showed normal blood chemistry values (this included normal ranges of complete blood count, liver and renal function, and thyroid hormones). Please see **Table 1** for characteristics of study participants.

The study was approved in accordance with the ethical standards of the responsible committee on human experimentation

[medical faculty of the Ludwig Maximilian University (LMU)] or with the Declaration of Helsinki 1975, revised Hong Kong 1989. Written informed consent was obtained from each participant.

Biochemical Analyses

Kynurenines were analyzed in serum obtained from fasting, early morning venous blood samples.

Tryptophan (TRP), kynurenine (KYN), and kynurenic acid (KYNA) were analyzed at the Psychoneuroimmunology Laboratory of the Department of Psychiatry and Psychotherapy, LMU, Munich, with high-performance liquid chromatography (HPLC) according to the method of Oades et al. (27). Analytes were extracted from the samples and calibrators/controls by using Waters Oasis MCX 1 cc (30 mg) extraction cartridges. The eluent was evaporated to dryness under nitrogen and reconstituted with 150 µl 0.1 M PBS. Reconstituted samples/calibrators/controls were analyzed with HPLC with 250 mm × 4 mm Supersphere 60 RP-select B, C8 column (Merck, Darmstadt, Germany). TRP (lex: 300 nm; lem: 350 nm) was measured by fluorescence detection, and KYN (365 nm) and KA (330 nm) were measured by UV detection.

Serum quinolinic acid (QUIN) was analyzed at the Laboratory of Immunology and Transfusion Medicine at the University of Greifswald on a Hewlett-Packard model 5988 quadrupole mass spectrometer operated in the electron capture negative chemical ionization mode with methane as the reagent gas (0.5 Torr). Sample extraction was performed according to Morrison et al. (28).

The coefficient of variation of the above analyses ranged from 7 to 10. Patient and control samples were analyzed in random order, and the technical assistants who analyzed the samples and read the chromatograms were blind to the diagnoses and treatment groups. Finally, in order to estimate TRP degradation, the KYN to TRP ratio (KYN/TRP) was calculated, which is an indirect marker for the activity of the enzyme indoleamine 2,3-dioxygenase. For the further degradation of KYN, the ratios KYNA/KYN and QUIN/KYN were calculated, as these ratios provide insights of the accumulation of neuroactive substrates.

TABLE 1 | Characteristics of study participants.

Patient's characteristics	Celecoxib group (n = 18)	Placebo group (n = 14)	Controls (n = 20)
Sex	11 males, 7 females	5 males, 9 females	15 males, 5 females
Mean age (years)	44.6 ± 11.5	43.9 ± 13.3	40.0 ± 10.4
Remitters	6 with HAMD-17 < 7	3 with HAMD-17 < 7	
Mean benzodiazepine dose	2.4 ± 3.0 mg/day	2.7 ± 3.1 mg/day	

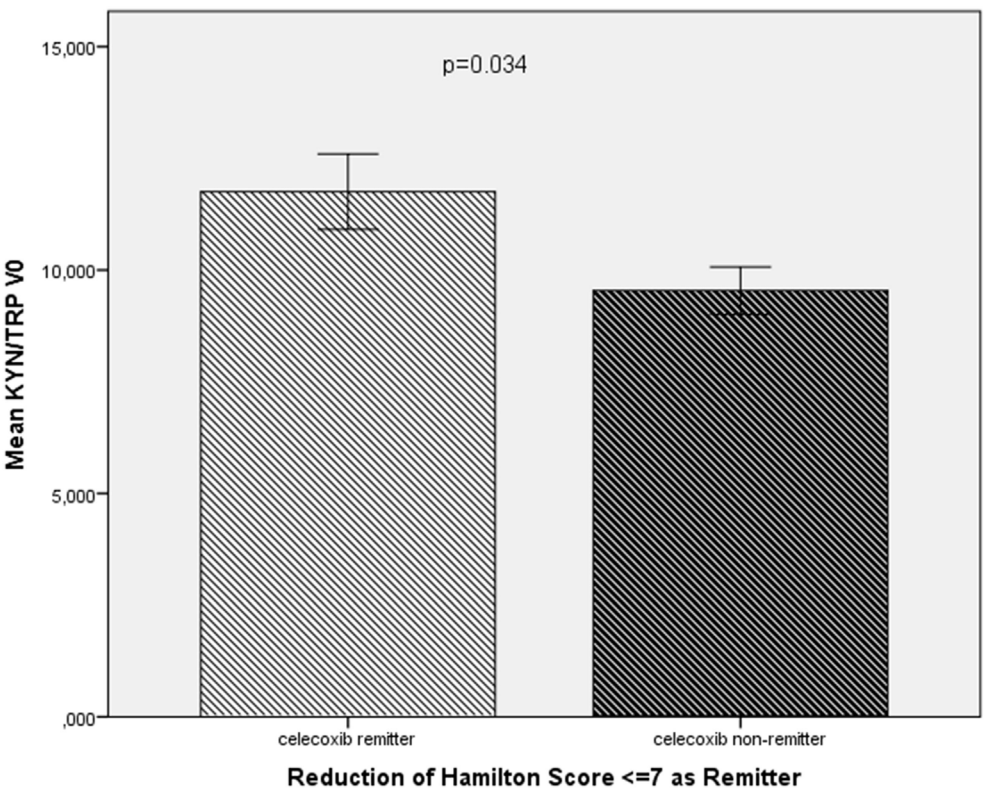

FIGURE 1 | Comparison of the KYN/TRP ratio at baseline (KYN/TRP V0) between remitters (n = 6) and non-remitters (n = 12) in the celecoxib add-on group.

Statistical Analyses

Student's *t*-test was used to compare normally distributed data between patients and controls and between different subgroups. Linear regression analysis was performed to analyze the effect of the parameters of TRP/KYN metabolism on remission to treatment (HAMD $\leq$ 7) in treatment subgroups, and multivariate analysis controlling for age and gender was performed to analyze the effect in the overall group. SPSS version 18.0 was used, and $p < 0.05$ was considered significant.

RESULTS

Patients vs Controls

Serum KYN levels were significantly lower (1.78 ± 0.35 vs 2.04 ± 0.35 μg/ml; $t = -2.78$, $p = 0.008$) in patients ($n = 32$) than in controls ($n = 20$), but QUIN/KYN ratios were significantly higher (0.17 ± 0.05 vs 0.14 ± 0.03; $t = -2.28$, $p = 0.028$). Tryptophan did not differ significantly between the two groups. Serum KYNA levels showed a trend toward being lower in the patient group (0.311 ± 0.054 vs 0.347 ± 0.087 ng/ml; $t = -1.808$, $p = 0.077$).

Celecoxib vs Placebo

Tryptophan metabolites did not differ significantly between the celecoxib ($n = 18$) and control groups ($n = 14$) at baseline or after 6 weeks of treatment.

Remitters vs Non-Remitters of the Depressed Patients

In the celecoxib group (add-on to reboxetine), remitters ($n = 6$) showed a higher KYN/TRP ratio (11.76 ± 2.07 vs 9.54 ± 1.68; $t = 2.2$, $p = 0.034$) at baseline (**Figure 1**). In the linear regression analysis, the baseline elevated KYN/TRP ratio was predictive for remission to celecoxib add-on treatment in terms of the percentage of patients showing a decrease in HAMD score to 7 or less at the end of the study ($B = 0.03$, CI = 0.001–0.059, $p = 0.04$).

In the placebo group (add-on to reboxetine), remitters ($n = 3$) showed a higher KYNA/QUIN ratio (1.36 ± 0.059 vs 1.07 ± 0.162; $t = -2.25$, $p = 0.032$).

In the overall group (celecoxib and placebo add-on to reboxetine), remitters ($n = 9$) showed a higher KYN/TRP ratio (11.51 ± 1.81 vs 9.31 ± 1.99; $t = -2.72$, $p = 0.011$) (**Figure 2**) and lower KYNA/KYN (0.163 ± 0.017 vs 0.19 ± 0.051; $t = -2.22$, $p = 0.035$) (**Figure 3**) and lower QUIN/KYN (0.139 ± 0.022 vs 0.179 ± 0.057; $t = -2.74$, $p = 0.011$) ratios. When age and gender were controlled for, higher KYN/TRP was predictive for remission to antidepressant treatment with or without celecoxib add-on in terms of reduction of HAMD score to 7 or below ($B = 33.012$, $F = 10.312$, $p = 0.004$).

DISCUSSION

Our results show that initial serum kynurenine levels predict remission after add-on treatment with the COX-2 inhibitor

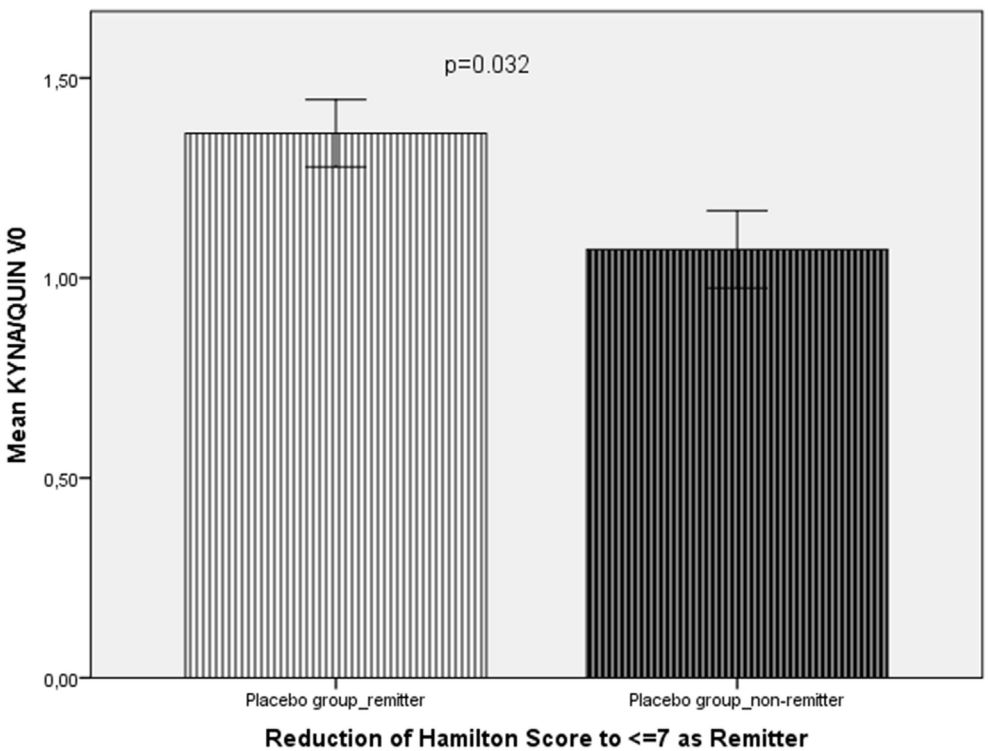

FIGURE 2 | Comparison of the KYNA/QUIN ratio at baseline (KYNA/QUIN V0) between remitters ($n = 3$) and non-remitters ($n = 11$) from the placebo group.

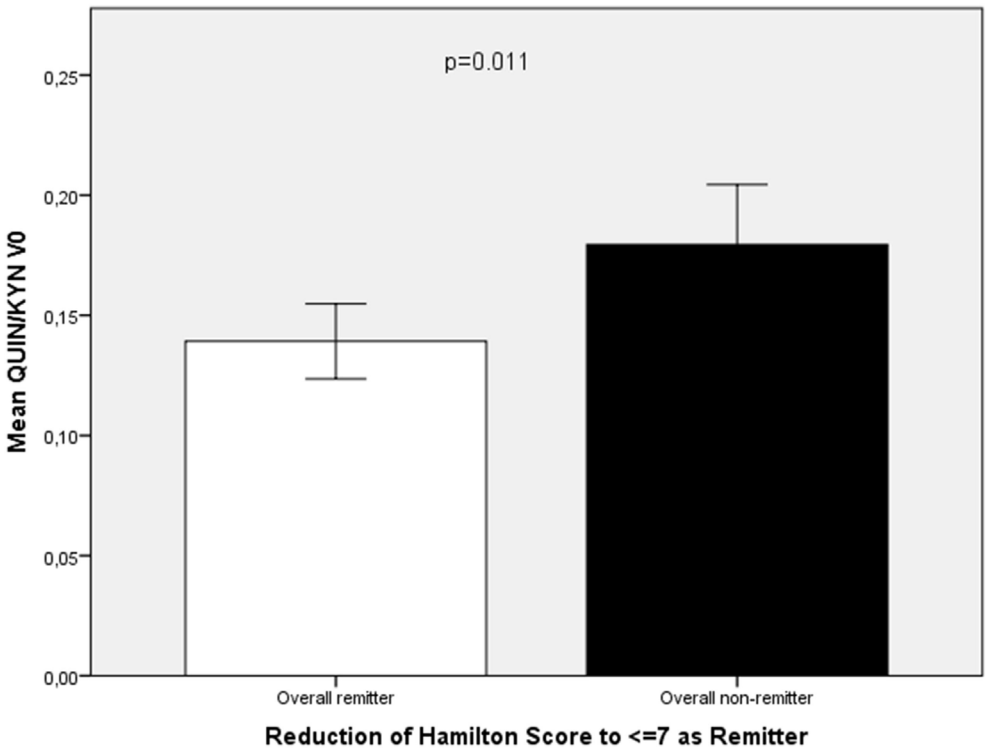

FIGURE 3 | Comparison of QUIN/KYN ratio at baseline (QUIN/KYN V0) between remitters ($n = 9$) and non-remitters ($n = 23$) from the overall group of patients with major depression.

celecoxib. More precisely, the higher KYN/TRP ratio at baseline predicted both remission in the celecoxib add-on group and in the whole (reboxetine) group. The KYN/TRP ratio indicates the activity of the enzymes tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), which reflect a response of the inflammatory system. Circulating monocytes have already been identified to overexpress inflammatory genes in depressed patients (6). Therefore, we conclude that the activity of the inflammatory response system predicts the outcome of treatment with the anti-inflammatory COX-2 inhibitor celecoxib: the greater the inflammatory response, the better the outcome after treatment with anti-inflammatory medication. However, a greater inflammatory response was associated also with better outcome after treatment with the antidepressant reboxetine alone. From a statistical point of view, it is relevant that two-thirds of the remitters were in the celecoxib group; therefore, the effect in the whole group is primarily due to the effect in the celecoxib group.

The ratios between the metabolites are intriguing (1) the ratio between KYNA—a downstream metabolite of KYN—and KYN (KYNA/KYN), and (2) between QUIN—another downstream metabolite of KYN—and KYN (QUIN/KYN) were significantly lower in the overall group of remitters. Also, the remitters in the placebo group showed a higher KYNA/QUIN ratio. QUIN is an NMDA-R agonist and excitotoxic (29), and KYNA is an NMDA-R antagonist (26). Overall, the results also suggest that the lower the formation of downstream metabolites, especially the excitotoxic QUIN, the better the treatment outcome. From the findings concerning the lower KYNA/KYN and lower QUIN/KYN ratios in remitters, it follows that the lower the degradation rate of KYN is to the downstream metabolites, the more KYN accumulates, and the higher the ratio is between KYN and TRP. A higher KYN/TRP ratio thus would mean less formation of downstream neurotoxic metabolites such as QUIN and subsequently would be associated with a better treatment outcome.

Our study shows that the KYN/TRP ratio may predict remission after antidepressant treatment with add-on anti-inflammatory medication using a COX-2 inhibitor, and this finding is also significant for the overall patient group. Our study also highlighted the fact that an increased formation of the downstream neuroactive KYN metabolites such as QUIN may negatively influence treatment outcome in depression. This finding is in line with the view that QUIN might be a pathogenetic factor in MD (30).

The increasing discussion of the role of inflammation and the increasing number of reports on beneficial effects of COX-2 inhibitors in MD demand the identification not only of biomarkers to characterize the immunopathology of MD but also of immune markers for treatment response and remission. Another study of celecoxib add-on reported that decreased serum levels of the pro-inflammatory cytokine IL-6 predict the response to treatment (31). Our findings and the IL-6 report strengthen not only the view that inflammation and the TRP/KYN metabolites play a role in the pathogenesis of MD but also that markers of this system may be suitable to predict treatment remission with anti-inflammatory compounds.

The present study also compared patients with MD to healthy individuals. We observed a higher QUIN/KYN ratio in the overall group of patients than in the healthy controls. This finding is in line with hypotheses previously proposed to explain the link between enhanced tryptophan degradation induced by activation of the inflammatory response system and subsequent enhanced formation of neurotoxic QUIN, thereby resulting in clinical symptoms such as depression (23, 32).

Our study has a major limitation: the overall sample size is rather small, thereby resulting in very small subgroups within each treatment group, e.g., of remitters and non-remitters. This small sample size has also some implications for statistics: no control for multiple comparisons has been performed. Therefore, the current results can only be interpreted with caution, and it is not yet possible to generalize them. Meaning, the present study should be seen as a pilot study for the identification of tryptophan metabolites as predictors for treatment remission and further studies with a larger sample size properly designed for a biomarker study should be performed. This future study could eventually divide patients into groups with high or low inflammatory markers. Nevertheless, our study can be seen as a first attempt on the way for a possible application of TRP/KYN metabolites to predict treatment outcome.

AUTHOR CONTRIBUTIONS

DK, NM, A-MM, MR, VA, and MS planned the study, wrote the study protocol, and prepared the manuscript. A-MM, MS, and MR calculated the statistics. MS, CS, and A-MM performed the lab work. DK, SD, and AC recruited the patients and samples and helped for interpretation of the data.

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Social Phobia Is Associated with Delayed Onset of Chickenpox, Measles, and Mumps Infections

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Objective: Evidence showing that infectious diseases in childhood play an important role in the etiopathogenesis of neurodevelopmental and other mental disorders is growing. The aim of this study was to explore the timing of common childhood diseases in early-onset anxiety disorders.

Materials and methods: We analyzed data from PsyCoLaus, a large Swiss Population Cohort Study (N = 3720). In this study, we regressed overanxious disorder, separation anxiety disorder, social phobia, and specific phobias on the age of onset of several childhood diseases, always adjusting for the other anxiety disorders listed above and for sex.

Results: The timing of viral childhood diseases (chickenpox, measles, and mumps) was consistently delayed in social phobia, notably both in men and women. We found no evidence for a reversed sequence of onset of phobia symptoms before that of the infections included.

Conclusion: Social phobia was the only early anxiety disorder to show an association with a delayed onset of common viral childhood diseases.

Keywords: social phobia, anxiety disorders, childhood diseases, infectious diseases, epidemiology

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INTRODUCTION

Evidence showing that infectious diseases in childhood can play a crucial role in the etiopathogenesis of mental disorders is growing (1). This relation has been a long-standing issue in psychiatric epidemiology, but research activity has recently intensified. Current topics include prenatal infections with *Toxoplasma gondii*, rubella, and other infectious diseases (2, 3) as risk factors for neurodevelopmental disorders and schizophrenia. Furthermore, a series of postnatal infectious agents was reported in association with schizophrenia/psychosis (4–6). The pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS)

model also postulates a crucial role for autoantibodies against basal ganglia tissue appearing after infections with group A streptococci in the development of attention deficit hyperactivity disorder, Gilles de la Tourette syndrome, and obsessive–compulsive disorder (OCD) (7, 8). Finally, recent analyses have shed new light on the role of infectious diseases in anxiety disorders (9).

On a formal level, there are four models of how infectious diseases could affect the risk for mental disorders. The models can be illustrated using the associations between Epstein–Barr virus (EBV) and multiple sclerosis (MS) or psychotic disorders. The first model relates to the mere occurrence of an infection. For example, an EBV infection is a necessary precondition for later MS onset: while the seroprevalence for EBV is high, i.e., it covers 90% of the adult population or more (10), almost no one from the negative seroprevalence group develops MS. The second model includes the severity of an infection, for example, due to a dysfunctional immune response and/or due to a combination with genetic or other types of vulnerabilities. The third model considers the timing of childhood infections. Deviations from typical timing, in particular delayed childhood infectious diseases such as chickenpox, measles, mumps, or rubella, are well known to lead to a more severe disease course with more complications (11–14). This also applies to delayed EBV infections, which increase the risk for mononucleosis and probably also for MS (15). Finally, the fourth model focuses on the sequence of childhood infections. This is a possible reason for why infectious disease occurring earlier in life than typically expected might have harmful effects. For example, EBV infections in the first years of life have been linked to psychotic experiences (16). Both the timing and sequence of childhood infections can be assumed to be subjected to evolutionary optimization.

There is a lack of research systematically examining how the timing of infections and other inflammatory insults (17) impacts the risk for mental disorders. This study focused on the timing of childhood diseases with regard to early-onset anxiety disorders (separation anxiety disorder, overanxious disorder, specific phobias, and social phobia). The aim of the study was to examine whether deviations in age of onset of childhood infectious diseases are associated with the early-onset anxiety disorders. We used a two-step approach. First, we examined whether the age of onset of infections differs in cases with and without a specific disorder. The analyses were adjusted for common confounding factors and other early-onset anxiety disorders. In the case of delayed onset, we examined whether the sequence of a childhood infectious disease and the first symptoms of an anxiety disorder might have induced the result.

MATERIALS AND METHODS

The PsyCoLaus Study

The analysis was carried out within the framework of PsyCoLaus, a large epidemiological study conducted in Switzerland. The PsyCoLaus study (18) is the psychiatric part of the population-based CoLaus study (19). The participants in the CoLaus study were randomly selected from the population of the city of Lausanne (Switzerland). The assessment of the subjects took place between 2003 and 2006 in an outpatient clinic (19, 20). It included an interview with a semi-structured questionnaire, as well as the collection of clinical data and blood samples. One year after their initial assessment, CoLaus participants aged between 35 and 66 were asked to participate in the PsyCoLaus study. Subsequently, a total of 3,720 individuals (67%) agreed to participate (18). One major goal of PsyCoLaus was to collect data on the prevalence of psychiatric syndromes/disorders.

A French version of the semi-structured diagnostic interview for genetic studies (DIGS) (21, 22) was used in the PsyCoLaus study to assess a broad spectrum of DSM-IV Axis I criteria. Moreover, the DIGS allowed for gathering additional information about the course and chronology of comorbid features (18). However, the brief phobia section of the DIGS was replaced by the corresponding sections from the Schedule for Affective Disorders and Schizophrenia-Lifetime Version (23).

The study was approved by the Ethics Committee of the University of Lausanne. All participants gave their written informed consent at study enrollment in accordance with the Declaration of Helsinki.

Childhood Diseases/Disorders and Age of Onset

The early-onset anxiety disorders are characterized by an early age of onset of first symptoms, i.e., mostly before adolescence. While separation anxiety disorder is defined by excessive anxiety of being separated from parents or significant others, overanxious disorder was introduced as a childhood form of general anxiety disorder and in DSM-IV subsumed within this disorder. Specific phobias are a heterogeneous group of disorders (24) related to specific animals, objects, or situations. In social phobia, these are social situations. Typically, girls and women are more frequently affected by early-onset anxiety disorders. The sex ratios can reach a factor of 3 (specific phobias).

The following childhood infections were included in the analyses: pertussis, chickenpox, measles, mumps, rubella, and scarlet fever. The information on infectious diseases and other related conditions was derived using an extended version of the medical history part of the DIGS and was based on self-reporting. In the interview, participants were asked questions about ever having been diagnosed with various infectious diseases, diseases of the nervous system, cardiovascular, respiratory, gastrointestinal, metabolic and dermatological conditions, as well as allergies and hormonal problems. For each disease group, a screening question was asked, followed by more specific questions in the case of an affirmative response. In the section on childhood diseases, chickenpox, measles, and mumps were explicitely asked, whereas information regarding pertussis, rubella, and scarlet fever was extracted from the free text field of this section. These questions routinely included the age of onset of a disease or the first occurrence of symptoms of a mental disorder. Information such as "so early that I cannot remember" or "since I can remember" or age below 2 was replaced by a random value between 2 and 5.

Statistical Analysis

The analyses followed the conventional analysis design of univariate, bivariate, and multivariate analyses. In bi- and multivariate regression models, the age of onset of infectious diseases was implemented as a predictor of any of the examined early-onset anxiety disorders. In regression analyses, the onset age of infectious diseases was limited to 16 years in order to exclude outliers (14.8% in rubella, and 2.3–6.6% in other infectious diseases). In addition, the age data were smoothed by square root transformation to approach a normal distribution. Each regression analysis relied on a subsample of subjects who reported the infectious disease in question (and not on the whole sample). The analyses were routinely adjusted for:

- sex: in the analysis of overall data;
- other early-onset anxiety disorders (separation anxiety disorder, overanxious disorder, specific phobias, and social phobia) apart from the dependent variable.

Additional adjustments introduced in a one-by-one manner included the education level (three categories), the age of the participants (because of the possibility of recalling a higher age for any events among older age groups), and childhood adversities (fear of parental punishment, parental quarrels, growing up in children's home).

In the case of statistically significant and consistent results, the analysis design was supplemented by an additional comparison of the observed and expected cases relating to the sequence of a childhood disease and the first symptoms of an early-onset anxiety disorder in order to ascertain that the sequence remained unchanged. Since the relevant information on the age of onset was available only for a subset of subjects (those reporting the respective infectious disease and simultaneously indicating the age of onset of symptoms of an early-onset anxiety disorder), a consistent framework for inference statistics was missing and had to be replaced by a bootstrapping procedure (25). In this procedure, we created for each run a subsample of 1:1 matched controls from the overall sample positively reporting the infectious disease in question. For each run, the hypothetical number of controls was determined in whom an infectious disease occurred before or in the same year as in the corresponding case (relating to the year of onset of first symptoms of an anxiety disorder). We performed 1,000 runs in order to fix the hypothetical sequence and treated it, for the sake of simplicity, as the underlying population sequence. Therefore, the current sequence of infectious diseases and the first symptoms of an early-onset anxiety disorder were tested as the comparison between observed and theoretical frequencies.

Furthermore, in the case of statistically significant and consistent results, we additionally examined the associations between the early-onset anxiety disorders and the frequencies of reported chickenpox, mumps, and measles infections.

The analyses were routinely carried out with the overall data as well as for males and females separately. Moreover, they were repeated for measles after excluding those born in or after 1963 (decreasing endemicity of measles in part due to due to a national vaccination policy). The basic analyses and programming were carried out using SPSS Statistics (version 21). The bootstrapping procedure was programed in a SAS (version 9.3) macro.

RESULTS

The mean age of onset of first symptoms was 7.0 (SD 3.5) years in overanxious disorder, 5.2 (SD 2.2) years in separation anxiety disorder, 12.1 (SD 9.8) years in specific phobias and 10.9 (SD 8.2) years in social phobia. **Table 1** displays the average onset age of common viral and bacterial childhood diseases in early anxiety disorders.

Table 2 shows the results for social phobia regressed on the age of onset of chickenpox, measles, and mumps for the whole sample and the results for separation anxiety disorders (only males) regressed on the age of onset of mumps. The analyses in other anxiety disorders did not yield significant estimates (results not shown). Neither the adjustment for covariates (other early anxiety disorders and sex) and potential confounders (education level, age of subjects, and childhood adversities) nor the replication of the analyses after excluding subjects born in 1963 or later yielded any noteworthy differences. However, replication by sex-specific analyses showed that in social phobia the onset of chickenpox, measles, and mumps was consistently delayed in both sexes. The odds ratios based on square root smoothed values for age were 1.8 (1.2–2.7), 2.1 (1.3–3.2), and 2.0 (1.3–3.0) in males, and 1.4 (1.0–1.9), 1.7 (1.2–2.2), and 1.4 (1.0–2.0) in females.

The results from analyses addressing the sequence of each childhood disease and the onset of first symptoms of social phobia (or separation anxiety disorder) are displayed in **Table 3**. In social phobia, the delay in the onset of chickenpox, measles, and mumps did not induce any noteworthy shift between observed and expected cases. However, in separation anxiety disorders, the delay of the onset of mumps was accompanied by an increase of cases with a mumps infection after encountering preliminary separation anxiety symptoms thus indicating that an altered sequence of onsets was involved and induced artificial results.

TABLE 1 | Onset age of childhood infectious diseases overall and per anxiety disorder; outliers above 16 years excluded; mean and 95% confidence interval.

	Chickenpox *N* = 2566	Measles *N* = 2390	Mumps *N* = 1936	Rubella *N* = 201	Pertussis *N* = 256	Scarlet fever *N* = 146
Overall	6.05 (5.95–6.14)	6.06 (5.97–6.16)	7.04 (6.93–7.16)	7.55 (7.01–8.01)	6.39 (6.06–6.72)	7.46 (6.97–7.95)
Separation anxiety disorder	5.76 (5.41–6.12)	5.93 (5.53–6.33)	7.46 (6.90–8.01)	7.11 (5.42–8.80)	7.14 (4.86–9.43)	7.85 (6.07–9.62)
Overanxious disorder	5.98 (5.64–6.33)	6.30 (5.98–6.63)	7.38 (6.93–7.82)	7.89 (6.01–9.78)	6.18 (4.87–7.49)	7.50 (4.47–10.53)
Specific phobia	5.99 (5.76–6.22)	6.16 (5.93–6.39)	7.15 (6.86–7.44)	7.40 (6.36–8.43)	6.39 (5.68–7.10)	6.64 (5.52–7.76)
Social phobia	6.50 (6.22–6.77)	6.67 (6.40–6.93)	7.60 (7.25–7.95)	7.54 (6.07–9.00)	6.83 (5.70–7.96)	8.15 (6.84–9.46)

TABLE 2 | Regression analysis models with social phobia regressed on age of onset in chickenpox (model 1), measles (model 2), and mumps (model 3); separation anxiety disorder on age of onset in mumps (only males; model 4); age smoothed by square root transformation; odds ratios and 95% confidence interval adjusted in each model for other early anxiety disorders and sex.

	Model 1 social phobia on chickenpox age	Model 2 social phobia on measles age	Model 3 social phobia on mumps age	Model 4 (males) separation anxiety disorder on mumps age
Age at onset	1.57 (1.23–1.99)	1.76 (1.36–2.28)	1.55 (1.19–2.04)	2.89 (1.55–5.39)
Sex	1.44 (1.11–1.86)	1.39 (1.07–1.82)	1.54 (1.16–2.06)	*
Separation anxiety disorder	1.95 (1.28–2.97)	1.47 (0.93–2.33)	1.46 (0.88–2.42)	*
Overanxious disorder	2.42 (1.69–3.47)	2.47 (1.70–3.59)	2.69 (1.81–3.99)	*
Specific phobia	1.89 (1.31–2.71)	1.79 (1.22–2.63)	2.20 (1.46–3.31)	*

not applied (see text and **Table 3).*

TABLE 3 | Observed and expected cases with onset of selected childhood disease before or in the same year as the onset of first symptoms of social phobia (overall sample) and separation anxiety disorder (only males).

	Social phobia vs. chickenpox	Social phobia vs. measles	Social phobia vs. mumps	Separation anxiety disorder vs. mumps (males)
Subsample *N* total	325	288	247	43
N (%) observed	198 (60.9)	185 (64.2)	135 (54.7)	8 (18.6)
N (%) expected	208.4 (64.1)	189 (65.7)	140.4 (56.9)	15.3 (35.7)
χ^2	1.45 (n.s.)	0.27 (n.s.)	0.48 (n.s.)	5.41*

**$p < 0.05$.*

Additional analyses examining the associations between social phobia and reported chickenpox, mumps, and measles infections consistently yielded odds ratios above one, but reached significant levels only regarding mumps (OR = 1.26, CI 1.01–1.57).

DISCUSSION

This study was the first to examine the timing of childhood diseases in early-onset anxiety disorders (separation anxiety disorder, overanxious disorder, specific phobias, and social phobia). We found that social phobia was associated with a delayed age of viral infectious diseases, in particular with respect to chickenpox, measles, and mumps. Notably, these results were separately replicable in males and females and could not be explained by a reciprocal sequence of reported conditions.

Thus, we offer four possible interpretations of our results. First, the timing of the viral infections could interfere with the changing activity of the immune system, as hypothesized regarding EBV infection in/after adolescence and the EBV–MS link (15, 26). However, social phobia symptoms typically have an early onset before adolescence (27). Since the average delay of chickenpox, measles and mumps infections found in persons with social phobia is rather small (below 1 year), it cannot fill the gap between childhood and adolescence. Therefore, there is no support for a crucial role for adolescence and its concomitants in this disorder.

The second interpretation is more trivial, suggesting that the reporting of chickenpox, measles, and mumps basically relies on the visibility of symptoms such as exanthema. It is noteworthy that these childhood diseases had reached a high seroprevalence of above 90%, before the vaccination campaigns began, thus inducing a ceiling effect in the analyses (12, 14). As mentioned earlier, a higher age at infection is a risk factor for more severe symptoms. This is compatible with the increased frequencies of at least mumps infections reported by subjects with social phobia. With this interpretation, some viral infections are directly associated with the risk for social phobia and mediated by a more severe course and symptoms of the infectious disease.

The third interpretation is closely linked to the second one mentioned earlier. It cannot be excluded that an unknown common factor simultaneously increases the risk for more severe forms of childhood diseases and for social phobia. However, no such factor is currently apparent.

Last but not least, the association between social phobia and delayed age of viral infectious diseases could emerge as an implication of coping with early social phobia symptoms, for example, by avoiding social contact in free time and out-of-school activities. A similar rationale would also apply to separation anxiety and overanxious disorder. However, in these instances, it was not supported by the data.

Thus, the most plausible interpretation for the moment is that viral infections in childhood not only precede but also contribute to the risk for social phobia. The reporting of viral infections in a survey such as PsyCoLaus depends on the perception of manifest symptoms by the subjects. In turn, manifest symptoms are related to a more severe course of these infections and thus also to a stronger involvement of the immune system. Therefore, we hypothesize that the increased risk for social phobia is mediated by a relatively stronger involvement of the immune system. This is in line with models linking infectious diseases with neurodevelopmental disorders—for example, the PANDAS model (8)—or with postinfectious fatigue and depression (28–30). However, in contrast to the PANDAS model, viral—and not, or not only, bacterial—pathogens are involved in social phobia. Moreover, the small delay suggests that apart from the severity of viral childhood diseases a specific age stage, i.e., a specific stage of brain development, should also be considered in social phobia.

While the results of this study show a new facet of how the immune system interferes in the etiopathogenesis of social

phobia, they should not be generalized to the whole spectrum of social phobia subtypes. As in other phobias (24) or OCD (31), the analysis of epidemiological parameters, risk factors, and comorbidity patterns split by sex and age at onset of first symptoms indicates some hidden heterogeneity of this disorder, as do clinical studies (32).

Limitations

As customary in population surveys, the information used was based on self-reporting, including all information on childhood diseases. The participants in PsyCoLaus were adults up to 66 years of age. Therefore, the analyses might be biased by telescoping effects such as those found typically regarding onset of substance use (27, 28) and display an age-dependent recall-bias regarding symptoms and diseases occurring in childhood and youth. However, no evidence for this was found in the analyses adjusted for age.

The diagnosis of social phobias and other disorders was based on information from epidemiological instruments and not on clinical assessment. In some instances, the analyses might also have failed to reveal significant estimates due to small frequencies of cases. While some infectious diseases were directly documented in the DIGS, others were covered by a more general question. Thus, the recall bias might interfere differently, depending on the question format.

The interpretation is solely based on association analyses and results from regression analyses. Therefore, it includes a strong theoretical or speculative component and should be adopted with caution. Ongoing studies covering other infectious diseases and other mental disorders will facilitate a more precise and comprehensive interpretation of the specific link between viral childhood diseases and social phobia.

CONCLUSION

The analysis of the timing of childhood diseases in early anxiety disorders has shed new light on the connection between viral childhood diseases—chickenpox, measles, and mumps—and social phobia. While the clinical implications of this study are minor, its theoretical implications are challenging. The results suggest a role for the immune system in impacting the development of early onset anxiety disorders.

AUTHOR CONTRIBUTIONS

MP, EC, and CV designed the PsyCoLaus study and acquired the data. VA-G, AA, SR, and MM carried out the analysis. WK, WR, RK, MargM, and RL discussed the preliminary results. VA-G and AA wrote the paper. All the authors contributed to the interpretation of the results and to the critical revision of the manuscript.

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Effects of Immune Activation during Early or Late Gestation on *N*-Methyl-D-Aspartate Receptor Measures in Adult Rat Offspring

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Background: Glutamatergic receptor [*N*-methyl-D-aspartate receptor (NMDAR)] alterations within cortex, hippocampus, and striatum are linked to schizophrenia pathology. Maternal immune activation (MIA) is an environmental risk factor for the development of schizophrenia in offspring. In rodents, gestational timing of MIA may result in distinct behavioral outcomes in adulthood, but how timing of MIA may impact the nature and extent of NMDAR-related changes in brain is not known. We hypothesize that NMDAR-related molecular changes in rat cortex, striatum, and hippocampus are induced by MIA and are dependent on the timing of gestational inflammation and sex of the offspring.

Methods: Wistar dams were treated the with viral mimic, polyriboinosinic:polyribocytidylic acid (polyI:C), or vehicle on either gestational day 10 or 19. Fresh-frozen coronal brain sections were collected from offspring between postnatal day 63–91. Autoradiographic binding was used to infer levels of the NMDAR channel, and NR2A and NR2B subunits in cortex [cingulate (Cg), motor, auditory], hippocampus (dentate gyrus, cornu ammonis area 3, cornu ammonis area 1), and striatum [dorsal striatum, nucleus accumbens core, and nucleus accumbens shell (AS)]. NR1 and NR2A mRNA levels were measured by *in situ* hybridization in cortex, hippocampus, and striatum in male offspring only.

Results: In the total sample, NMDAR channel binding was elevated in the Cg of polyI:C offspring. NR2A binding was elevated, while NR2B binding was unchanged, in all brain regions of polyI:C offspring overall. Male, but not female, polyI:C offspring exhibited increased NMDAR channel and NR2A binding in the striatum overall, and increased

Abbreviations: Aud, auditory cortex; Cg, cingulate cortex; DS, dorsal striatum; M1, motor cortex; MIA, maternal immune activation; AC, nucleus accumbens core; AS, nucleus accumbens shell; DG, dentage gyrus; CA1, cornu ammonis area 1; CA3, cornu ammonis area 3; so, stratum oriens; sr, stratum radiatum; PBS, phosphate-buffered saline; NMDAR, *N*-methyl-D-aspartate receptor; polyI:C, polyriboinosinic:polyribocytidilic acid; GD, gestational day; PND, postnatal day; RM-ANOVA, repeated measures two-way analysis of variance.

NR2A binding in the cortex overall. Male polyI:C offspring exhibited increased NR1 mRNA in the AS, and increased NR2A mRNA in cortex and subregions of the hippocampus.

Conclusion: MIA may alter glutamatergic signaling in cortical and hippocampal regions *via* alterations in NMDAR indices; however, this was independent of gestational timing. Male MIA offspring have exaggerated changes in NMDAR compared to females in both the cortex and striatum. The MIA-induced increase in NR2A may decrease brain plasticity and contribute to the exacerbated behavioral changes reported in males and indicate that the brains of male offspring are more susceptible to long-lasting changes in glutamate neurotransmission induced by developmental inflammation.

Keywords: striatum, NMDA, polyriboinosinic:polyribocytidylic acid, maternal immune activation, Wistar rat, schizophrenia, rat model

INTRODUCTION

Epidemiological investigations implicate gestational inflammation as a significant risk factor in the manifestation of psychiatric disorders, including schizophrenia, in offspring (1). Studies suggest either the first (2) or second (1) trimester as the critical window of vulnerability for maternal infection to increase schizophrenia risk in people. Maternal immune activation (MIA), as modeled in rodents and non-human primates, suggests pro-inflammatory cytokines that result from MIA, rather than the pathogen itself, are critical in the development of schizophrenia-like behaviors and neuropathology in adult offspring (3–6). Deficits in sensorimotor gating and working memory are prevalent in schizophrenia (7, 8) and found in MIA rodents. The use of MIA in animal models has therefore been pivotal to establish a causal link between gestational inflammation and the development of neuropsychiatric-related phenotypes; however, only limited changes in brain cytokines are still found in the adult (9), suggesting that molecular alterations in neurotransmitter pathways including monoamines (10, 11) and GABA (12) may be more long lasting. Since pharmacological manipulations of glutamate neurotransmission are known to cause deficits in sensorimotor gating and in working memory, which are reminiscent of changes found in schizophrenia, we predict that widespread changes in glutamate receptors may be found in adult offspring of mothers who experienced immune activation while pregnant. While there is a report of a reduction in one subunit of the *N*-methyl-D-aspartate receptor (NMDAR) in one brain region (the hippocampus) (13), there are still gaps in our knowledge regarding the putative changes in other NMDAR subunits and the extent of NMDAR changes outside the hippocampus. It is important to determine the nature and extent of NMDAR changes more fully to not only correlate them with behavior but to also aid in the design of treatments aimed to either prevent or ameliorate changes in NMDAR-mediated neurotransmission that may underlie symptoms and cognitive deficits resulting from developmental overactivation of the immune system. This knowledge will expand our understanding of how gestational inflammation impacts glutamatergic signaling in psychiatric disorders with a neurodevelopmental etiology.

The timing of MIA can elicit specific behavioral phenotypes in rodent offspring during development and adulthood (6, 14). This phenomenon has been demonstrated in a mouse model that used the viral mimic, synthetic double-stranded RNA polyriboinosinic:polyribocytidilic acid (polyI:C, henceforth polyI:C offspring). Early polyI:C exposure on gestational day (GD) 9 results in adult offspring with behaviors that are used to index schizophrenia-like behaviors, such as sensorimotor gating deficits (13) and primarily dopaminergic-related behavioral alterations concomitant with a reduction in dopamine receptor D1 levels in cortex (13, 15, 16). Late polyI:C exposure on GD17 results in offspring with behavioral changes consistent with schizophrenia-like deficits in cognitive function, including working memory and reversal learning deficits (13, 17). Late polyI:C exposure results in offspring with changes in the hippocampus that include NMDAR-related alterations (13). In rats, mid-late gestation (GD14–17) MIA produces a range of schizophrenia-like phenotypes that include both increased and decreased locomotor response to the NMDAR antagonist MK-801 (18–21), deficits in sensorimotor gating (22–25), impaired cognition (23, 26), and reduced brain volume in cortical, hippocampal, and striatal regions (27, 28). We have recently reported that early (GD10) and late (GD19) MIA in rats, at developmentally equivalent times to mice (29), may not result in differential schizophrenia-like behaviors. We found that males with early polyI:C exhibit sensorimotor gating deficits and that males with late (GD19) polyI:C exhibit sensorimotor gating and working memory deficits (30), suggesting that there may be long-lasting alterations in cortical glutamate neurotransmission especially after late MIA.

A key glutamate receptor involved in learning and memory is the NMDAR. NMDAR hypofunction is a prevailing hypothesis in the pathophysiology of schizophrenia (31) and NMDAR1 is reduced in the frontal cortex of people with schizophrenia compared to controls (32). NMDARs are ionotropic glutamate receptors essential for synaptic plasticity throughout the brain (33). NMDARs consist of two obligatory NR1 subunits and two regulatory subunits (i.e., NR2A and/or NR2B) functioning as a membrane heterotetramer (34). NR2B-rich NMDARs predominate throughout the brain during early development, and NR2A-containing NMDARs gradually increase expression after birth (34). NMDARs rich in NR2A have shorter channel opening time and an increased probability to open compared to NR2B-rich NMDARs (35, 36). Additionally, the ratio between NR2A- and NR2B-containing NMDARs in the hippocampus is critical in activity-dependent plasticity, where increased NR2A:NR2B is

associated with less plasticity and reduced signal transduction (37–39). PolyI:C offspring exhibit decreased neonatal neuronal excitability (40), and decreased NR1 immunoreactivity (13) and synaptic plasticity in the hippocampus in adulthood. The extent to which other NMDAR regulatory subunits (2A and 2B) are changed due to MIA and the relationship of NMDAR changes to the time of exposure and sex of the offspring will provide information needed to help design ways to ameliorate the neurobiological impact of MIA.

In the current study, we sought to elucidate the effects of early (GD10) versus late (GD19) polyI:C treatment on NMDA receptor subunits in a range of cortical, hippocampal, and striatal subregions of male and female adult offspring. We hypothesize that MIA will induce NMDAR-related molecular changes in rat cortex, striatum, and hippocampus in adult offspring and changes will be dependent both on the timing of gestational inflammation and the sex of the offspring. We specifically aimed to extend the scope of previous MIA studies, which examined NR1 mRNA only (13), to include quantitative binding assays of the whole NMDA channel, and NR2A and NR2B subunits. As our largest changes were primarily NMDAR binding alterations in male polyI:C offspring, we investigated related mRNAs in the same brain regions from male offspring only.

MATERIALS AND METHODS

Animals and Prenatal PolyI:C Administration

Experiments were performed in accordance with the National Health and Medical Research Council's *Australian code for the care and use of animals for scientific purposes*. The current study was approved by the University of Newcastle's Animal Care and Ethics Committee (Approval number A-2009-108). Rats were sourced from the University of Newcastle's Central Animal House and housed with *ad libitum* food and water and 12 h light exposure in the University of Newcastle's Behavioral Sciences Animal Facility.

Postnatal day (PND) 70–90 Wistar rats were time-mated and day of vaginal plug detection was designated as GD0. Pregnant rats were assigned to two groups: GD10 (control, $n = 8$) or GD19 ($n = 7$). On the appropriate GD, pregnant rats were weighed, lightly anesthetized with isoflurane, and injected intravenously through the tail vein with 0.1 M phosphate-buffered saline (PBS) (control; $n = 8$) or 4 mg/mL of polyI:C (P9582, Sigma-Aldrich; $n = 7$) in PBS at a volume of 1 mL/kg body weight. To confirm successful MIA, saphenous vein blood samples were collected 2 h after treatment injections. Plasma was used for interleukin-6 (IL-6) measurement using rat IL-6 Quantikine ELISA (R&D Systems, MN, USA). PolyI:C treated dams had significantly increased IL-6 levels (624.7 ± 57.0 pg/mL) compared to saline-injected dams (68.4 ± 57.0 pg/mL) [$F_{(1,8)} = 47.646$, $p < 0.001$]. There was no effect of gestational timing [$F_{(1,8)} = 0.218$, $p > 0.05$] or interaction between gestational timing and treatment [$F_{(1,8)} = 0.211$, $p > 0.05$] on maternal plasma IL-6 levels (data not shown).

Offspring were weaned on PND 21, separated into same-sex cages in pairs, and euthanized by isoflurane anesthesia and decapitation between PND 63–91 ($n = 6$–8 for each subgroup). At time of euthanasia, there was no effect of treatment on weight between control (350 ± 6 g) and polyI:C offspring (341 ± 7 g) [$F_{(1,50)} = 0.975$, $p > 0.05$]. There was a significant effect of sex on weight where female offspring (264 ± 6 g) weighed less than males (426 ± 7 g) [$F_{(1,50)} = 302.0$, $p < 0.05$]. There were no other

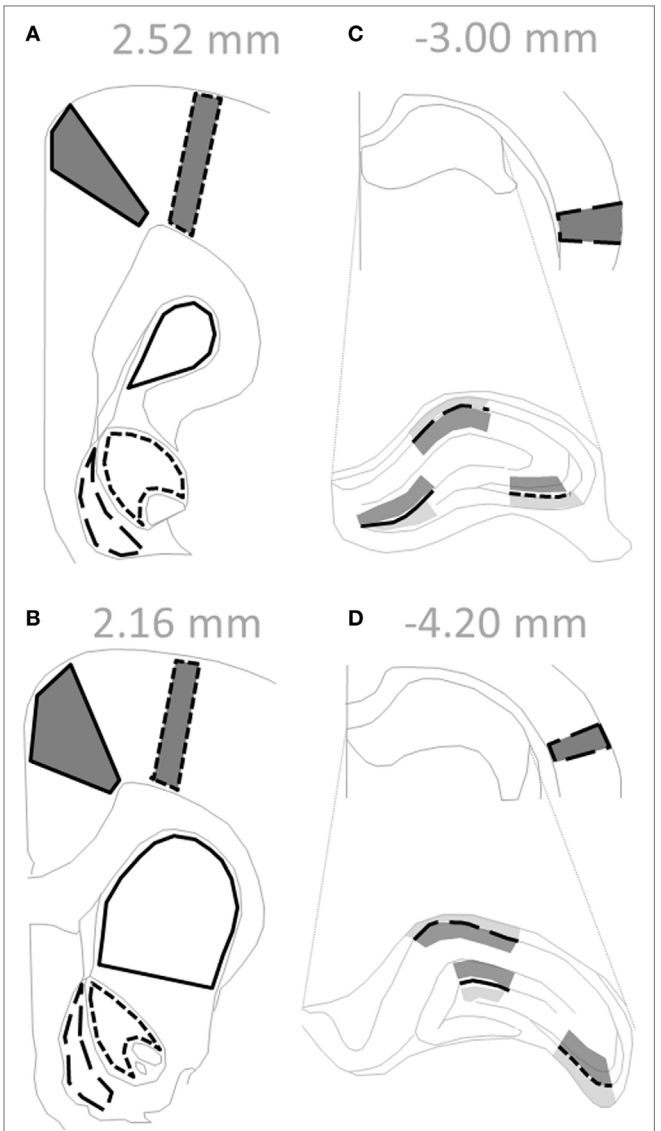

FIGURE 1 | Regions within coronal rat sections used for autoradiographic binding and *in situ* hybridization. **(A–D)** Solid gray polygons outlined in black depict cortical subregions quantified: cingulate [**(A,B)** solid line], motor [**(A,B)** short dashed line], and auditory [**(C,D)** long dashed line]. **(C,D)** Hippocampus is enlarged to delineate subregions, where black lines depict: dentate gyrus (solid line), cornu ammonis field 3 (short dashed line), and cornu ammonis field 1 (long dashed line). Shaded regions indicate levels quantified for binding data: afferent (dark gray; molecular layer, stratum radiatum) and efferent (light gray; polymorph layer, stratum oriens). **(A,B)** Empty black outlines depict striatal subregions quantified: dorsal striatum (solid line), nucleus accumbens core (short dashed line), and nucleus accumbens shell (long dashed line). Text in gray indicates Bregma level of section. Adapted from Paxinos and Watson sixth edition Rat Atlas (41).

interaction effects [$F_{(1,50)} < 1.50$, $p > 0.05$; data not shown]. Whole brains were snap-frozen in isopentane at −40°C and stored at −80°C. Coronal sections (14 µm; **Figure 1**) were prepared using a cryostat (Leica, Wetzlar, Germany) and mounted onto gelatin-coated glass slides.

In Situ Radioligand Binding

Receptors were identified using autoradiography and tritiated specific ligands. Following the autoradiographic assays (**Table 1**), slides were dipped in ice-cold distilled H_2O, dried in a stream of cold air, and exposed to BioMax MR (Kodak, Rochester, NY, USA) autoradiographic film for 75 ([^{3}H]MK-801), 76 ([^{3}H] CGP39653), or 69 ([^{3}H]Ifenprodil) days with tritium standards (American Radiolabeled Chemicals, St. Louis, MO, USA). Films were developed using standard procedures.

Quantification of Autoradiographic Images

Developed films were digitized and calibrated using the NIH imaging software (v1.56[1]) to produce nCi/mg tissue equivalent values based on the standard Rodbard curve obtained from the ^{3}H standards (American Radiolabeled Chemicals, St. Louis, MO, USA). Optical density values were quantitated using ImageJ (v1.48[2]). Non-specific binding was at background levels for all ligands examined; therefore, total binding values were averaged from between two and four consecutive sections for each measure. Cortical [cingulate (Cg), motor (M1), auditory (Aud)], hippocampal [dentate gyrus (DG), cornu ammonis area 3 (CA3), cornu ammonis area 1 (CA1)], and striatal [dorsal striatum (DS), nucleus accumbens core (AC), nucleus accumbens shell (AS)] regions were quantified (**Figure 1**). For hippocampus, afferent and efferent (neuropil) regions were quantified for binding, while the soma layer was quantified for mRNA. All brain regions were identified as per Paxinos and Watson sixth edition Rat Atlas (41).

In Situ Hybridization

Sections from control and MIA male offspring were investigated for related mRNAs. Riboprobes (**Table 2**) were generated with ^{35}S-UTP (PerkinElmer) using an *in vitro* transcription kit (Promega, Madison, WI, USA). *In situ* hybridization was performed as previously described (44), using 5 ng/mL radiolabeled riboprobes in hybridization buffer, and ^{35}S-UTP labeled sense riboprobes as a negative control. Slides were exposed to BioMax MR (Kodak, Rochester, NY, USA) autoradiographic film (details in **Table 2**) alongside a ^{14}C standard slide (American Radiolabeled Chemicals, St. Louis, MO, USA). Quantification of mRNAs was completed as mentioned above (see Quantification of Autoradiographic Images) with the standard Rodbard curve from the ^{14}C standard slide.

Statistical Analysis

Analysis was performed with IBM SPSS statistics (v23). Graphs were plotted using GraphPad Prism (v6). Data for each measure in each region (cortex, hippocampus, striatum) were analyzed using repeated measures two-way analysis of variance (RM-ANOVA) separately. Within-subject factors were the three subregions. Between-subject factors were prenatal treatment (polyI:C or vehicle), offspring sex (male or female), and gestational timing (GD10 or GD19) for the binding, and prenatal and gestational timing for the mRNAs (in male offspring only). Fisher's least significant differences were used for pairwise comparisons by treatment

[1] http://rsb.info.nih.gov/nih-image.
[2] https://imagej.nih.gov/ij/.

TABLE 1 | Summary of autoradiographic binding methods.

Radioligand (target)	Preincubation	Specific binding conditions	Non-specific binding condition	Wash
[^{3}H]MK-801 (42) (*N*-methyl-D-aspartate receptor channel)		2.5 h at RT 30 mM HEPES buffer with 100 µM glycine, 100 µM glutamate, 1 mM EDTA, and 20 nM [^{3}H]MK-801 (s.a. 17.1 Ci/mmol, PerkinElmer, USA), pH 7.5	20 µM non-tritiated MK-801 (Sigma)	2 × 20 min at 4°C 30 mM HEPES buffer with 1 mM EDTA, pH 7.5
[^{3}H]CGP39653 (43) (NR2A subunit)	45 min at RT 50 mM Tris–HCl, pH 8	45 min at RT 50 mM Tris–HCl with 20 nM [^{3}H]CGP39653, pH 8	1 mM L-glutamic acid	30 s at 4°C 50 mM Tris–HCl, pH 8
[^{3}H]Ifenprodil (NRB subunit)		3 h at 4°C 50 mM Tris–HCl buffer with 3 µM R(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride, 30 µM GBR-12909, 100 µM 20 nM [^{3}H] Ifenprodil trifluoperazine, pH 7.4	10 µM non-tritiated Ifenprodil	3 × 5 min at 4°C 50 mM Tris–HCl buffer, pH 7.4

TABLE 2 | *In situ* hybridization riboprobe details.

Target gene	Bp region from origin (0)	LOCUS ID	Autoradiographic exposure time on film (days)	Specific activity (cpm/µg) of riboprobe	
				Sense (+)	Antisense (–)
GRIN1 (NR1)	1,840–2,081	NM_008169.3	10	1.69×10^9	1.42×10^9
GRIN2A (NR2A)	3,218–3,418	NM_000833.2	21	1.61×10^9	1.87×10^9

when ANOVA analyses were significant. The Greenhouse Geisser correction was used if Mauchley's sphericity test was violated for within-subjects interaction effects. Overall treatment effects or effects of sex, region, or GD of exposure are presented graphically only when a significant effect or interaction was identified. In all cases, data are expressed as the mean ± SEM and $p \leq 0.05$ was deemed statistically significant. NR2A:NR2B ratio for hippocampus was calculated by division of [^{3}H]CGP39653 binding values (NR2A subunit) with [^{3}H]Ifenprodil binding values (NR2B subunit) in each subregion and level for each animal.

RESULTS

Effect of Maternal PolyI:C Treatment and Timing on Ligand Binding in Adult Male and Female Offspring

Representative autoradiographs of binding assays are shown in **Figure 2** and show the expected pattern of NMDAR channel, NR2A subunit, and NR2B subunit (**Figures 2A–C**, respectively) binding and distribution in rodent brain (45–47). Briefly, signals are evenly distributed across the cortex and striatum. White matter tracts (i.e., corpus callosum) are not labeled and hippocampal architecture including neuropil areas surrounding ammon's horns region are labeled. Overall, autoradiographic signals were less in cortex and striatum (a,b) in comparison to hippocampus (c,d).

[^{3}H]MK-801 Binding (NMDAR Channel)

Although there was no overall effect of polyI:C on NMDAR channel binding ([^{3}H]MK-801 binding) in the cortex [Treatment effect: $F_{(1,45)} = 1.86$, $p > 0.10$], polyI:C offspring had ~13% more NMDAR channel expression than control offspring in Cg [Treatment × Subregion effect: $F_{(2,90)} = 3.27$, $p < 0.05$; Control versus polyI:C in cingulate: $F_{(1,45)} = 5.34$, $p < 0.05$; **Figures 2A** and **3A**]. PolyI:C did not affect NMDAR channel binding in the M1 or Aud (**Figure 3A**). PolyI:C did not affect NMDAR channel binding in the hippocampus [Treatment effect: $F_{(1,45)} = 1.13$, $p > 0.10$; **Figure 2A**; **Table 3**]. There was no effect of offspring sex or GD of exposure on NMDAR channel binding in either the cortex or the hippocampus [both, $F_{(1,42)} < 2.65$, $p > 0.10$].

In the striatum, polyI:C offspring had ~20% more NMDAR channel binding than control offspring [Treatment effect: $F_{(1,45)} = 17.63$, $p < 0.001$; **Table 3**; **Figure 2A**]. In addition, male polyI:C offspring had ~34% more NMDAR channel binding than male control offspring [Treatment × Sex effect: $F_{(1,45)} = 6.68$, $p < 0.05$; Pairwise contrast: $F_{(1,45)} = 22.68$, $p < 0.001$; **Figure 3B**]. No change was observed in polyI:C-exposed compared to control females [$F_{(1,45)} = 1.322$, $p > 0.05$; **Figure 3B**]. There was no effect of GD of exposure on NMDAR channel binding in the striatum [$F_{(1,45)} = 3.473$, $p < 0.10$].

[^{3}H]CGP39653 Binding (NR2A Subunit)

In total hippocampus, polyI:C offspring had ~30% more NR2A binding than control offspring [Treatment effect: $F_{(1,44)} = 6.22$, $p < 0.05$; **Table 3**; **Figure 2B**], the magnitude of this increase was moderate to strong (31–44% increases) in all subregions [Treatment × Subregion × Level effect: $F_{(1.7,45)} = 3.68$, $p < 0.05$; Pairwise contrasts: $F_{(1,44)} > 5.41$, $p < 0.05$; **Figure 4A**] except the molecular layer of the DG [Pairwise contrast: $F_{(1,44)} = 2.74$, $p > 0.10$; **Figure 4A**]. There was no effect of gestation day of exposure on NR2A binding in the hippocampus, cortex, or striatum [$F_{(1,45-47)} < 0.178$, $p > 0.10$].

Overall, polyI:C offspring had ~57% more NR2A binding than control offspring in the cortex [Treatment effect: $F_{(1,45)} = 11.004$, $p < 0.01$; **Table 3**; **Figure 2B**]. In addition, we detected a sex by treatment interaction effect, where male polyI:C offspring had

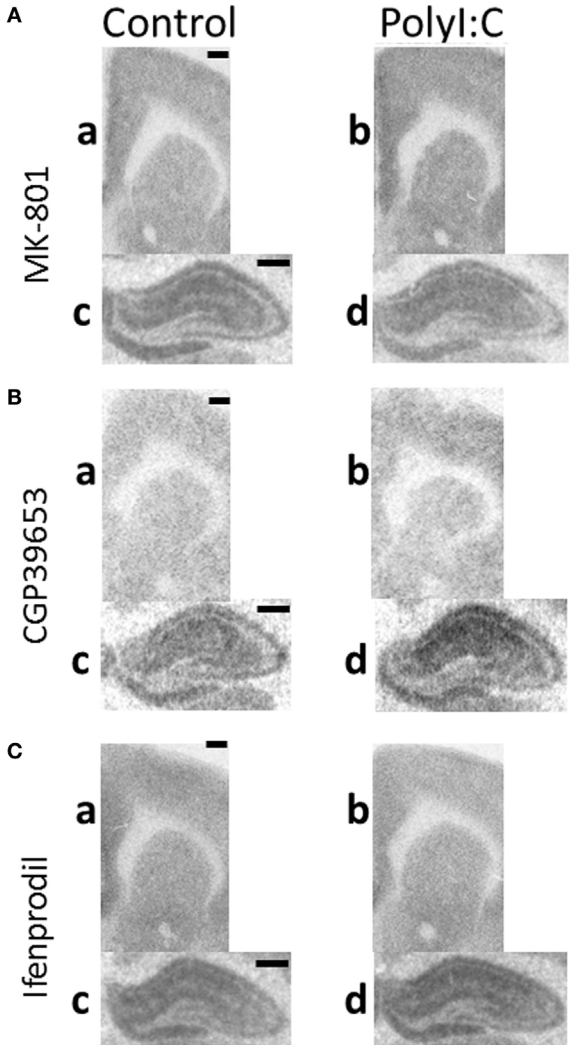

FIGURE 2 | Representative autoradiographs of regions from maternal immune activation offspring. Coronal sections from adult (postnatal day 63–91) male offspring from vehicle (control: a,c) or polyI:C (b,d) treated dams were processed with tritiated radioligands to detect **(A)** *N*-methyl-D-aspartate receptor (NMDAR) channel (MK-801), **(B)** NR2A (CGP39653), and **(C)** NR2B (Ifenprodil) binding in (a,b: ~2.50 mm bregma) cortical, striatal, and (c,d: ~−3.10 mm bregma) hippocampal regions. **(A)** PolyI:C offspring showed significantly increased NMDAR channel binding in (a,b) cingulate and striatum, but not (c,d) hippocampal regions. **(B)** PolyI:C offspring showed significant increases in NR2A binding overall in all quantified regions. **(C)** PolyI:C offspring showed no significant change in NR2B binding overall in any quantified region. Scale bars represent 500 µm.

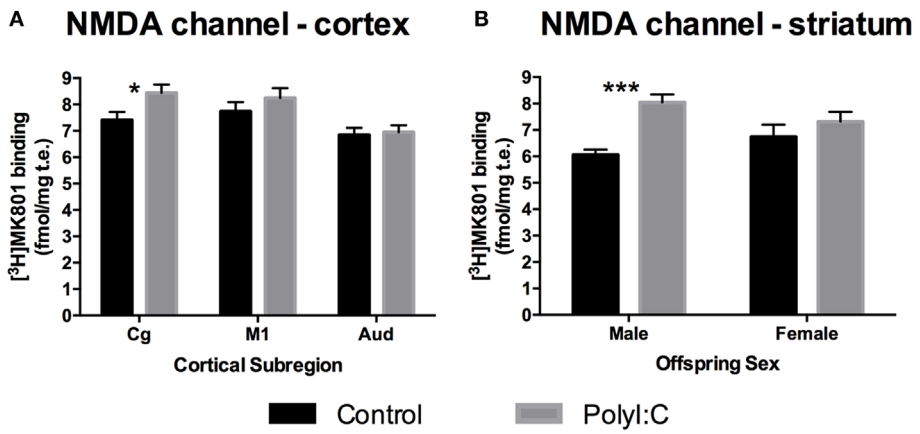

FIGURE 3 | *N*-methyl-d-aspartate receptor (NMDAR) channel binding alterations in cortex and striatum from adult offspring exposed to vehicle (control; black) or polyI:C (gray) during early (GD10) or late (GD19) gestation. Tritiated MK-801 binding was used to quantify NMDAR channel levels. Significantly increased NMDAR binding was found in the cingulate subregion of polyI:C offspring compared to controls **(A)**. Male, but not female, polyI:C offspring specifically exhibited significantly increased NMDAR channel binding in the striatum **(B)**. Bars represent mean ± SEM (n = 15–28 rats per group). Cg, cingulate cortex; M1, motor cortex; Aud, auditory cortex (*$p < 0.05$, ***$p < 0.001$).

TABLE 3 | Overall effect of gestational polyI:C exposure on NMDA receptor channel and subunit binding.

Binding (fmol/mg tissue equivalent)		Mean ± SEM (*n*)		
		Control	**Maternal immune activation**	**Treatment effect**
N-methyl-D-aspartate receptor channel [^{3}H]MK-801	Cortex	7.33 ± 0.29 (30)	7.87 ± 0.28 (23)	$F_{(1,45)} = 1.86$, $p > 0.10$
	Hippocampus	8.74 ± 0.3 (30)	9.15 ± 0.27 (23)	$F_{(1,45)} = 1.13$, $p > 0.10$
	Striatum	**6.5 ± 0.23 (29)**	**7.62 ± 0.27 (25)*** **	$F_{(1,45)} = 17.63$, $p < 0.001$
NR2A [^{3}H]CGP39653	Cortex	**2.69 ± 0.31 (28)**	**4.24 ± 0.36 (25)** **	$F_{(1,45)} = 11.004$, $p < 0.01$
	Hippocampus	**4.05 ± 0.28 (28)**	**5.3 ± 0.42 (25)***	$F_{(1,44)} = 6.22$, $p < 0.05$
	Striatum	**1.69 ± 0.14 (26)**	**2.3 ± 0.22 (25)***	$F_{(1,47)} = 6.62$, $p < 0.05$
NR2B [^{3}H]Ifenprodil	Cortex	11.03 ± 0.36 (31)	10.77 ± 0.32 (27)	$F_{(1,50)} = 0.24$, $p > 0.10$
	Hippocampus	13.81 ± 0.38 (31)	13.58 ± 0.35 (25)	$F_{(1,48)} = 0.11$, $p > 0.10$
	Striatum	13.26 ± 0.6 (30)	13.14 ± 0.5 (24)	$F_{(1,47)} = 0.08$, $p > 0.10$

*$p < 0.05$
**$p < 0.01$
***$p < 0.001$

~132% more NR2A binding than male control offspring in cortical tissue [Treatment × Sex effect: $F_{(1,45)} = 4.794$, $p < 0.05$; Pairwise contrast: $F_{(1,42)} = 24.61$, $p < 0.001$; **Figure 4B**], an effect that was not observed for females [$F_{(1,45)} = 0.483$, $p > 0.10$; **Figure 4B**].

Overall, polyI:C offspring had ~43% more NR2A binding than control offspring in the striatum [Treatment effect: $F_{(1,47)} = 6.62$, $p < 0.05$; **Table 3**; **Figure 2B**]. Similar to the cortex, polyI:C-exposed male offspring had ~86% more NR2A binding than male control offspring [Treatment × Sex effect: $F_{(1,47)} = 5.38$, $p < 0.05$; Pairwise comparison: $F_{(1,47)} = 11.79$, $p < 0.01$; **Figure 4C**], an effect which was not observed in females [$F_{(1,45)} = 0.03$, $p > 0.10$; **Figure 4C**].

[^{3}H]Ifenprodil Binding (NR2B Subunit)

There was a consistent lack of change in [^{3}H]Ifenprodil expression across all regions in polyI:C offspring compared to control offspring (**Table 3**; **Figure 2C**). There was no effect of treatment, or any interaction of treatment with offspring sex, GD of exposure, or anatomical region in the cortex [all $F_{(1,50)}$ and $F_{(2,100)} < 0.50$, $p > 0.10$; **Table 3**; **Figure 2C**], hippocampus [all $F_{(1,48)} < 0.40$, $F_{(1.4,62.8)} < 1.50$, $F_{(2,96)} < 2.21$, $p > 0.10$; **Table 3**; **Figure 2C**], or striatum [all $F_{(1,47)}$ and $F_{(2,94)} < 0.50$, $p > 0.05$; **Table 3**; **Figure 2C**] on NR2B binding.

NR2A:NR2B Ratio in Hippocampus

PolyI:C offspring had ~31% increased NR2A:NR2B ratio compared to control offspring [Treatment effect: $F_{(1,46)} = 5.40$, $p < 0.05$; **Figure 5A**]. In addition, compared to control offspring, polyI:C offspring had ~25% higher NR2A:NR2B ratio in hippocampal afferent levels [$F_{(1,46)} = 4.35$, $p < 0.05$] and ~38% higher NR2A:NR2B ratio in hippocampal efferent levels [$F_{(1,46)} = 6.37$, $p < 0.05$] than control offspring [Treatment × Level effect: $F_{(1,46)} = 5.09$, $p < 0.05$; **Figure 5B**].

Effect of Maternal PolyI:C Treatment on NR1 and NR2A mRNA in Adult Male Offspring

Representative *in situ* autoradiographs are shown in **Figure 6** and the expected pattern of NR1 mRNA (GRIN1, **Figure 6A**)

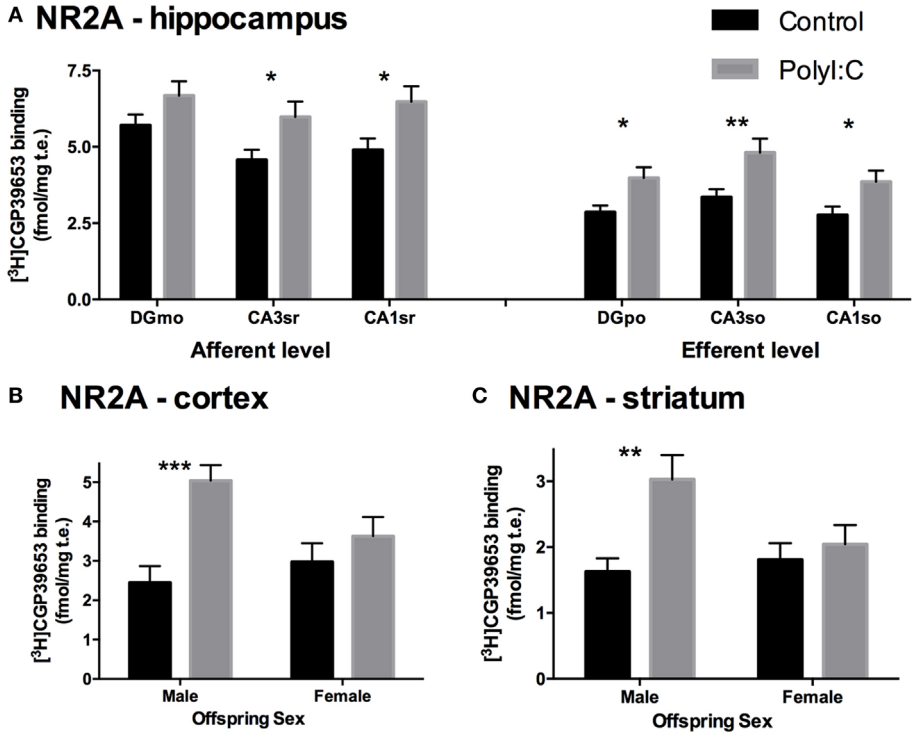

FIGURE 4 | NR2A subunit binding alterations in hippocampus, cortex, and striatum from adult offspring exposed to vehicle (control; black) or polyI:C (gray) during early (GD10) or late (GD19) gestation. Tritiated CGP39653 was used to quantify NR2A subunit levels. PolyI:C offspring had significantly increased NR2A binding in all hippocampal subregional levels, except the DGmo **(A)**. Male, but not female, rats exposed to polyI:C during gestation had increased NR2A binding in the cortex **(B)** and striatum **(C)**. Bars represent mean ± SEM (n = 15–28 rats per group). DGmo, molecular layer of dentate gyrus; CA3sr, stratum radiatum of cornu ammonis 3; CA1sr, stratum radiatum of cornu ammonis 1; DGpo, polymorph layer of dentate gyrus; CA3so, stratum oriens of cornu ammonis 3; CA1so, stratum oriens of cornu ammonis 1 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

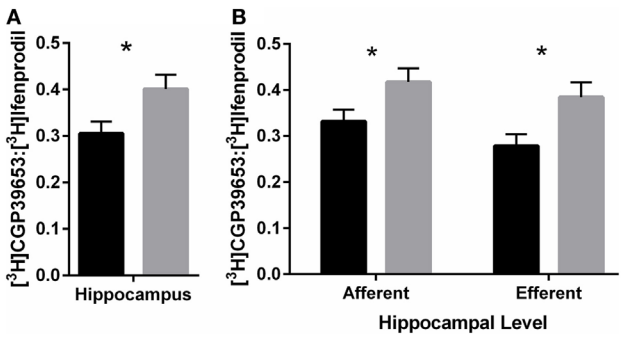

FIGURE 5 | NR2A:NR2B binding ratio in hippocampus of adult offspring exposed to vehicle (control; black) or polyI:C (gray) during early (GD10) or late (GD19) gestation. Tritiated CGP39653 and Ifenprodil were used to calculate the ratio of NR2A:NR2B subunit binding. **(A)** The ratio of NR2A:NR2B ratio binding in the hippocampus was significantly increased in polyI:C offspring. **(B)** This increased ratio was elevated at both afferent and efferent levels of each hippocampal subregion. Bars represent mean ± SEM (n = 25–28 rats per group) (*$p < 0.05$).

and NR2A mRNA (GRIN2A, **Figure 6B**) distribution was identified in rodent brain (37). Briefly, signals were evenly distributed across the cortex and striatum (a,b). In the hippocampus, the granular layer of the DG and the pyramidal neuronal layers within ammons-horn (CA3–CA1) were darkly labeled for both NMDAR1 and NMDAR2A mRNAs (c,d), whereas the hilar region of CA4 had intermediate levels of both mRNAs. NR2A mRNA (**Figure 6B**) was less in the striatum relative to the cortex (a,b). White matter tracts were clearly identifiable (corpus callosum and anterior commissure) with no mRNA signal. Overall, all autoradiographic signals were less intense and more homogenous in cortex and striatum (a,b) in comparison to the hippocampus (c,d).

Maternal immune activation did not affect NR1 mRNA levels in cortical tissue [all Treatment effects/interactions: $F_{(1,22)} < 2.88$, $p > 0.05$; **Figures 6A** and **7A**]. Similarly, no treatment main effects or interactions were observed for NR1 mRNA in hippocampus [all $F_{(1,24)} < 2.64$, all $F_{(1.4,24)} < 1.0$, $p > 0.05$; **Figure 7A**]. In the striatum, polyI:C offspring had a small, but statistically significant, increase (~11%) in NR1 mRNA in the AS compared to controls [Treatment × Subregion: $F_{(2,44)} = 6.08$, $p < 0.01$; Pairwise contrast: $F_{(1,22)} = 8.11$, $p < 0.01$; **Figures 6B** and **7B**].

In contrast to the overall lack of large changes in NR1 mRNA, polyI:C offspring had widespread increases in NR2A mRNA. Compared to controls, polyI:C offspring had ~25% more NR2A mRNA in cortex [Treatment effect: $F_{(1,24)} = 4.20$, $p < 0.05$] and ~22% more NR2A mRNA in hippocampus [Treatment effect: $F_{(1,24)} = 9.02$, $p < 0.01$; **Figures 6B** and **7C**]. In addition, polyI:C offspring had ~18% more NR2A mRNA in DG [$F_{(1,24)} = 4.53$, $p < 0.05$], and ~29% more NR2A mRNA in CA1 [$F_{(1,49)} = 16.61$,

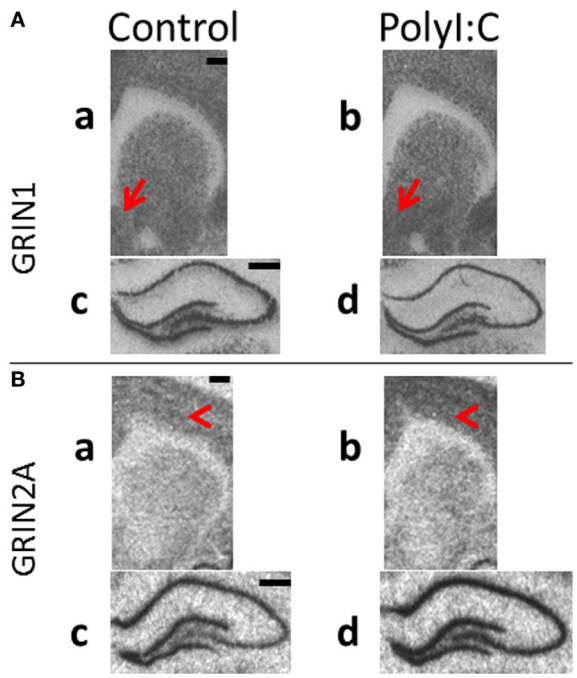

FIGURE 6 | Representative *in situ* hybridization films of *N*-methyl-D-aspartate receptor subunit gene expression in brain regions from male polyI:C offspring. Coronal sections from adult (postnatal day 63–91) male offspring from vehicle (control: a,c) or polyI:C (b,d) treated dams were processed to detect **(A)** GRIN1 (NR1) and **(B)** GRIN2A (NR2A) mRNA in (a,b: ~2.30 mm bregma) cortical, striatal and (c,d: ~−3.10 mm bregma) hippocampal regions. **(A)** PolyI:C offspring showed significant increases in NR1 mRNA in (a,b) nucleus accumbens shell (red arrows), with no significant changes in cortex or (c,d) hippocampus. **(B)** PolyI:C offspring showed (a,b) no significant change in NR2A mRNA in striatum, but significantly elevated NR2A mRNA in cortex (red arrowheads) and (c,d) hippocampal regions. Scale bars represent 500 µm.

$p < 0.01$; Subregion × Treatment effect: $F_{(2,48)} = 3.28$, $p < 0.05$; **Figures 6B** and **7D**]. The increase of NR2A mRNA in CA3 of polyI:C offspring did not reach significance [$F_{(1,24)} = 3.32$, $p < 0.10$] (**Figure 7D**). In striatal tissue, there was no significant treatment or between-groups interaction effect [$F_{(1,23)} < 3.15$, $p > 0.05$; **Figures 6B** and **7C**].

DISCUSSION

We found NMDAR-related molecular alterations in multiple brain regions from polyI:C offspring that potentially could contribute to early and late MIA behavioral changes previously reported in this model. However, contrary to our expectations, we did not find any interaction effects between the impact of MIA and GD. Additionally, while we would have expected decreased NMDAR binding overall, we found *increased* NMDAR channel binding, specifically in the Cg of polyI:C offspring. We also found novel and widespread increases in NR2A binding supported by significant increases in NR2A mRNA, along with no changes in NR2B binding, in polyI:C offspring. As hypothesized, we found more exaggerated molecular alterations in male polyI:C offspring, compared to female polyI:C offspring. Thus, we found that MIA elicited coordinated NMDAR alterations that were primarily in male offspring, in line with the exaggerated behavioral change found in males after MIA in our previous investigation (30).

Our most robust finding was increased NR2A binding in the brain of adult male polyI:C offspring. NR2A-rich NMDARs have relatively shorter channel opening duration (38), and faster decay kinetics (37, 48). We did not detect corresponding altered NMDAR channel binding in the hippocampus of polyI:C offspring, suggesting that more NMDARs would be of the NR1–NR2A containing type but may not be dramatically changed in overall numbers. This concurs with our finding of increased NR2A mRNA, and no change in NR1 mRNA, in several brain regions. The lack of interaction between treatment and gestational timing indicates that hippocampal NMDARs are potentially vulnerable to MIA at either gestational stage and suggests that increases in NMDAR2A could be a common brain response to developmental activation of the immune system. Previous studies report increased NR2A mRNA in the hippocampus of adult rats (7–8 weeks of age) exposed postnatally (at 2 weeks of age) to polyI:C (49) or the bacterial mimic LPS (50), suggesting that this NMDAR2A response to immune activation can even occur if the inflammation occurred after birth. This demonstrates that the mechanisms that govern NR2A expression in adulthood are potentially vulnerable to long-lasting effects of inflammation, regardless of the precise timing of inflammatory stress. In contrast to our lack of change in NR1 mRNA in the hippocampus, previous studies have reported ~20% reduced NR1 mRNA in the hippocampus of polyI:C mouse offspring exposed to late MIA, but not early MIA (13). Our data in rats suggest that polyI:C treatment during gestation may not affect NR1 gene expression to the same extent or as consistently as it does NR2A gene expression in the hippocampus. These variable findings could be due to subtle differences in the timing of vulnerable neurodevelopmental epochs, or species differences.

Our results show that the CA1 subregion exhibited increased NR2A mRNA and increased NR2A binding and NR2A:NR2B ratio in the apical dendritic region (afferent) in polyI:C offspring, regardless of gestational timing of MIA and offspring sex. This suggests that synaptic input coming from CA3, which is critically dependant on NMDAR function for learning and memory (Schaffer collaterals), is significantly changed after developmental immune activation. Indeed, increases in NR2A:NR2B ratio reduces both long-term potentiation and long-term depression in the CA3-to-CA1 circuitry [see Ref. (38) for details], limiting plasticity and making it more difficult to learn and remember. Our present findings therefore suggest that polyI:C offspring have associated molecular alterations in CA1 that perhaps would produce longer term memory retrieval deficits, consistent with long-term memory deficits found in adult polyI:C rat offspring (26).

We find localized increases in NMDAR binding in the Cg of polyI:C offspring, suggesting that MIA can elicit long-lasting changes in NMDAR expression within cortical regions of adult offspring, regardless of sex or gestational timing of MIA. Although systemic MK-801 did not alter locomotion in polyI:C offspring in our previous studies (30), other studies have reported

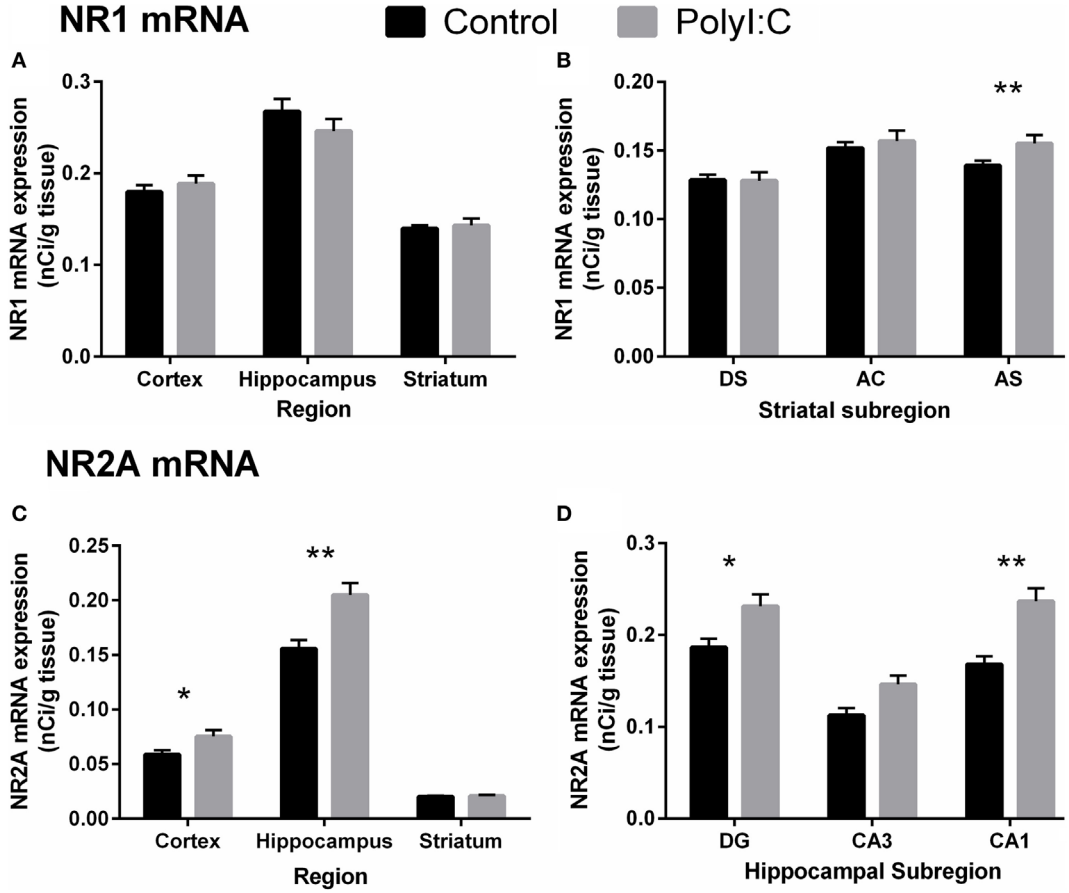

FIGURE 7 | *N*-methyl-D-aspartate receptor-related mRNA alterations in cortex, hippocampus, and striatum from male adult offspring exposed to vehicle (control; black) or polyI:C (gray) during early (GD10) or late (GD19) gestation. *In situ* hybridization was used to quantify NR1 mRNA (GRIN1; **A,B**) and NR2A mRNA (GRIN2A; **C,D**). Male polyI:C offspring had **(A)** no change in NR1 mRNA overall in any quantified region, but **(C)** significantly increased NR2A mRNA in cortex and hippocampus. **(B)** Male polyI:C offspring had significantly elevated NR1 mRNA in, the striatal subregion, AS. **(D)** PolyI:C offspring had significantly increased NR2A mRNA, in the hippocampal subregions, DG, and CA1. Bars represent mean ± SEM (n = 25–28 rats per group). DG, dentate gyrus; CA3, cornu ammonis 3; CA1, cornu ammonis 1; DS, dorsal striatum; AC, nucleus accumbens core; AS, nucleus accumbens shell (*$p < 0.05$, **$p < 0.01$).

both increased (18) and decreased (19–21) locomotion in polyI:C offspring in response to systemic MK-801. The mechanism by which MK-801 elicits behavioral changes, and the contribution of other neurotransmitter systems, is unclear (51–54). Although the increased NMDARs in the cingulate may not be sufficient to elicit consistent MK-801 locomotion alterations in all cohorts, it may impact cognition dependent on Cg. Indeed, previous studies report increased glutamatergic processes (55) and NMDAR channel binding in anterior (42) and posterior (56) Cg of people with schizophrenia.

We found some interesting changes within the striatal brain areas examined. Our current investigation of exaggerated molecular changes in striatal NMDAR of males appears to align with our previous behavioral investigation that showed that male, but not female, polyI:C rat offspring exhibit sensorimotor gating deficits (30). Our current molecular data shows that male, but not female, polyI:C offspring had increased NMDAR channel binding in the striatum, and elevated NR2A binding in both the cortex and striatum. Given that these molecular and behavioral alterations were exaggerated in male polyI:C offspring, one interpretation would be that these cortical and striatal changes contribute to the sensorimotor gating deficits that male polyI:C offspring exhibit at adulthood. However, further research is needed to elucidate how MIA-induced changes in NR2A-rich NMDARs are modified by sex and how NMDARs may contribute to sensorimotor gating function. In the present study, the coordinated increase in striatal NR2A and NMDAR channel binding in male polyI:C offspring indicates they potentially have increased NR2A-rich NMDARs in the striatum. In normal rodents, the "indirect" striatopallidal pathway is primarily mediated by NR1–NR2A-rich NMDARs, while the direct "striatonigral–entopeduncular" pathway is mediated by NR1–NR2B rich NMDARs (57–59). The changes in NMDARs in the MIA model could suggest more activity in the indirect striatopallidal pathway, which is also known to be enriched in D2 receptors. However, how the striatal changes found here map onto these distinct striatal pathways needs to be determined. PolyI:C offspring show increased dopamine turnover and reduced D2-like

receptor levels in the striatum (60), supporting that the indirect striatal pathway may have more pathology in response to MIA, especially in male offspring. However, our previous investigation found no change in D2 receptor mRNA in the striatum of polyI:C offspring (30). This suggests that the NMDAR changes may precede or be independent of D2 receptor changes. Previously, we found increased D1 dopamine receptor mRNA in the nucleus accumbens of male GD10 polyI:C offspring (30). In this study, we found that male polyI:C offspring had increased NR1 mRNA in the nucleus accumbens shell only, regardless of gestational timing but with overall increases in striatal NMDAR channel binding. This suggests male polyI:C offspring may have elevated NMDAR synthesis and activity in the striatum, particularly in the ventral portion. Taken together, the results on striatal abnormalities suggest dopaminergic and glutamatergic alterations found in early and/or late MIA rat offspring may interact and perhaps be rescued by pharmacotherapies that target either dopamine [as evidenced in MIA rat offspring treated with antipsychotics (18, 27)] or NMDAR, particularly NMDAR2A.

The primary finding of this study is that immune challenge *in utero* irrespective of timing of the challenge has an effect on glutamatergic signaling in adult offspring, an effect that seems to be largely restricted, or more extreme, in male offspring. In terms of the validity of the MIA model of schizophrenia, the sex differences are consistent with epidemiological and clinical evidence of a small increased incidence of schizophrenia in males and a more chronic course of the disorder in males (61). The long-term changes in NMDAR, particularly the consistent, anatomically widespread and quite robust increase in the NMDAR2A subunit may have deleterious effects on synaptic plasticity, learning and memory. We suggest that even transient developmental immune activation mediates neural and possibly behavioral changes, through adult changes in the synthesis and function of NMDAR. The clear and robust increases in NMDAR2A, whose gene has been recently linked to the increased risk of schizophrenia (62), suggest a possible point of convergence between genetic and environmental risk (maternal infection) factors in schizophrenia. Our study that identifies increased NMDAR2A after MIA suggests that future efforts could focus on determining when this NMDAR2A change occurs developmentally and could test if and when reversing the NMDAR2A increase could restore normal behavior.

ETHICS STATEMENT

Experiments were performed in accordance with the National Health and Medical Research Council's *Australian code for the care and use of animals for scientific purposes*. The current study was approved by the University of Newcastle's Animal Care and Ethics Committee (Approval number A-2009-108).

AVAILABILITY OF DATA AND MATERIAL

The data analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

KZ, US, JT, DH, and PM conceived and designed the experiments. CW assisted in the design of the experiments. LH and CM undertook the PolyI:C treatments and provided the rat brain tissue. KZ undertook the binding experiments, GRIN1 in situ experiment, contributed to the analysis of the hippocampus data, and provided a first draft based on hippocampus data only. TR undertook the GRIN2A experiment and analyzed the data. TR, TP-T, and CW wrote the final draft. TP-T and LH checked the data and edited the manuscript. All authors contributed to the interpretation and approval of the final manuscript.

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Hydrogen Sulfide Affects Radical Formation in the Hippocampus of LPS Treated Rats and the Effect of Antipsychotics on Hydrogen Sulfide Forming Enzymes in Human Cell Lines

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Objectives: Psychiatric disorders, such as schizophrenia and other neuroinflammatory diseases are accompanied by an increase in the oxidative stress and changes in the immune system and in the metabolic, hormonal and neurological components of the central nervous system (CNS). Hydrogen sulfide (H_2S) is a gaseous molecule that is endogenously produced in the peripheral and central nervous system through cysteine by the following major H_2S producing enzymes in the brain: cystathionine-γlyase (CSE), cystathionine ß-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST). The physiological effects of H_2S are broad, with antioxidative properties being a major role in the body. The aims of our investigation were to analyze the central nervous antioxidant, metabolic and neuronal effects in the hippocampus of the rat after inflammatory peripheral lipopolysaccharide (LPS) treatment; and to examine the effects of antipsychotics on the expression of these enzymes in human cell lines.

Material and Methods: Male Lewis rats (250 g) received an i.p. LPS injection (1 mg/kg) 24 h before microdialysis experiments. Conscious rats were infused via these probes (1.5 μl/min) with a radical scavenger 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) in Krebs-Ringer solution. Sodiumhydrogensulfide (NaHS, 10 μg/min) was infused after a 2-h baseline for 1 h. Corticosterone, glutamate, glucose and lactate were measured by Elisa. Reactive oxygen species (ROS) were detected by electron spin resonance spectroscopy (ESR). The impact of the antipsychotics haloperidol, clozapine, olanzapine and risperidone on the expression of genes encoding the key enzymes of H_2S synthesis was studied at the human neuroblastoma SH-SY5Y and monocytic U-937 cell lines. The cells were incubated for 24 h with 30 μM antipsychotic following which mRNA levels were measured by polymerase chain reaction.

Results: Microdialysate glucose and lactate levels dramatically increased in the hippocampus of LPS untreated rats by local application of NaHS. By contrast, in the LPS pretreated rats, there was no effect of NaHS infusion on glucose but a further significant increase in microdialysate lactate was found. It was LPS pretreatment alone that particularly enhanced lactate levels. There was a marked increase in hippocampal microdialysate glutamate levels after local NaHS infusion in LPS untreated animals. In LPS treated rats, no change was observed by NaHS, but LPS itself had the strongest effect on microdialysate glutamate levels. Microdialysate corticosterone levels were reduced by NaHS in both LPS pretreated and untreated rats. The formation of free radicals in the hippocampus significantly reduced in LPS pretreated rats, while in LPS untreated rats a significant increase was observed after NaHS infusion. In human SH-SY5Y and U-937 cells, all three major enzymes of H_2S-Synthesis, namely cystathionine-γ-lyase, cystathione ß-synthase and 3-mercaptopyruvate sulfurtransferase, could be detected by PCR. The antipsychotics haloperidol, clozapine, olanzapine and risperidone affected all three enzymes in different ways; with haloperidol and risperidone showing major effects that led to reductions in CBS or CSE expression.

Discussion: The local application of NaHS in the hippocampus of the rat strongly affected glucose, lactate and glutamate release. Contrastingly, in LPS pretreated rats, a decreased radical formation was the only effect found. H_2S synthetizing enzymes may be involved in antipsychotic mechanisms, although no clear common mechanism could be found.

Keywords: schizophrenia, electron spin resonance spectroscopy (ESR), reactive oxygen species (ROS), hydrogensulfide (H_2S), lipopolysaccharide (LPS)

INTRODUCTION

Psychiatric disorders, such as schizophrenia and other neuroinflammatory diseases are accompanied by an increased oxidative stress, changes in the immune system and in metabolic, hormonal and neurological components of the central nervous system (1–22). More and more evidence suggests various dysregulations of the hypothalamus-pituitary-adrenal (HPA)-Axis in the course of numerous mental disorders, such as affective disorders (23–25), and schizophrenia (18–21).

The response to antipsychotics in schizophrenia shows a high variability. There are several patient cases that are resistant to clozapine, which is called "ultra-resistance to treatments in schizophrenia" (UTRS). Peripheral inflammations are associated with UTRS (26).

Hydrogen sulfide (H_2S) is a gaseous molecule that is endogenously and enzymatically produced in the peripheral and central nervous system by three major H_2S producing enzymes: cystathionine-γ-lyase (CSE) (27), cystathionine ß-synthase (CBS) (28) and 3-mercaptopyruvate sulfurtransferase (MPST) (29, 30); besides other mechanisms (31). High endogenous concentrations of H_2S were found in the hippocampus and cerebellum which are parts of the human brain where CBS seems to be the most important enzyme for the synthesis (32, 33).

H_2S is known to regulate a multitude of physiological and pathophysiological functions in the vascular, immune and nervous system [for review see (34, 35)]. There is evidence for a role of H_2S in neurodegeneration (36), but also radical scavenging effects regarding nitric oxide (37) or glutamate mediated oxidative stress (38). Other authors report a role for H_2S in Alzheimer's disease (39) or Parkinson's disease (40).

Promising studies have demonstrated the therapeutic effect of H_2S donation in various disease models of cancer, inflammation or neuroinflammation (41, 42). Additionally, the pharmacological inhibition of H_2S production (43–47) or the genetic deficiency of H_2S producing enzymes (48–50) results in beneficial effects.

Xiong et al. could show that the H_2S levels of patients with schizophrenia are significantly low. H_2S is a regulator for the N-methyl-D-aspartate receptor (NMDAR) function and low H_2S levels can cause a hypofunction of NMDAR. Given that a hypofunction of the NMDAR receptor is related to the pathogenesis of this disorder, H_2S levels in the liquor are also likely to have an effect on the pathophysiology of this disorder (51).

The aims of our investigation were to analyze antioxidant, metabolic, hormonal and neuronal changes of NaHS application in the hippocampus in naive and LPS pretreated rats. Furthermore, we aim to show the effects of different antipsychotics on the expression of several enzymes related with the H_2S synthesis in a neuronal and an immunological human cell lines. Especially the role of exogenous H_2S as a possible

therapeutic mediator in inflammatory and neurodegenerative diseases will be analyzed.

MATERIALS AND METHODS

Animals

Animal experiments were carried out at the Department of Surgical Research, University Hospital, Freiburg, Germany. All procedures were performed in accordance with the German animal protection law, FELASA, the national animal welfare body, GV-SOLAS and the NIH guide for the care and use of laboratory animals; and were approved by the animal welfare committee of the University of Freiburg (AZ: G-10/44). Male Lewis rats (250–350 g; Charles River, Germany) were housed at a temperature of 21° C in plastic cages with lights turned on from 06:00 to 19:00 h and with free access to food and water. Experiments were conducted during the light phase after at least 1 week of adaptation.

In vivo Microdialysis Experimental Design

CMA/12 microdialysis probes were implanted under isoflurane anesthesia (3%) using stereotaxic coordinates according to (52) A: +5.2 mm; L: +2.0 mm; V: −4.4 mm from Cortex top (53). The microdialysis experiments started after awakening using the CMA freely moving system in groups of 8 animals. Microdialysis samples were collected every 30 min for a period of 4 h at a constant flow rate of 1.5 μL/min (Krebs-Ringer). Microdialysis samples were collected and stored at −20°C until analysis. Electron spin resonance (ESR) measurements were performed immediately. The same effluents were used to measure Corticosterone (Enzyme immunoassay, IBL, Hamburg, Germany), Glucose, Lactate and Glutamate (Colorimetric Assays, BioCat, Heidelberg, Germany).

To detect central nervous protective effects of hydrogen sulfide, a series of rats received an intraperitoneal injection of 1 mg/kg body weight Lipopolysaccharide from *E. coli* (LPS; Sigma Aldrich Chemicals, Steinheim, Germany). The injections were made 24 h before microdialysis probes were implanted. NaHS was infused intrahippocampal via the microdialysis cannula at a flowrate and dose of 10 μg/min 2 h after beginning of sampling. ROS, Glutamate, Corticosterone, Lactate and Glucose-Uptake (difference between glucose inflow concentration and outflow concentration) were measured.

Histology

At the end of the experiment, one group of animals was euthanized with CO_2. To verify probe placement, the brain was removed and stored at −20°C. Subsequently, serial coronal brain sections (thickness: 20 μm) were cut on a freezing microtome at −16°C and sections were stained with a 0.5% cresyl violet solution.

Chemicals

Krebs-Ringer's solution was obtained from Delta-Select, Pfullingen, Germany; LPS (Lipopolysaccharides from Escherichia coli, Serotype 0127:B8 purified by trichloroacetic acid extraction) from Sigma, Steinheim, Germany; 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) from Noxygen, Elzach, Germany; Isofluorane from Abbott, Wiesbaden, Germany; Primers were obtained from MWG Biotech, Ebersberg, Germany.

ROS Measurements

Hippocampal detection of reactive oxygen species is based on the reaction of CMH and ROS as reactant in dialysates. CMH solution (1 mg/ml Krebs-Ringer) was prepared fresh every day. The spin probe CMH was applied by infusion via a microdialysis cannula at a flow rate of 1.5 μl/min. The oxidation of spin probe CMH by reactive oxygen species generates stable 3-methoxycarbonyl-proxyl radicals (CM) as shown by Dikalov et al. (54, 55). Autoxidation was found to be in the range of 1–2%. The amount of CM. radical is equivalent to the formation of reactive oxygen species *in vivo*. The amount of reacted ROS was determined from the ESR amplitude according to a calibration curve using standard CM. solutions. ESR measurements were performed at room temperature using an EMX ESR spectrometer (MiniScope MS 200, Magnettech, Berlin, Germany). The ESR had the following settings: center field $g = 2.001$, sweep wide 60 G, sweep time 5 ms over 10 scans, modulation amplitude 2.4 G, microwave power 20 mW. The total spin probe concentration was measured to determine the concentration of free radicals (56).

Cells and Culture Conditions

U-937 cells were maintained from the Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco/BRL and Seromed, Berlin, Germany), 0.5% glutamine, and 1% gentamycine at 37°C in a 5% CO_2 atmosphere. For the studies, cells were plated at a concentration of 225,000 cells in 3 ml medium per well into six-well culture plates (Greiner, Frickenhausen, Germany).

Cells were preincubated for 24 h and then treated with the antipsychotics, dissolved in ethanol at a concentration of 30 μM. These were added to the plates at a quantity of 300 μl to each well. The same procedure was performed with the ethanol controls without antipsychotics. Antipsychotics were obtained from Sigma, Deisenhofen, Germany.

Neuroblastoma SH-SY5Y cells were cultured in heat-inactivated Roswell Park Memorial Institute medium (RPMI) (Gibco/BRL, Eggenstein, Germany) supplemented with 15% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1% penicillin-streptomycin and 1% glutamine in a 5% CO2 atmosphere. For further studies, cells were plated at a number of 225.000 cells/dish in 10 mm culture dishes. Antipsychotics and ethanol-control treatments were performed at a density of 450.000 cells/dish. The antipsychotics were dissolved in ethanol and were further diluted in culture medium. The cells were exposed to antipsychotics at 30 μM for 24 h at 37°C.

RNA Extraction

After incubation, the cells were collected from the culture dishes and total RNA was extracted by the use of Trizol® reagent in accordance with the manufacturer's instructions. The amount

of extracted total RNA was quantified by established optical methods at A260/A280 (Genequant II, Pharmacia Biotech, Freiburg, Germany) and structural integrity checked by agarose-gel electrophoresis [1.5% agarose (Gibco/BRL)].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to analyse the transcription of the GRs and the house-keeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 1 mg of cellular total RNA was reverse-transcribed with 40 U of Superscript II (Gibco/BRL) and 1 mg oligo-(dT) in a volume of 20 ml following the manufacturer's protocol.

The following primer pairs were used to amplify the cDNA's:

CBS: Sense 5′-CGATGGGTACCATATGCAGAAAAGACG CCTCCTCACAAGG-3′, Antisense 5′-CGGTACCTCGAGTTA CTACTGTGATTCCACTTGGAGGGTGTGCTGCC-3′. CSE: Sense 5′-GGCCTGAAGTGTGAGCTCTT-3′, Antisense 5′-TTG GGGATTTCGTTCTTCAG-3′. MPST: Sense 5′-GACCCC GCCTTCATCAAG-3′, Antisense 5′-CATGTACCACTCCAC CCA-3′. GAPDH: Sense 5′-CGTCTTCACCACCATGGAGA-3′, Antisense 5′-CGGCCATCACGCCACAGTTT-3′.

Aliquots of 1 ml cDNA were amplified with a PCR cycler (Biometra Trio, Göttingen, Germany) for the enzymes and GAPDH using the primers described above with the following cycling program: denaturation for 45 s at 95°C, annealing for 60 s at 59°C, and extension for 60 s at 72°C. PCR products were analyzed for all enzymes after amplification with 28 cycles and gel electrophoresis in 1.5% agarose gels. Semi-quantitative determination was achieved by digitization of gels with a Polaroid video system (Rothaar & Schroeder, Heidelberg, Germany) and further densitometric evaluation achieved with the Gelscan 4.0 Professional Program (LTF/BioSciTec, Landau/Frankfurt, Germany).

Quantification of mRNA by RT-PCR and Densitometry

Different approaches were used to minimize variations and to ensure the reliability of the quantification procedure: first, the purity of mRNA probes was determined by measuring the optical density at A260/A280. This revealed a ratio of 1.56 ± 0.05 for all extractable probes. Second, integrity and amounts of mRNA measured were checked by gel electrophoresis. Third, relevant impurities of DNA were routinely excluded by PCR-amplification of extracted mRNA probes after omission of the RT reaction. The reliability of the further steps of quantification (PCR reaction, gel densitometry and evaluation of results) including non-saturating conditions of the PCR was determined using different amounts of RT products. This yielded a near-linear dose-product relationship in the gel electrophoresis.

Statistical Analyses

The statistical analysis of the microdialysis data was performed using Microsoft Excel 8.0 and SPSS version 9.0 (SPSS Inc., Chicago, USA). The given data comprises mean ± standard deviation (SD). For microdialysis experiments significance was assessed by unpaired Student *t*-test, one-tailed (since we had a clear hypothesis from the previous *in-vitro* studies). An α-level of $p < 0.05$ was considered significant (57). Data are presented in percent of the mean of the respective control samples and are shown as mean ± SD values. The results from at least three independent different experiments were pooled and analyzed by Mann-Whitney Rank Sum tests. A $p < 0.05$ (*) was accepted as a statistically significant difference. For statistical evaluations, Sigmastat (Jandel Scientific, Kerpenich, Germany) was applied (58).

RESULTS

The local intrahippocampal application of NaHS via microdialysis cannula (10 μg/min. at 1.5 ml/min. for 1 h) affected different neuronal parameters. In LPS untreated rats, NaHS infusion exerted massive increases in both microdialysis glucose and lactate release in the hippocampus. These effects were not seen in LPS treated rats (1 mg/kg i.p. 24 h before microdialysis experiments), **Figure 1**.

Microdialysis monitoring of Glutamate and Corticosterone showed a highly significant increase in Glutamate and a diminished formation of Corticosterone after 10 μg/min NaHS compared to the previous control periods. In the LPS treated rats no further increase in Glutamate could be observed. The decreasing effect of NaHS on Corticosterone was also observed in LPS treated animals (**Figure 2**).

ROS are also affected significantly by NaHS (**Figure 3**). The infusion of NaHS, 10 μg/min, led to a significant increase in free radical formation in LPS-untreated animals. In contrary, in LPS treated animals we saw a free radical decrease of NaHS.

Our present study demonstrated effects of typical (haloperidol) as well as atypical antipsychotics (clozapine, olanzapine, risperidone) on the main H_2S synthesizing enzymes CBS, CBE, and MPST in SH-SY5Y cells, and U-937 cells (**Figure 4**). In essence, all measured significant effects of the used antipsychotics in the employed concentration were reductions in the expression of both enzymes CBS and CSE in the neuronal cell line. Haloperidol significantly reduced the expression of CBS and CSE in SH-SY5Y cells, but the effect of haloperidol was not significant in U-937 cells. In U-937 cells the antipsychotics olanzapine and risperidone reduced the m-RNA of CSE significantly. The expression of MPST was only affected in U-937 cells and only by olanzapine. Clozapine, the most important antipsychotic did not show any effect in both cell lines.

DISCUSSION

Our data indicate that H_2S affects parameters involved in the pathophysiology of inflammatory psychiatric illnesses, such as depression and schizophrenia. Especially in normal Lewis rats, extracellular glucose, lactate release and glutamate release are highly increased in the hippocampus after local application of NaHS via microdialysis cannula. Hippocampal corticosterone levels are found to be significantly reduced under these conditions. At this concentration, an increase in free radical formation could also be observed, whereas after

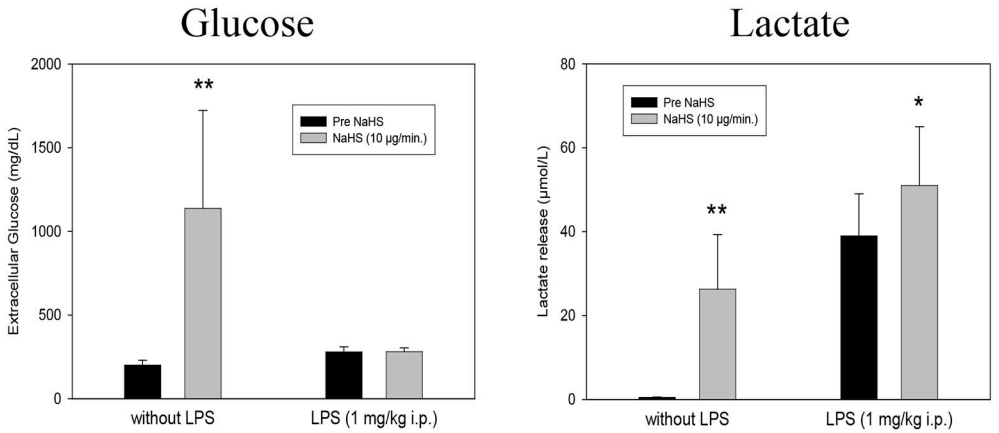

FIGURE 1 | Effect of intrahippocampal NaHS infusion (10 µg/min) via CMA/12 micordialysis cannula on Glucose uptake (**Left**) and Lactate release (**Right**) with and without LPS treatment (1 mg/kg i.p., 24 h before microdialysis, LPS) as compared to control. Means ± SD, $n = 8$, $^{*}p < 0.05$; $^{**}p < 0.01$, Student *t*-test.

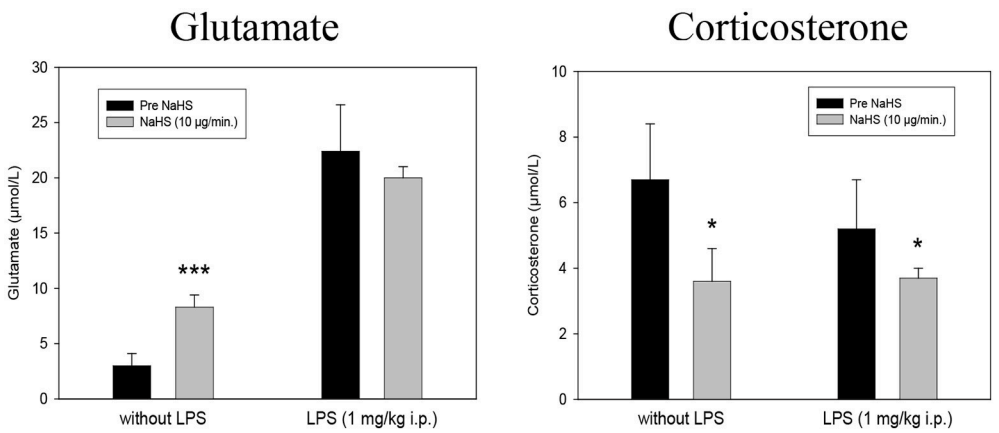

FIGURE 2 | Effect of intrahippocampal NaHS infusion (10 µg/min) via CMA/12 micordialysis cannula on Glutamate release (**Left**) and Corticosterone release (**Right**) with and without LPS treatment (1 mg/kg i.p., 24 h before microdialysis, LPS) as compared to control. Means ± SD, $n = 8$, $^{*}p < 0.05$; $^{***}p < 0.001$, Student *t*-test.

LPS pretreatment of animals a significant reduction could be observed. Under the conditions of LPS pretreatment the effects of NaHS on glutamate, corticosterone, glucose, and lactate release were missing. Antipsychotics seem to have individual effects on the expression of the major H_2S-synthetizing enzymes with haloperidol exerting mostly reducing effects, while clozapine was ineffective in both SH-SY5Y and U-937 cell lines.

Hayden et al. (59) have also shown elevated blood glucose levels after exposure with H_2S. Pichette and Gagnon summarize the literature on the effects of H_2S and glucose regulation, indicating that mechanisms besides insulin regulation, such as glucagon-like-peptide (GLP)-1 or peptide YY are involved (60). Our data confirm findings of Lin et al. (61) that exogenous H_2S protects cells by its glucose reducing activity.

The increase in lactate by NaHS-Application may be supported by the observation that in fish exposed to H_2S, an increase in whole blood lactate could be observed (62). Without LPS pretreatment, our results suggest that the local application of NaHS leads to a general metabolic activation of neurons and glial cells in the hippocampus.

Our findings of the significant release of glutamate in the hippocampus of the rat induced by local NaHS application might be due to postsynaptic effects of H_2S on the membrane potential, and thus excitability of CNS neurons, as it has been reported in dorsal raphe (63), paraventricular nucleus (64), subfornical organs (65) and dorsal root ganglion neurons (66). The effects in the dorsal raphe have been suggested to be the result of direct modulatory actions of H_2S on calcium dependent potassium channels (63).

Significant alterations in the HPA axis activity occur in course of the schizophrenia, regarding basal cortisol secretion, probably in response to a decrease in the amount of glucocorticoid receptors (25, 67, 68). Walder et al. (69) revealed a significant positive correlation between salivary cortisol concentrations and symptoms severity. A study of Walker et al. (70) revealed that increased cortisol levels in patients who developed psychosis.

Therefore, the hypothesis that HPA axis distortion is closely bound with symptoms and pathophysiology of the schizophrenia is becoming increasingly recognized. Antipsychotic medication leads to the reduction of cortisol concentrations in patients as well as in healthy controls (71, 72). Flores et al. (73) suggested that this might be responsible for their effectivity (17). Wang et al. (74) showed that inhibitors of the H_2S producing enzymes, such as CBS or CSE, or the application of small interfering RNAs lead to mitochondrial oxidative stress and dysfunction, resulting in an even blunted corticosterone response to ACTH in adrenal glands. These effects were significantly attenuated by the treatment of H_2S donor GYY4137. As shown by Navarra et al. (75), NaHS application is associated with the inhibition of the stimulated release of corticotropin-releasing hormone from rat hypothalamic explants.

In our experiments we found a significant reduction on free radical production in the hippocampus induced by the local NaHS application in the LPS pretreated rats. These findings support the role of H_2S as an important antioxidative anti-inflammatory mediator. H_2S was found to increase the antioxidative properties of cells in different ways (76). It decreases lipid peroxidation induced by homocysteine (77). H_2S is able to directly react with reactive oxygen but its endogenous concentrations are too low to act as an important endogenous antioxidant. On the other hand H_2S has a cytoprotective effect in brain cells by elevating the reduced glutathione (GSH) production via activation of cystine/cysteine transporters and redistribution of GSH to mitochondria (38, 78, 79). On the molecular level, several mechanisms seem to be involved in the antioxidant and neuroprotective role of H_2S, such as the nuclear factor (NF)-κB pathway (80). H_2S significantly reduced levels of malondialdehyde and 4-hydroxynonenal and elevated levels of superoxide dismutase and reduced glutathione in the hippocampus of streptozotocin (STZ)-induced diabetic rats (81). They also found that H_2S alleviated depressive-like behaviors of STZ-induced diabetic rats in the forced swimming and tail suspension tests and reduced their anxiety-like behaviors in the elevated plus maze test. The results provide evidence for antidepressant-like and anxiolytic-like effects of H_2S in STZ-induced diabetic rats and suggest that the therapeutic effects may result from inhibition of hippocampal oxidative stress (81). In HT22 neuronal cells, Kimura et al. (82) observed a cytoprotective role of H_2S by activating ATP-dependent K+ (KATP) and Cl- channels, in addition to increasing the levels of glutathione. In LPS-treated mice the findings of (48) indicated that a deficiency in MPST does not significantly affect endotoxemia but a deficiency in CBS or CSE slightly ameliorates the outcome of LPS-induced endotoxemia *in vivo*.

In summary our data support Huang and Moore's (83) notion, that H_2S is not only a toxic agent but also a gasotransmitter with growing therapeutic potential. Here we could demonstrate that it might be a double sided sword and that the body condition is important for the effects of H_2S.

We here observed that the neuroleptics haloperidol, clozapine, olanzapine and risperidone have different effects on human SH-SY5Y and U-937 cell lines. Several drugs or even vitamins, such as

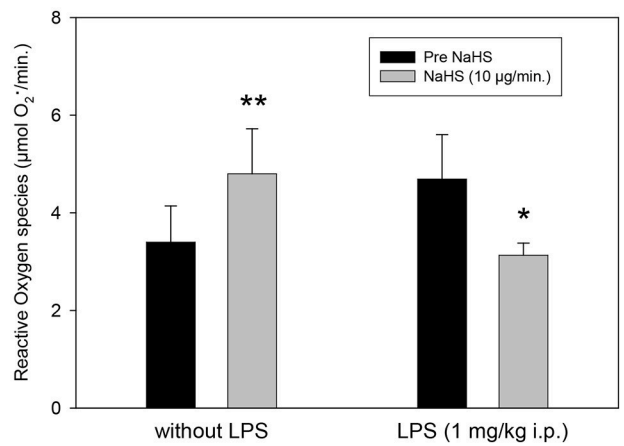

FIGURE 3 | Effect of intrahippocampal NaHS infusion (10 µg/min) via CMA/12 micordialysis cannula on ROS production (as measured by CM.) with and without LPS treatment (1 mg/kg i.p., 24 h before microdialysis, LPS) as compared to control. Means ± SD, $n = 8$, $^*p < 0.05$; $^{**}p < 0.01$, Student *t*-test.

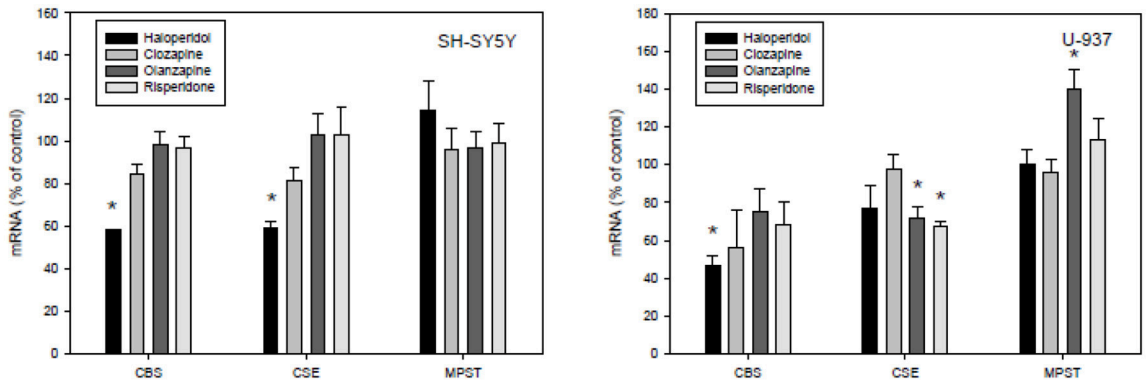

FIGURE 4 | Effects of antipsychotic treatment on cystathione-y-lyase (CSE), cystathione ß-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3MST) in human SH-SY5Y and U-937 cell lines. Means ± SD, $n = 8$, $^*p < 0.05$; Mann-Whitney Rank Sum test.

vitamin D_3 (84) are able to change the concentrations of H_2S and these effects seem to be tissue dependent (35). The mechanisms through which these effects occur are still unclear. The regulation of gene expression seems to be different in respect to tissue and cell type since antipsychotics, such as haloperidol and quetiapine seem to reduce genes encoding antioxidant enzyme expression (58), the reduction of H_2S-forming enzymes, CBS and CSE, especially by haloperidol in neuronal cell line. It cannot be excluded that the inhibition of CSE in U-937 cells by olanzapine and risperidone is combined not only with beneficial effects of these drugs but also with shared side effects. Furthermore, Fond et al. (26) recently found that ultra-resistance to treatment in schizophrenia (URTS) is independently associated with peripheral low-grade inflammation in schizophrenia patients (26).

H_2S does not seem to be a classical neurotransmitter as specific receptors are not reported until now. Changes in cytokine production, such as IL-1 or TNF-a in the LPS model are reported (85). Although several mechanisms report that H_2S can exert its physiological effects, such as sulfhydration or hemeprotein interactions [for review see (31)], other mechanisms, such as the influence on receptor heterocomplexes that are important in the pathophysiology of schizophrenia cannot be excluded from consideration (86, 87).

Schizophrenic Patients have olfactory impairments as was shown by Turetsky et al. (88) and show higher depolarization responses after stimulation with H_2S indicating a special role for H_2S in schizophrenia. Nevertheless, it is still unclear whether there is avoidance or an even beneficial effect (88).

Taken together our data confirm that hydrogen sulfide affects mechanisms involved in the pathophysiology of neuroinflammatory diseases, such as schizophrenia and depression and antipsychotic treatment might alter H_2S-related mechanisms. We could show that H_2S has effects on several brain metabolites and hormones, and our data confirm the idea that H_2S can have toxic and protective properties depending on the state the body is in, here demonstrated for the case of inflammation. Nevertheless, the collected results of the study have not adequately clarified the therapeutic benefits H_2S has for neurodegenerative diseases in brain, and the conditions and concentrations under which the beneficial properties H_2S exceed the toxicity of this gasotransmitter. Further beneficial effects of H_2S and related compounds might be studied in animal models of schizophrenia.

AUTHOR CONTRIBUTIONS

All the authors meet the following criteria: made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and drafted the work or revised it critically for important intellectual content; and approved the final the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. In addition OS performed the animal experiments and AS performed the cell culture experiments.

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CASE REPORT
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Schizophrenia or Atypical Lupus Erythematosus with Predominant Psychiatric Manifestations over 25 Years: Case Analysis and Review

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We observed a case over 25 years of relapsing–remitting schizophrenic spectrum disorder, varying regarding the main symptomatology between more depressive or more schizoaffective or rather typical schizophrenic syndrome. Diseased phases were repeatedly accompanied by minor skin lesions, which were initially classified as mixed tissue disorder. Psychotic phases were waxing–waning over years. During one later relapse, skin involvement was severe, classified to likely represent an allergic reaction to psychopharmaca; this generalized exanthema remitted rapidly with cortisone treatment and azathioprine. Under continued azathioprine and low dose neuroleptics, the patient remitted completely, appearing psychiatrically healthy for 16 years. When azathioprine was set off due to pregnancy, an extraordinary severe relapse of schizophrenia like psychosis accompanied by most severe skin lesions developed within a few weeks, then requiring 2 years of psychiatric inpatient treatment. Finally, a diagnosis of systemic lupus erythematodes plus neuropsychiatric lupus was made. A single CSF sample in 2013 showed suspicious biomarkers, matching with CSF cytokine profiling in schizophrenic and affective spectrum disorder patients and indicated mild neuroinflammation. Complex immune suppressive treatment was reinitiated short after relapse, but was only partially successful. However, surprisingly the psychosis and skin lesions remitted (in parallel) when belimumab was given (add-on). The very details of this complicated, long-term disease course are discussed also with regard to general ideas, in particular with respect to the question if this case of seemingly comorbid schizophrenia with minor autoimmunity signs represented a case of one emerging autoimmune disorder with variant manifestations systemically and within the CNS, though atypically with predominant appearance as a schizophrenia spectrum disorder.

Keywords: mild encephalitis, neuroinflammation, chronic schizophrenia, autoimmune encephalitis, autoimmune diseases, neuropsychiatric lupus erythematosus, lupus erythematosus, systemic

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INTRODUCTION

Schizophrenia is understood as a partly heritable brain disease, recent interesting findings showing alleles of the complement component C4 genes play a role in both in the CNS and the immune system (1). Autoimmune diseases and severe infections are associated with a later increased risk of schizophrenia and other psychiatric disorders (2, 3). The relative risk of schizophrenia for an

individual with a history of autoimmune disease in themselves or in their family is elevated by about 45%. Inversely schizophrenia is associated with a nearly 50% elevated lifetime prevalence of autoimmune diseases (4).

We observed a case of a female patient with a 25-year psychiatric history, her disease beginning at the age of 24. As the medical records show, the diagnosis was changing several times, from depression (bipolar disorder hypothesized) to schizoaffective to schizophrenic disorder, this was the predominating diagnosis over disease cause. Besides psychiatric process, the disease course in this patient was atypical, as her psychotic phases were often accompanied by appearance of minor non-specific skin lesions on the trunk and limbs. It has been suggested that there might be an autoimmune process; however, she did not fulfill any criteria for a defined autoimmune disorder. During one particular psychotic relapse she developed severe skin efflorescences, which were treated with cortisone and later azathioprine. Under this regimen skin improved rapidly and surprisingly after this particular inpatient treatment, the patient remained psychiatrically well with few exceptions over many years. Sixteen years later, this patient became pregnant and azathioprine had been set off because of possible teratogenic side effects. Soon, after discontinuing azathioprine, she rapidly relapsed and for the first time the criteria of an established autoimmune disorder, systemic lupus erythematosus (SLE), were fulfilled and could be even extended to neuropsychiatric lupus erythematosus (NPSLE).

The question discussed in this single case, is whether the patient suffered from the two unrelated disorders of schizophrenia and SLE, or an atypical course of a not clearly defined autoimmune disorder with early predominant psychiatric and late neuropsychiatric manifestations, with minor systemic manifestations and late fulfilling criteria of SLE/NPSLE.

BACKGROUND

In previous CSF studies, we accumulated evidence of minor neuroinflammation and immune activation in large subgroups of both schizophrenic and affective disorders (5). These findings were recently confirmed by others (6). In addition we recently found high CSF cytokines, especially at IL-8 in each patient (7), also increased CSF neopterin (8). All these findings supported the recently updated mild encephalitis (ME) hypothesis (9). Also epidemiological studies are well compatible with ME hypothesis, in that infections and autoimmune disorders are additive risk factors for a spectrum of severe psychiatric disorders (3). Discrete features of neuroinflammation are seen in a variety of CNS disorders, including degenerative diseases like Alzheimer's disease, where neuroinflammation seems to represent a disease escalating factor (10). Most interesting is that in single case studies of acute psychosis, despite normal magnetic resonance imaging scans of the brain and normal CSF and without detection of CNS autoantibodies, there was nevertheless proof of mild neuroinflammation in the cortex biopsy (11). These findings point to the difficulties in detecting mild inflammatory processes and show the limitations of available diagnostic methods including CSF diagnostics (12).

The study was approved by the ethics committee of the University of Ulm (the patient gave written informed consent to publication) was to gain a better insight into possible relationships between a seemingly primary psychiatric disorder and a poorly defined autoimmune process. By using a careful retrospective analysis of an unusual case with a long-standing disease course and considerable available clinical material, the possibility of a unifying diagnosis over the disease course, against the established assumption of two separate disorders (schizophrenia and autoimmune disorder) was tested.

FAMILY HISTORY

Within the framework of the patient's sixth hospitalization, the suspicious family history for both psychiatric and autoimmune disorders became apparent: both parents and the maternal grandmother suffered from a longtime depression with several psychiatric hospitalizations; the grandmother committed suicide. The patient's only sibling has a schizophrenic disorder as well as Crohn's disease.

RETROSPECTIVE CASE ANALYSIS OF 25 YEARS

In 1989, a 24-year-old woman suffering from severe depression (ICD-10 F32.3) with predominant loss of interest, hypersomnia, feelings of guilt, and worthlessness and diminished ability to concentrate was hospitalized in our clinic for the first time. The physical findings were unremarkable except slight anemia, increased blood sedimentation rate and positive rheumatoid factor. Besides the psychiatric symptomatology no signs of autoimmunity were registered. The patient was treated successfully with a combination of pimozide, flupentixol, and amitriptyline and released after 3 months inpatient stay in good mental condition.

During 1990, now 25 years old, the patient again demonstrated emerging anxiety, thought disorder (derailment and thought blocking), disorganized speech and for the first time acoustic hallucinations (commenting voices) and initially catatonic stupor. In the required second psychiatric hospital stay she showed slight leukopenia and hypochromic microcytic anemia. One month after hospitalization some non-specific skin manifestations on her chest and back appeared. Internal and dermatological consultancies happened, tissue samples were made, but neither serological nor histopathological results could name the disorder. Now the differential diagnosis of collagenosis was considered and further blood testing was undertaken. For the first time, antinuclear antibodies (ANA) tested positive. Bone marrow examinations showing no hematological disorder, brain MRI exposed no cerebral pathology. There was no evidence of infection, electrolyte disturbances or metabolic derangements. Established criteria of a defined autoimmune disorder were not fulfilled and therefore no immune suppressive therapy was started. With a combination of bromperidol and clorazepate, she distanced from productive psychotic content. During the cause, more depressive symptoms developed, tranylcypromine were added. The patient was dismissed in acceptable mental status.

In 1993, a third severe relapse occurred requiring hospitalization again. Main symptoms were restlessness, anxiety and extensive productive psychotic symptoms. One month after hospitalization, additional symptoms of severe maculopapular exanthema on the trunk and limbs appeared. This was diagnosed as a Stevens–Johnson syndrome and thought to be caused by the psychopharmacological drugs, propyphenazone ergotamine or acetylsalicylic acid (her own headache medication). The patient was transferred to the internal medicine department, where high-dose cortisone (100 mg/day) was initiated, 11 days later the exanthema had remitted, and patient was transferred back to the psychiatric ward. Cortisone was gradually phased out until the patient was discharged from the hospital. A few months later, in outpatient treatment the patient attempted suicide during a psychotic episode and was readmitted to the hospital. Clinical examination now demonstrated speckled exanthema on her chest, which was rapidly progressing, then confluating and spreading over the limbs (see **Figure 1**). Blood testing showed high ANA Titer and for the first time autoantibodies against Ro/SSA and La/SSB (see **Table 1**). In October 1993, an atypical collagenosis was hypothesized. Under this assumption, prednisolone was administered for over 6 months. During this period, skin and psychosis improved, though some symptoms continued to fluctuate.

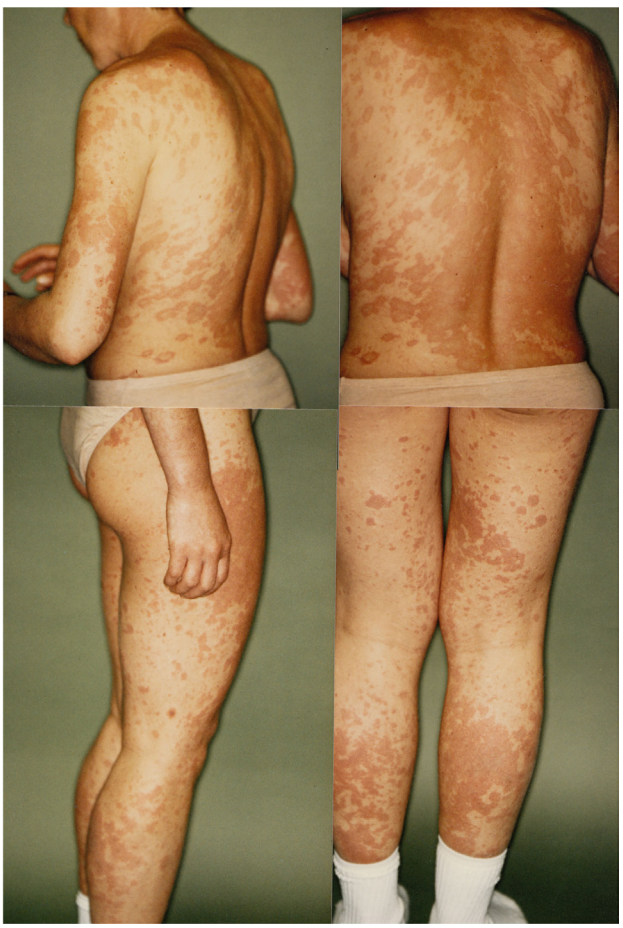

FIGURE 1 | Skin manifestations of the patient in April 1993, hypothesized as Stevens–Johnson Syndrome.

SURPRISING REMISSION UNDER AZATHIOPRINE TREATMENT FOR ALMOST 16 YEARS

In the time period between 1994 and 1996 no severe problems were stated, according to the patient's own evaluation "skin and mind came to rest." However, in June 1996 another relapse of psychosis occurred: she presented initially symptoms of thought disorder, delusional ideas, paranoia, depersonalization and later acoustic hallucinations. Thioridazine, fluphenazine, and diazepam were added. In the course, fluphenazine was reduced and because of emerging comorbid depressive symptoms and affective instability, amitriptyline and later lithium was added. Because of suspected drug-induced leukopenia, fluphenazine was replaced by olanzapine. Skin eruptions on the trunk followed psychiatric symptoms about 5 months later. Another therapy trial with prednisolone was initiated, with worsening of her skin and mental status, so that treatment on a protected psychiatric ward became necessary. CSF examination ruled out any brain infection, however, did show unspecific inflammation. A diagnosis of mixed connective tissue disorder was then made. Azathioprine (Imurek®) was prescribed, starting in January 1997 and continued for nearly 16 years. About 2 weeks after the start of azathioprine, the exanthema regressed and prednisolone was tapered out. As the psychiatric symptoms gradually improved, with a complex psychopharmacological treatment including lithium, olanzapine, amitriptyline, the patient was discharged from hospital. Azathioprine was reduced from 100 to 50 mg future years. Because of her good mental health, olanzapine and amitriptyline were gradually reduced but maintained until 2004. Lithium was phased out in subsequent 2 years. Under this treatment the patient remained stable with regard to mental status and skin status for nearly 16 years (except for one exception in 2004). After release she was in psychiatric and sporadic rheumatologic supervision. Interim, she married a second time. She chose early retirement but was able to work part-time in a fashion store. The ambulatory protocols in this time noted occasionally social anxieties, but predominant an "emotionally and psychologically stable" status.

In 2004, a brief hospitalization became necessary, because of restlessness and affective tension, probably as a consequence of life events (suicide attempt of her mother and sister and the sisters diagnosis schizophrenic disorder). During inpatient stay, acoustic hallucinations were noted and quetiapine was added next to amitriptyline, olanzapine, and azathioprine. She was released after 2 months in good condition and was mentally stable for another 8 years.

SEVERE RELAPSE AFTER DISCONTINUANCE OF AZATHIOPRINE

In 2012, because of pregnancy, the patient decided together with the gynecologist to stop azathioprine medication immediately,

TABLE 1 | Exemplary serological immunoinflammatory markers of the patient over last 25 years.

Laboratory marker	Reference range/ unit	Treatment	ST (no. 1)	AMB	ST (no. 2)	ST (no. 3)	ST (no. 4)	AMB	ST (no. 5)	ST (no. 6)	AMB		ST (no. 7)	ST (no. 8)	ST (no. 9)
		Period of time	14.04.–23.06.1989	01.01.2000	15.02.–11.07.1990	11.03.–27.07.1990	20.08.1993–30.03.1994	20.02.1995	11.06.1996–12.06.1997	19.07.–25.09.2004	30.09.2008–30.04.2009	01.02.201020.02.2011	29.05.2012–03.05.2013	29.05.–13.12.2013	03.01.–06.05.2014
Leukocytes	4.0–10.0 × 10^3/µL		Normal	Normal	3.3	3.4	NDA	4.9	3.88	4.9	NDA	NDA	2.8	2.1	2,8
Hemoglobin	12.0–16.0 g/dl		Normal	Normal	11.2	12.2	NDA	17.3	12.3	13.0	NDA	NDA	11.0	11.3	11.5
ESR	<10/20 mm		38/76	29/64	26/54	NDA	33/65	56/60	18/55	19/46	14/33	14/35	NDA	NDA	17/39
Alpha 2 macroglobulin	7.4–12.6 rel%		High	NDA	NDA	NDA	NDA	9.1	8.1	NDA	NDA	NDA	NDA	13.2	NDA
Gamma-globulin	8.0–15.8 rel%		25.4	NDA	NDA	NDA	NDA	24.4	19.5	NDA	NDA	NDA	NDA	10.8	NDA
ANA	<1:80 Titer		Negative	Negative	NDA	NDA	1:10,240	1:2,400	1:10,000	1:1,200	1:9,600	NDA	NDA	1:2,560	1:2,560
Anti-dsDNA	<10 or <100 U/ml		NDA	Negative	62	NDA	<40	<3	NDA	10	<2	<3	NDA	<0.5	NDA
Anticentromere	<7 U/ml		NDA	NDA	NDA	NDA	Negative	NDA	NDA	Negative	NDA	NDA	NDA	<0.4	NDA
Anti-histone AB	<25 U/ml		NDA	NDA	NDA	NDA	Negative	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
Anti-Jo-1-AB	<7 U/ml		NDA	NDA	NDA	NDA	Negative	NDA	NDA	Negative	NDA	NDA	NDA	<0.3	NDA
Anti-U1-RNP-AB	<25 U/ml		NDA	NDA	NDA	NDA	Negative	NDA	NDA	Negative	NDA	NDA	NDA	0.3	NDA
Anti-Scl-70-AB	<25 U/ml		NDA	NDA	NDA	NDA	Negative	NDA	NDA	Negative	NDA	NDA	NDA	<0.4	NDA
Anti-Smith-AB	<25 U/ml		NDA	NDA	NDA	NDA	Negative	NDA	NDA	Negative	NDA	NDA	NDA	0.3	NDA
Anti-SSA/Ro-AB	Negative U/ml		NDA	NDA	NDA	NDA	182.5	NDA	NDA	4.4	NDA	NDA	NDA	>240.0	>240.0
Anti-SSB/La-AB	Negative U/ml		NDA	NDA	NDA	NDA	117.5	NDA	NDA	2.0	NDA	NDA	NDA	2.5	NDA
Rheumatoid factor	<20 IU/ml		Positive	112	176	NDA	48.4	NDA	NDA	44	NDA	NDA	NDA	NDA	NDA
Anti-CCP-AB	<7 U/ml		NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
aPL-AB Lupus anticoagulant	<1.08 (Ratio)		NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
Anti-cardiolipin-AB	MPL-U/ml		NDA	Negative	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	Negative
β_2-Glycoprotein 1-AB	<7 U/ml		NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
Antiphosphatidylserine-AB	<10 IU/ml		NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
Complement component 3	0.90–1.70 g/l		Low	Normal	NDA	NDA	1.13	NDA	NDA	NDA	NDA	NDA	NDA	0,89	1,03
Complement component 4	0.11–0.34 g/l		NDA	Normal	NDA	NDA	0.18	NDA	NDA	NDA	NDA	NDA	NDA	0.18	0,25
ANCAs c-ANCA	1:<10		NDA	NDA	NDA	NDA	NDA	NDA	NDA	1:<10	NDA	NDA	NDA	NDA	NDA
p-ANCA	1:<10		NDA	NDA	NDA	NDA	NDA	NDA	NDA	1:<10	NDA	NDA	NDA	NDA	NDA

Laboratory marker and reference range/unit: AB, antibody; ANA, antinuclear antibody; CCP, (anti) citrullinated protein antibodies; DsDNA, double stranded deoxyribonucleic acid; ESR, erythrocyte sedimentation rate; aPL, antiphospolipid (antibody); ANCA, antineutrophil cytoplasmic antibodies.

Treatment: ST, stationary treatment (number of hospitalization in brackets); AMB, ambulatory treatment.

NDA, no data available; green, pathologically elevated; red, pathologically elevated.

balancing the teratogenic potential versus the need for continuing azathioprine. Shortly after, the pregnancy was terminated at the request of the patient, but azathioprine was still left out. As a result, an unexpected and dramatic change in the overall health status of the patient reoccurred. Within 4 weeks the patient developed signs of restlessness, insomnia and mania, all symptoms rapidly deteriorating further to a full blown psychosis, and again requiring hospitalization in May 2012. She soon developed progressive disorganized behavior, thought disorder overall incoherence and paranoid ideation. The initial therapy was undergoing a continual adjustment, including benperidol (stopped because of side effects), later fluphenazine, quetiapine, asenapine, and because of depressive symptoms trimipramine and amitriptyline. Finally, a combination of risperidone, olanzapine, chlorprothixene, and valproate was given. Electroconvulsive therapy was tried. Besides psychotropic medication, cortisone and azathioprine were restarted from the beginning. Exactly 19 days after admission, an exanthema appeared on her cleavage and upper back. Simultaneously, the patient reported to suffer from light sensitivity and arthralgia. ANA titer and antibodies against SSA–Ro were rising, C3 complement was low (compare **Table 1**). Severe skin eruptions were constantly treated in the department of Dermatology, at Ulm University.

In January 2013, the disease fulfilled the criteria of subacute cutaneous lupus erythematosus (psychosis, discoid lesions, photosensitivity, leukopenia, and ANA titer). Despite immediate resumption of immune suppression, the clinical situation was unchanged if not worsening. Because of self injurious behavior she was taken to the protected ward for weeks, partly medical restraint become necessary. Subsequently, instead of azathioprine, mycophenolate mofetil (CellCept® 3 g/day) was prescribed. Nevertheless, there was little therapeutic success to be observed. In March 2013, additionally even the scalp and face were affected by skin lesions. After 10 months of inpatient stay, the patient agreed to another CSF examination, which showed oligoclonal IgG bands (compare **Tables 2** and **3**). There were no neuronal antibodies. Also levels of tumor necrosis factor (TNF)-α and SCDs 25 were increased in the blood and elevated biomarkers in CSF were found (compare **Table 2**). The results of CSF examination now justified a diagnosis of NPSLE in accordance with established criteria (12–15).

OVERCOMING THERAPY RESISTANCE WITH MONOCLONAL ANTIBODY THERAPY

Because of therapy resistance for more than 1 year and the initial diagnoses of SCLE with NPSLE, actual immune suppressive therapy was reconsidered, and intravenous therapy with the monoclonal antibody belimumab (Benlysta®) was started as an add-on treatment to the previous therapy (see **Figure 2**). Gradually, the patient improved over the next 2 months in every aspect and was discharged in December 2013. However, 2 weeks later she again attempted suicide by drug intoxication, requiring treatment in the intensive care unit, where all psychiatric and immune suppressive treatments were stopped. Eight weeks later, severe skin eruptions again emerged. In spite of hydroxychloroquine (Quensyl® 400 mg/day) being prescribed now, the skin eruptions worsened further, and psychiatric hospitalization was again required because of her severe psychosis relapse. Subsequently belimumab was restarted again. With the combination of mycophenolate mofetil (1,000 mg/day), hydroxychloroquine (400 mg/day), urbason (2 mg/day), and belimumab (10 mg kg/bodyweight per cycle) a rapid remission was achieved. Both, psychotic symptoms and skin manifestations improved in parallel. An attempt at pausing hydroxychloroquine was discarded because of repeated erupting skin manifestations. The patient was discharged from hospital treatment in an acceptable clinical status. During the next continuous outpatient treatment months she improved to full remission in psychosis and skin manifestations.

Following dismissal, she remained clinically stable with a complex of psychopharmacological and immune suppressive medications (for details see **Figure 2**).

DISCUSSION

We conducted a detailed analysis case study of a patient with schizophrenia in regard to the question of whether autoimmunity and schizophrenia were unrelated comorbid disorders, or whether both disorders could be interpreted as a unified disorder, representing in fact the varied manifestations of an atypical

TABLE 2 | Plasma cytokines and CSF cytokines (red framed) of the patient in 2011–2015 based on research done by Prof. Dr. E. Marion Schneider, Experimental Anesthesiology Section, University Clinic Ulm, 89075 Ulm, Germany.

	29.03.2011	18.06.2012	26.06.2012	10.12.2012	12.03.2013	15.03.2013	09.04.2013	23.05.2013	13.06.2013	13.06.2013 (CSF)	21.01.2015	Unit
EPO		5.55	6.39	11.00	7.03	10.20	21.10	3.74	15.50		5.77	mU/ml
IL-10	0.96	0.92	0.47	0.48	2.20	3.85	1.41	0.88	0.00	2.64	6.91	pg/ml
IL-1β	4.40	2.12	3.44	1.85	1.98	0.92	1.68	0.43	38.70	1.08	3.98	pg/ml
IL-6	0.28	0.62	0.215	4.77	3.40	2.33	3.11	0.94	131.00	8.41	1.34	pg/ml
IL-8	16.80	4.63	1.61	7.70	18.20	11.60	6.84	6.45	8.84	53.70	4.47	pg/ml
TNF-α	23.60	8.05	8.90	15.30	18.50	19.60	15.80	9.93	14.90	7.26	24.40	pg/ml
sCD25	588.00	603.00	591.00	421.00	865.00	986.00	1,422.00	1,246.0	1,126.00	1.32	849.00	U/ml
LBP	7.70	5.15	7.11	4.34	11.80	11.80	9.23	9.04	4.06	0.76	3.41	ng/ml
Ferritin	53.20	79.60	77.00	59.20	163.00	160.00	65.00	17.80	14.50	3.23	124.00	ng/ml

Normal range: erythropoietin (EPO) <30 mU/ml; IL-10 <3–15 pg/ml; IL-1β <1–3 pg/ml; IL-6 <6–10 pg/ml; IL-8 <70.0 pg/l; tumor necrosis factor (TNF)-α <3–15 pg/ml; sCD25 = 200–500 U/ml (adult); plasma ferritin <50 ng/ml; lipopolysaccharide binding protein (LBP) <15 μg.

course of SLE, predominantly with psychiatric symptoms and accompanying skin manifestations most over the time. The latter hypothesis was plausible from a first look because of concurrent minor autoimmune signs and findings with the psychotic phases. To assume indeed a unifying disease hypothesis, we need, however, more arguments. The following points support the unifying perspective:

TABLE 3 | CSF parameters in our patients sample (13 June 2013).

Cells			
CSF leukocytes, 1/µl		**CSF erythrocytes, 0/µl**	
Proteins			
	CSF	**Serum**	***Q* (CSF/serum) <10³**
Total protein	320 mg/l		
Albumin	169.00 mg/l	40.7 g/l	4.2
IgG	26.0 mg/l	11.2 g/l	2.3
IgA	1.77 mg/l	1.63 g/l	1.1
IgM	<0.142 mg/l	0.604 g/l	<0.2
Oligoclonal IgG in CSF with additional identical bands in CSF and serum			
Lactate	1.4 mmol/l		

1. Good clinical improvement of skin and psyche under short time corticosteroids in 1993 with repeated relapses after corticosteroids was discontinued.
2. Repeated temporal parallelism of the appearance of skin manifestations and psychiatric manifestations consistently over the disease course of 25 years.
3. Some temporal relationship between high titer of autoimmune/inflammatory markers and antinuclear antibodies to the severity of psychiatric symptoms.
4. Striking temporal parallelism of improvement of most severe psychiatric and skin manifestations with complex immune suppression during the last severe relapse.
5. Close temporal relationship between longtime full remission under azathioprine medication, followed by the most severe relapse ever after discontinuance. In addition, there is a plausible delay of a few weeks after discontinuance of azathioprine and onset of first psychotic symptoms.
6. Demonstration of mild neuroinflammation by CSF analysis during the most severe disease phase, when fulfilling criteria of SCLE, fulfilling thus criteria of NPSLE, the latter appearing as an acute severe schizophreniform psychosis.
7. Suspicious family history with both severe psychiatric [depression in both parents (ICD F 33.3); sister with

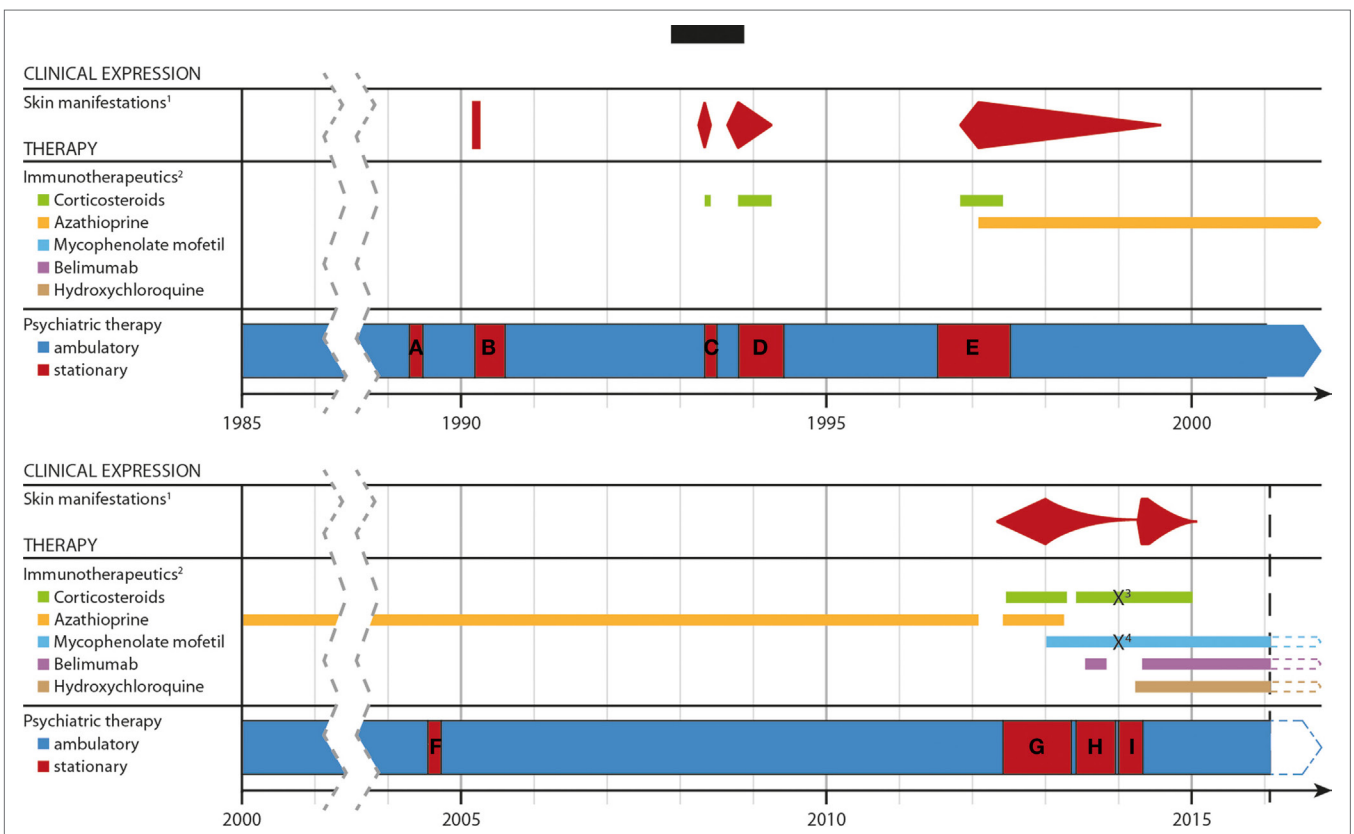

FIGURE 2 | Timeline of the development of the disease, the severity of the skin lesons and immunotherapeutic treatments over time. Abbreviations and explanations: [1]well documentated, specific cutaneous, and mucosal lesions; [2]except for topical application; [3/4]methylprednisolone/mycophenolate mofetil was paused due to a suicide attempt. **(A–G)** period of stationary treatment: **(A)** 14.04.–23.06.89; **(B)** 15.02.–11.07.90; **(C)** 11.03.–27.07.90; **(D)** 20.08.93–30.03.94; **(E)** 11.06.96–12.06.97; **(F)** 19.07.–25.09.2004; **(G)** 29.05.12–03.05.13; **(H)** 29.05.–13.12.1; **(I)** 03.01.–30.04.14 and 01.05.–06.05.14.

depression and paranoid schizophrenia (ICD-10 F20.0, F32.2)] and autoimmune disorders [sister with Crohn's disease (ICD-10 K50.1)].

8. Unnoticed autoimmune signs were retrospectively assessed with a newly developed questionnaire [details in Ref. (16), in review], yet detected in the prepsychotic phase repeated arthralgia and swelling of limbs and fingers, which happened in our patient.
9. Clinical improvement of psychosis repeatedly seemed associated with immune or anti-inflammatory therapies, including lithium with immune suppressive reactions.
10. Temporal relationship of increasing serum cytokines, especially TNF-α, during the most severe psychotic relapse and uniquely elevated CSF cytokines fitting to observations in schizophrenic patients (see **Table 2**; see below).

Because our patient rejected repeatedly CSF diagnostics, there is only a single CSF sample with cytokine profiling. The sampling was done in June 2013, during a phase of severe psychotic symptoms. In this CSF sample, we found suspicious biomarkers (compare **Table 2**): chemokine IL-8 was selectively higher than in the corresponding serum samples, other CSF cytokines were marginally elevated, TNF-α was not increased. These results match with CSF and serum cytokine profiling in schizophrenic and affective spectrum disorder patients (7). The presence of oligoclonal bands in CSF, few of them also in serum (compare **Table 3**), indicates in agreement with established criteria (13, 14) neuroinflammation, here overall mild and such was found in small groups of patients with affective and schizophrenic spectrum disorders in several studies (5, 6).

We conclude from our case analysis of 25 years disease course, that our case represented an atypical case of autoimmune disorder, lately diagnosed as LE, beginning with a focus on the CNS, explaining the variant psychiatric Syndrome from variant autoimmune-inflammatory disease activity. We could only partially define the apparently variant disease activity and only partially detect the immune inflammatory pathomechanisms involved. Overall the case represents a prototype of what was preliminary defined and hypothesized as ME. Cases with incomplete diagnostic autoimmune disorders over years, which later may develop a classified autoimmune disorder, are well-known, for example, presenting with mixed tissue disorder years before (17–20). Comparable cases of NPSLE were not described according to our knowledge. Our many arguments to assume an atypical case of remitting relapsing, or chronic when without immune suppressive treatment, NPSLE may be strong, though proof is missing: there were no definite, in part from circumstantial reasons, signs of neuroinflammation by imaging or CSF analysis in the early course. However, such was not unexpected from many arguments (9) and in rare single cases of schizophrenic syndromes with normal or quite normal CSF, nevertheless brain biopsy demonstrated mild inflammation (11, 21). We did not find neuronal antibodies, but this does not argue against our conclusion, cases with brain biopsy proven ME described by Najjar et al. (11) did also not demonstrate neuronal autoantibodies. We had not the opportunity to take a brain biopsy. On the other hand, we had the advantage of a long-term observation including several time periods of immune suppressive treatment, given from somewhat only partially established diagnostic criteria, detecting in retrospect a rather plausible if not clear evidence of positive treatment effects from immune suppression, which continues up to the time point of writing this paper. In sum, our arguments appear to represent strong arguments in total, to interpret our case as an atypical course of NPSLE from the beginning focused on the CNS.

With this case several difficult scientific and ethical questions had to be considered: After remission of the debated severe skin disease, thought to represent an allergic reaction to pharmaca or an undefined autoimmune disorder, also the possibility of a unified autoimmune disorder, as discussed in this paper, was considered by author Karl Bechter and discussed with the patient, though by majority of psychiatric doctors in our hospital rather held implausible. The patient was clearly informed about these scientific uncertainties and the novelty of the hypothesis of mild neuroinflammation to possibly causally underlie the psychotic disorder, held by Karl Bechter. The question of a relative indication of long-term treatment with azathioprine was evaluated independently by specialist in internal medicine (Dr. Peter Müller), with the result that long time azathioprine was justified (defined as relative indication) by the case history and because of continued mild leukopenia. With this informed knowledge the patient preferred to be treated with azathioprine as documented and the psychopharmacological medication to be reduced. The patient continued to visit Drs. Karl Bechter and Peter Müller, all over the respective years reported, though visiting Karl Bechter only sporadically but continuous over many years with remitted psychiatric syndrome but immediately with incipient relapse after set off of azathioprine.

DIFFICULTIES IN DIAGNOSING AUTOIMMUNE DISORDERS IN PSYCHIATRIC PATIENTS

Psychiatric symptoms are rarely reported as an initial and isolated feature of SLE (22), in spite of many patients having the feeling that psychiatric symptoms occurred before they were diagnosed with SLE. However, central inflammation does not always coincide with systemic signs. Tests on mice suggest that NPSLE is not always a complication of SLE and can occur in absence of systemic autoimmunity (23). This supports the importance that early CSF diagnostics are done in longtime psychiatric patients. The European League Against Rheumatism emphasizes the increased risk of neuropsychiatric manifestations in SLE and requests early diagnostics including CSF examination in neuropsychiatric patients (even if only to exclude CNS infections) (24).

In our case, there was a delay of 4 years between initial psychiatric symptoms and first noticed mild skin manifestations. Accompanying systemic signs are maybe misinterpreted or remain undiagnosed, in our case even the established criteria of lupus were fulfilled only late in the course. This case shows the difficulties in establishing a diagnosis of common autoimmune disorders such as SLE, by regarding criteria like those of the American College of Rheumatology (ACR)-97 or Systemic Lupus

International Collaborating Criteria (SLICC)-12. Our patient never had any further neuropsychiatric symptoms like seizures or neuropathy. No cerebrovascular accidents were noticed. Missing manifestations or incomplete laboratory markers create major uncertainties for clinicians. The general problem of incomplete criteria is known in autoimmune disorders, the diagnosis of mixed tissue disorder, a respective phenomenon, number of cases later being diagnosed with classical autoimmune disorders (19). Also atypical courses in other organ systems, e.g., with visceral focus have been described (25).

CONCLUSION

Psychosis as the initial or predominant manifestation of SLE over years is an unusual course of the disease presented here. If our conclusion is correct, that indeed we observed one disorder with manifestations in different organ systems, there is an apparent need for improved diagnostic instruments and methods. For better detection of unnoticed clinical signs we recently developed a questionnaire called "FAISF" [(16), in review]. Another problem is, as our case strikingly demonstrates, that even known signs and minor findings of autoimmunity do not usually allow a definitive diagnosis of the defined autoimmune disorder, as established for example by the ACR or the SLICC. This problem of sub diagnostic cases has also been recognized in rheumatology.

A careful analysis of our case with 25 years disease including actual and previous material, clearly suggests that this case of seemingly comorbid schizophrenia with minor autoimmunity signs represented a case of one emerging autoimmune disorder with variant manifestations systemically and within the CNS, though atypically with predominant appearance as a schizophrenia spectrum disorder. We do not exclude, that more such cases exist, but were certainly inappropriately understood. This case demonstrates the therapeutic potential if detected in an early phase and treated appropriately including with immunosuppressive options. The consequences of our perspective would likely include psychoimmunological aspects in psychiatric routine examinations. Recommendations should be developed including clinical signs and findings.

AUTHOR CONTRIBUTIONS

AM—collecting data and summary of the patient. CP and KB—treatment of the patient. EMS—CSF analysis, serum analysis, and cytokine profiling.

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CASE REPORT
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Steroid-Responsive Chronic Schizophreniform Syndrome in the Context of Mildly Increased Antithyroid Peroxidase Antibodies

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Background: Schizophreniform syndromes can be divided into primary forms from polygenic causes or secondary forms due to immunological, epileptiform, monogenic, or degenerative causes. Steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT) is a secondary immunological form associated with increased thyroid antibodies, such as antithyroid peroxidase antibodies and shows a good response to corticosteroids.

Case presentation: We present the case of a 41-year-old woman suffering from a schizophreniform syndrome. Starting at the age of 35, she developed psychotic exacerbations with formal thought disorder, acoustic hallucinations, cenesthopathic experiences, and loss of ego boundaries. At the same time, she began to suffer from chronic sexual delusions and olfactory hallucinations, which did not respond to neuroleptic medication. Her levels of antithyroid peroxidase antibodies were slightly increased, and the blood–brain barrier was disturbed. An electroencephalogram (EEG) showed intermittent generalized slowing, and cerebral magnetic resonance imaging (cMRI) depicted mild temporolateral atrophy. High-dose corticosteroid treatment led to convincing improvement of attentional performance and the disappearance of delusions and olfactory hallucinations.

Conclusion: SREAT can mimic typical symptoms of schizophreniform syndromes. The increased titer of antithyroid peroxidase antibodies in combination with the EEG slowing, blood–brain barrier dysfunction, and the cMRI alterations were the basis for suspecting an immunological cause in our patient. Chronic delusions, olfactory hallucinations, and cognitive deficits were successfully treated with corticosteroids. The occurrence of secondary immunological forms of schizophreniform syndromes demonstrates the need for innovative immunosuppressive treatment options.

Keywords: Hashimoto encephalopathy, steroid-responsive encephalopathy associated with autoimmune thyroiditis, schizophrenia, thyroiditis, thyroid peroxidase, corticosteroids

BACKGROUND

Schizophreniform syndromes are common severe disorders that are characterized by delusions, hallucinations, loss of ego boundaries, cognitive deficits, impaired motivation, and social withdrawal (1). Primary schizophrenia, which has polygenic causes, can be distinguished from various secondary forms of schizophreniform syndromes caused by immunological, epileptiform, monogenic, or degenerative factors. Immunological encephalopathies can be associated with antibodies against thyroid tissue, such as steroid-responsive encephalopathy associated with autoimmune thyroiditis (SREAT) with antithyroid peroxidase antibodies, intracellular onconeural or synaptic antigens, such as limbic encephalitis with anti-Hu antibodies, or neuronal cell surface antigens, such as autoimmune encephalitis due to anti-*N*-methyl-D-aspartate receptor (NMDAR) antibodies (2, 3). SREAT should be considered in the context of neuropsychiatric symptoms, autoimmune thyroiditis, increased thyroid antibodies, such as antithyroid peroxidase or anti-thyroglobulin antibodies, and other organic alterations [e.g., blood–brain barrier dysfunction in the cerebrospinal fluid, abnormal encephalopathic patterns as identified by an electroencephalogram (EEG), and non-specific white matter lesions as identified by cerebral magnetic resonance imaging (cMRI) (4–6)]. SREAT can mimic schizophreniform and other psychiatric syndromes (3, 7).

CASE PRESENTATION

We present the case of a single 41-year-old female teacher and sports therapist suffering from a schizophreniform syndrome. In her third decade, she developed recurrent reactive depressive episodes. In her fourth decade, she developed a sexualized delusional system, which chronified. During this time, she suffered from repeated episodes with hallucinations.

The patient suffered reactive depressive episodes at 22, 28, and 32 years of age. At age 32, she experienced delusions for the first time. At age 35, she experienced her first schizophreniform episode with delusions, hallucinations, and promiscuity. During this episode, her mood was hypomanic. Since that time, she has suffered from a complex system of chronic delusions and olfactory hallucinations. At 40 and 41 years of age, she developed exacerbations with hallucinations. During these episodes, she suffered from formal thought disorder (long, incoherent train of thought) and auditory hallucinations. During the auditory hallucinations, she heard the voices of her neighbors ask her to have sexual intercourse and masturbate, the voice of a man who talked about the sexual lives of her friends, and a computer voice that told her which clothes to wear and that she was a "porn queen." She also had cenesthopathic experiences in which she felt that she was being irradiated, her breasts were tense, and her intestines were pulsating, and felt that she was being externally controlled. In the latter case, she thought that she was being irradiated by the neighbors, her thoughts were being tracked, and she could feel the bodily sensations of her neighbors. These symptoms disappeared after she received neuroleptic treatment.

However, simultaneously at age 35, she developed a complex system of chronic delusions in which she attributed sexual body parts to everyday gestures. In this sexualized delusional system, the nostrils represented the buttocks, the mouth was the vagina, the tongue was the penis, and the ears were a combination of the bottom and the genitals. If a person scratched at their nose, our patient identified this gesture as proof that the person would like to have or that they had anal intercourse. The precise area of the nose that the person scratched was attributed to different positions of sexual intercourse. She had similar convictions if a person touched the mouth, tongue, or ears. Wrinkles at different locations in the face were assigned specific meanings with respect to sexual preferences (**Figure 1**). The patient was absolutely convinced of her interpretations. She also believed that she had the ability to smell whether people had had sexual intercourse and that other people could smell whether she had had sex. In addition, she had olfactory hallucinations in which she smelled urine, feces, and vaginal secretions in different situations. She took care not to wear provocative clothes to avoid attracting the sexual attention of men. All these symptoms persisted under treatment with neuroleptic medication.

Developmental, Somatic, and Family History

The patient reported no history of *in utero* or birth complications, febrile convulsions, inflammatory brain diseases, or cerebral contusions. Her childhood development was normal, and there was no evidence of any neurodevelopmental disorder, such as attention-deficit hyperactivity disorder or autism. There was also no evidence of a personality disorder. Her somatic history was unremarkable, except that she had been diagnosed with Hashimoto's thyroiditis. She also had no history of alcohol or drug abuse. There were no psychotic or neurological disorders in her family history, although her father did have a washing obsession, while her mother abused alcohol and her sister was diagnosed with recurrent depression.

Investigations

A neurological examination of the patient was normal. Serum analysis showed increased antithyroid peroxidase antibodies (35–55 IU/mL over the course of the disease and before steroid treatment; normal range <34 IU/mL). Levels of antithyroglobulin and antithyroid-stimulating hormone receptor antibodies were unremarkable. Thyroid-stimulating hormone levels were in the upper range; triiodothyronine and thyroxine

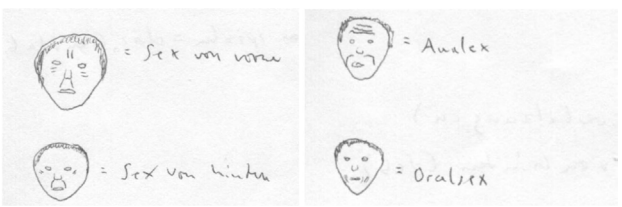

FIGURE 1 | **The meaning of different wrinkles**. Top left: sexual intercourse from the front; top right: anal sex; lower left: sexual intercourse from behind; lower right: oral sex.

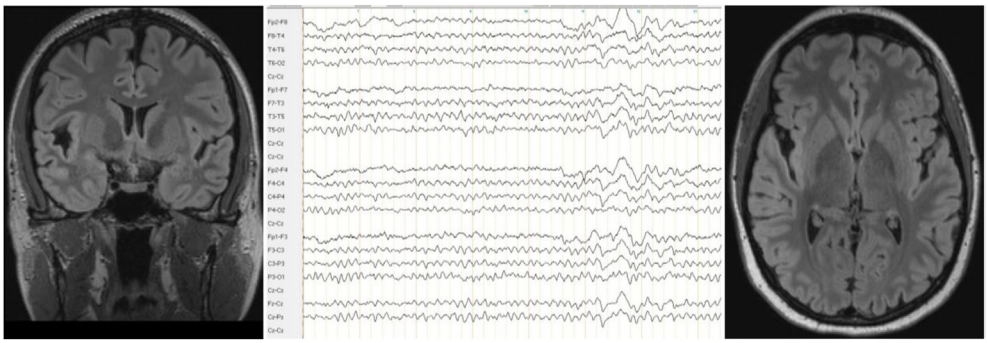

FIGURE 2 | **The cerebral magnetic resonance imaging showed mild temporolateral atrophy, and the electroencephalogram showed intermittent generalized slow waves**.

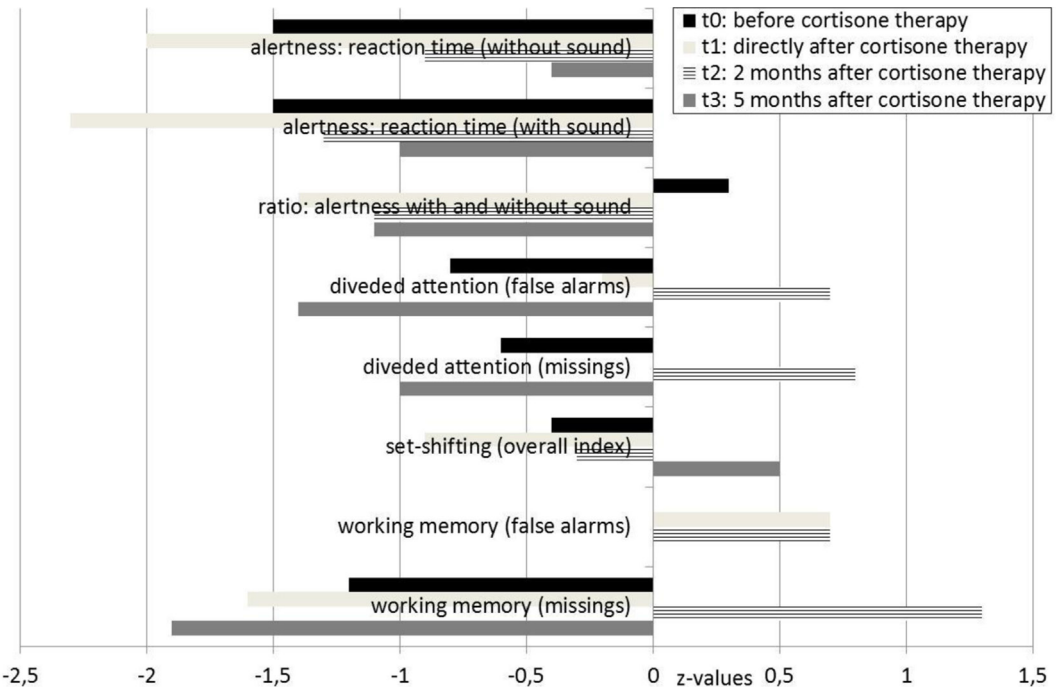

FIGURE 3 | **Test for attentional performance**.

levels were normal. Screening for rheumatoid factors, antinuclear antibodies, and antineutrophil cytoplasmic antibodies, as well as infectious diseases, such as *Borrelia*, lues, and HIV, showed no relevant abnormalities. In the cerebrospinal fluid analyses, we detected mild blood–brain barrier impairment and increased age-dependent albumin quotient (7.5; age-dependent reference $<6.7 \times 10^{-3}$). The white cell count (2/μL) and IgG index (0.43) were normal. No cerebrospinal fluid-specific oligoclonal bands were found. Antibodies against neuronal cell surface antigens [NMDAR, AMPA-R, GABA-B-R, and VGKC-complex (LGI1, Caspr2)] were negative in the cerebrospinal fluid. No antibodies against intracellular onconeural or synaptic antigens (Yo, Hu, CV2/CRMP5, Ri, Ma1/2, SOX1, GAD, and amphiphysin) were found in the serum. The cMRI showed a few right-accentuated hippocampus cysts in the cortical region. No white matter lesions were found. Mild temporolateral atrophy was identified. Repeated EEGs showed intermittent rhythmic delta activity (**Figure 2**). The neuropsychological test of attentional performance (TAP) revealed severe deficits in alertness and working memory (**Figure 3**).

Differential Diagnosis

The symptoms met the ICD-10 criteria for paranoid hallucinatory schizophrenia (F20.0). Because the patient had Hashimoto's thyroiditis and increased antithyroid peroxidase antibodies, we also considered SREAT.

Treatment

Different neuroleptics (amisulpride, aripiprazole, clozapine, flupentixol, olanzapine, quetiapine, and risperidone) were used

to successfully treat our patient's acoustic and cenesthopathic experiences and loss of ego boundaries. However, average doses were not tolerated by the patient, and the neuroleptics were not able to treat the chronic delusional system and olfactory hallucinations. The same was true for additional treatment with lithium and valproic acid. The patient's hypothyroidism was successfully treated with 100 µg thyroxine daily. Because of treatment resistance and organic abnormalities due to potential SREAT, we started treatment with 500 mg methylprednisolone administered intravenously once per day for five consecutive days. Methylprednisolone treatment was continued with 100 mg administered orally each day, which was then gradually tapered over nearly 3 months. Treatment with amisulpride (400 mg), lithium (450 mg), pipamperone (20 mg), thyroxine (100 µg), selenium (0.1 mg), and cholecalciferol (0.025 mg) was continued.

Outcome and Follow-up

Following the high-dose methylprednisolone treatment, the patient experienced full remission of all psychotic symptoms, including the delusional system and olfactory hallucinations. After realizing that the former system was a delusion, our patient developed depression. She felt sad and embarrassed about her previous misinterpretations and the "wasted years" of the disease. TAP at that time showed deterioration (t1, **Figure 3**).

Approximately 3 weeks later, her mood normalized. A TAP was performed again 2 months after starting high-dose methylprednisolone treatment and showed that the patient's alertness, divided attention, and working memory had all improved. However, the cMRI and EEG results were unchanged. Analysis of the cerebrospinal fluid showed that it normalized after steroid treatment; the blood–brain barrier dysfunction was no longer detected. Levels of antithyroid peroxidase antibodies were no longer elevated. Because of the positive response to the immunosuppressive treatment, we diagnosed SREAT encoded as an organic schizophreniform syndrome (ICD-10: F06.2). Six months later, the patient was still free of delusions, hallucinations, and loss of ego boundaries. Amisulpride treatment was reduced to 50 mg, pipamperone was stopped, and lithium, thyroxine, selenium, and cholecalciferol treatment remained unchanged. However, a TAP showed decreased results for divided attention and working memory (**Figure 3**). Antithyroid peroxidase antibody titers were still normal.

DISCUSSION

We present the case of a female patient with a chronic schizophreniform syndrome, who had side effects and insufficient responsiveness to neuroleptic medication, had mild organic alterations with increased levels of antithyroid peroxidase antibodies, and responded to treatment with corticosteroids. Therefore, we diagnosed the patient with SREAT, which is typically characterized by seizures (47%), confusion (46%), memory disturbances (43%), disordered speech (37%), gait disturbance (27%), delusions (25%), myoclonic jerks (22%), and depression (12%) (6). Clinical manifestations of isolated schizophreniform syndromes have been described for individual cases in the literature (8–12). Most case studies reported acute onset or repetitive episodes of SREAT (9–12).

The distinctive features of our case were chronic delusions and olfactory hallucinations, which were successfully treated with high doses of corticosteroids. The EEG slowing and blood–brain barrier dysfunction identified in our patient are frequent, but nonspecific alterations that are found in over 80% of SREAT patients on a review level (6). We suggested in earlier studies that the EEG slowing might lead to clinical symptoms *via* local area network inhibition (3, 13). Blood–brain barrier dysfunction might allow potentially pathogenic autoantibodies to enter the central nervous system (CNS), thereby causing subtle CNS inflammation. A similar cause has been proposed for anti-NMDAR encephalitis (14). One earlier study showed cross reactivity between antithyroid peroxidase antibodies and cerebellar astrocytes (15). The role of the cerebellum in psychiatric symptoms has also been described (16, 17). Therefore, anti-thyroideal antibodies might have a direct pathophysiological role in the development of neuropsychiatric symptoms. However, Blanchin et al. (15) only showed antibody binding, but no neuronal damage. Therefore, the thyroid antibodies might alternatively function as an epiphenomenon, similar to the MRZ reaction in patients with multiple sclerosis (18), along with increased susceptibility to autoimmune conditions.

Most researchers favor the idea that thyroid antibodies do not play a relevant role in the development of neuropsychiatric symptoms (19, 20). Thyroid antibodies have been found in the serum of 13% of healthy individuals (21, 22). Isolated elevated levels of antithyroid peroxidase antibodies were found in 34% of SREAT cases (6). In our patient, autoantibody titers were only slightly increased; however, earlier studies have shown that antibody titers are not correlated with clinical severity (4–6). Our patient had no white matter lesions, which have been described in up to 52% of SREAT patients (6). However, our patient did have mild temporolateral atrophy; atrophy has been described in earlier SREAT cases (7). Following the current diagnostic recommendations, SREAT can only be diagnosed by exclusion (22). Therefore, the presence of other well-characterized neuronal antibodies was excluded in our patient. However, it is possible that our patient's symptoms might also be due to new or unknown antineuronal antibodies. An unbiased search on rodent brain sections could be an additional tool in future cases.

We treated our patient in a probatory manner with high-dose corticosteroids. According to prior research, in cases of autoimmune encephalitis, first-line therapy should include corticosteroids, intravenous immunoglobulins, or plasmapheresis, whereas second-line therapy should include rituximab or cyclophosphamide (23). In our patient, the blood–brain barrier disturbance and antithyroid peroxidase antibody titer normalized following corticosteroid therapy. Cognitive testing showed substantial improvement within 2 months, and the psychotic symptoms disappeared. If the autoantibodies played a direct pathophysiological role, closure of the blood–brain barrier would have helped avoid direct autoantibody-mediated effects in the CNS. Furthermore, one could speculate about alternative mechanisms. For example, the corticosteroid treatment could have led to epigenetic effects

that modulated the genome functionality of different neural, glial, and immunological cell populations.

The worsening of neurocognitive testing after 5 months raises a question regarding the need for repeated or long-term immunosuppressive treatment. Patients with SREAT that have an insufficient response to corticosteroids are mostly treated with a second course of corticosteroids alone or combined with other immunosuppressive agents, such as azathioprine, intravenous immunoglobulins, or even plasmapheresis (6). For long-term immunosuppressive maintenance therapy, low-dose corticosteroids, azathioprine, methotrexate, cyclosporine A, mycophenolate mofetil, cyclophosphamide, rituximab, and intravenous immunoglobulins are available (23). Presently, there are no clear recommendations regarding long-term treatment for patients with SREAT. Therefore, further research is necessary in this field. Independent from such considerations, we found that neuroleptic treatment was initially sufficient for treating our patient's acoustic hallucinations, cenesthopathic experiences, and loss of ego boundaries. Therefore, an (initial) combination therapy with neuroleptics and corticosteroids seems a reasonable treatment for patients with SREAT.

CONCLUSION

Single cases of chronic schizophreniform symptoms in the context of autoimmune thyroiditis and slightly increased antithyroid peroxidase antibodies can be successfully treated with corticosteroids. However, these effects were only shown for a short-term follow-up in our patient; a diagnosis of cenesthopathic schizophrenia in our patient cannot be fully ruled out. We suggest that clinical screening for autoimmune conditions should become a routine procedure among patients with schizophreniform syndromes. The decision to administer immunosuppressive therapy in the presence of organ-specific autoantibodies should take into account other organic alterations, such as EEG and cMRI findings, or blood–brain barrier dysfunction. Therefore, cerebrospinal fluid analyses should also have a greater importance in patients with schizophreniform syndromes (24).

ETHICS STATEMENT

The patient has given her signed written informed consent for this case report, including the presented images, to be published.

AUTHOR CONTRIBUTIONS

DEndres and LTvE treated the patient and performed the data research. VM supported the data research. DEndres wrote the paper. IM performed the cMRI analyses. RD performed the EEG analyses. OS performed the CSF analyses. NV performed the rheumatological analyses. ANR performed the neuropsychological testing. EP and DErny critically reviewed the diagnostic results and contributed to the manuscript preparation. All authors were critically involved in the theoretical discussion and composition of the manuscript; read and approved the final version of the manuscript.

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frontiers in Psychiatry

CASE REPORT
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Schizophrenia Associated with Epileptiform Discharges without Seizures Successfully Treated with Levetiracetam

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Background: Schizophrenia-like disorders can be divided into endogenic or primary, idiopathic, polygenetic forms, and different secondary, organic subgroups [e.g., (para)epileptic, immunological, degenerative]. Epileptic and paraepileptic explanatory approaches have a long tradition due to the high rate of electroencephalography (EEG) alterations in patients with schizophrenia.

Case presentation: We present the case of a 23-year-old female patient suffering, since the age of 14 years, from a fluctuating paranoid-hallucinatory syndrome with formal thought disorder, fear, delusions of persecution, auditory, visual, and tactile hallucinations, as well as negative and cognitive symptoms. Laboratory measurements showed increased titers of antinuclear antibodies (ANAs) in the context of ulcerative colitis. While there was no clear history or evidence of epileptic seizures, the EEG showed generalized 3 Hz polyspike wave complexes. Under treatment with levetiracetam, the symptoms disappeared and the patient was able to complete vocational training.

Conclusion: The schizophrenia-like symptoms associated with epileptiform discharges but not overt seizures and the good response to antiepileptic treatment could be interpreted in the context of a (para)epileptic pathomechanism. The EEG alterations might be due to a polygenetic effect due to different genes. Mild immunological mechanisms in the framework of ulcerative colitis and increased ANA titers might have supported the network instability. This case report illustrates (1) the importance of EEG screenings in schizophrenia, (2) a potential pathogenetic role of epileptiform discharges in a subgroup of patients with schizophrenia-like symptoms, and (3) that antiepileptic medication with levetiracetam could be a successful treatment alternative in schizophrenia-like disorders with EEG alterations.

Keywords: epilepsy, schizophrenia, epileptiform discharges, levetiracetam, paraepileptic, LANI hypothesis

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Abbreviations: ACR, American College of Rheumatology; ANA, antinuclear antibody; CSF, cerebrospinal fluid; EEG, electroencephalography; FIRDA, frontal intermittent rhythmic delta activity; GABA, γ-aminobutyric acid; HV, hyperventilation; ICA, independent component analyses; IRDA; intermittent rhythmic delta activity; LANI, local area network inhibition; SLE, systemic lupus erythematosus; SV2A, synaptic vesicle glycoprotein 2A.

BACKGROUND

Schizophrenia-like disorders are characterized by delusional perception and delusions of control, hallucinations (e.g., commenting or discussing voices), thought insertion or withdrawal, cognitive impairment, thought disorders, or social withdrawal.[1] In addition to primary, endogenic or idiopathic, polygenetic forms, different secondary pathophysiological mechanisms [e.g., (para)epileptic, immunological, degenerative] can be assumed. Because of the high rates of electroencephalography (EEG) alterations, ranging from 7 to 60% in patients with schizophreniform syndromes, epileptic and paraepileptic explanatory approaches have a long tradition (1–3). In line with this assumption, we reported the first case of a young patient with a schizophrenia-like disorder, generalized spike-and-slow-wave complexes without epileptic seizures but with remission under treatment with valproate (4, 5). Immunological reasons might be due to autoantibody-associated autoimmune encephalitis, cerebral vasculitis, or collagenosis [e.g., systemic lupus erythematosus (SLE)] (6). Immunological effects might lead to network instability and therefore cause (para)epileptic phenomena (7). The detection of a (para)epileptic or immunological mechanism opens new treatment perspectives, in that antiepileptics or immunomodulators may be helpful (4, 5, 7–10).

CASE PRESENTATION

Clinical Presentation

We present the case of a 23-year-old female office clerk suffering from fluctuating paranoid-hallucinatory symptoms since the age of 14 years (2007). Therefore, the diagnosis of paranoid schizophrenia was made by different psychiatrists. Although taking neuroleptics, in the course of the disease, the patient developed five episodes (for several weeks) with paranoid-hallucinatory exacerbation. In these episodes, the patient suffered from formal thought disorder, fear, delusions of persecution, auditory hallucinations with commenting, discussing, and commanding voices, visual hallucinations with seeing maggots in her room, and tactile hallucinations with the feeling of being touched from behind. In parallel to these exacerbations, the patient developed severe negative and cognitive symptoms including attention and memory deficits, fatigue, depressive mood, and sleep disturbances thus completing the psychopathological features of comprehensive schizophrenia. Neurological and medical examinations were normal.

Family History

There was a positive family history for unipolar depression, which was diagnosed earlier in two sisters, both parents, and both grandmothers. There was no history for schizophrenia-like psychopathology, bipolar disorder, or epilepsy.

Somatic and Developmental History

Symptoms started 6 weeks after pain of the large joints. Therefore, a rheumatological disease was discussed. During an external work-up of repeated diarrhea, a chronic inflammation gut disease (ulcerative colitis) was diagnosed in 2014 and treated with mesalazine. No birth complications or *in utero* abnormalities were remembered; the birth was performed by cesarean section. The early childhood development was normal. No febrile convulsions or inflammatory brain diseases were remembered. The patient suffered mild cerebral contusions at the age of 4 and 12 years.

[1] http://apps.who.int/classifications/icd10/browse/2010/en#/F20-F29.

TABLE 1 | Diagnostic findings.

Serum basic diagnostics and blood count	• Normal renal, liver, and thyroid values; • Slightly increased C3d concentration (11.1 mg/l; reference value <9 mg/l); • Normal blood count.
Serum autoantibody analyses	• Normal thyroid autoantibodies (*against thyroglobulin, thyroid peroxidase, and thyroid-stimulating hormone*); • Rheumatological screening: increased antinuclear antibodies (titer: 1:400; reference value <1:50) without clear extractable nuclear antigens; the anti-nucleosome antibodies were weakly positive; • No antibodies against intracellular onconeural antigens (*Yo*, *Hu*, *CV2/CRMP5*, *Ri*, *Ma1/2*, *SOX1*), or the intracellular synaptic antigens (*GAD*, *amphiphysin*).
Cerebrospinal fluid (CSF) analyses	• Normal white cell count, no blood–brain barrier dysfunction (normal protein concentration and albumin quotient); • No CSF-specific oligoclonal bands, but a weak identical band in the CSF and serum; • Antibodies against neuronal cell surface antigens [*NMDAR*, *AMPA-R*, *GABA-B-R*, *VGKC complex* (*LGI1*, *Caspr2*)] were negative.
Cerebral magnetic resonance imaging (1.5 T)	• Normal brain findings; • Additional examination findings included a benign lesion of the right frontoparietal skull without contrast enhancement (*most likely equivalent with dermoid cysts; the criteria for monoclonal gammopathy of undetermined significance or multiple myeloma were not fulfilled*).
Electroencephalography (*during the first admission to our clinic in 2013, under the treatment with clozapine, aripiprazole, and citalopram*)	• Frontal accentuated intermittent rhythmic delta activity (FIRDA) and generalized 3 Hz polyspike wave complexes.

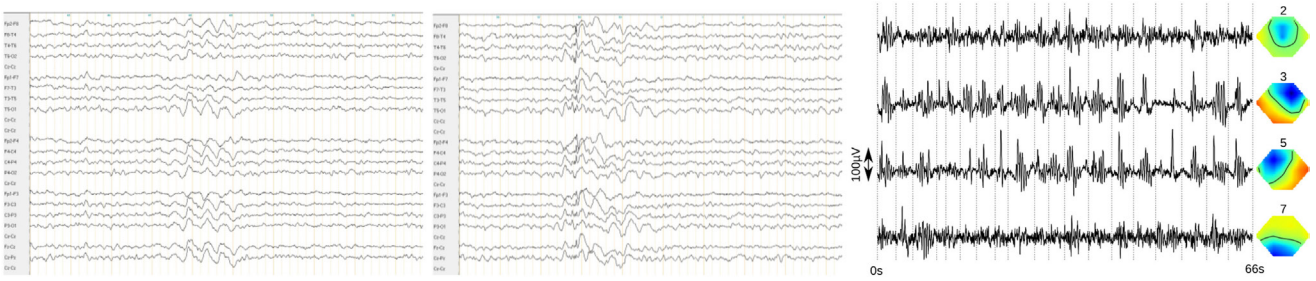

FIGURE 1 | Frontal accentuated intermittent rhythmic delta activity (FIRDA, left) and generalized 3 Hz polyspike wave complexes (middle) in the bipolar longitudinal rows (7 µV/mm, 0.3 s, 70 Hz). The findings of the independent component analysis are presented in the right picture [*the following four relevant components were found, left: activity traces, with examples of atypical activity cut from the clinical electroencephalography and appended at the dotted lines. Right: topographies, nose upward, negative (direction opposite of activity trace) blue, positive (direction same as activity trace) red. Right (component 3) and left (component 5) frontal activity show most prominent ~2.6 Hz bursts. Higher frequency activity frontocentral (component 2) and occipital (alpha component 7) are partially related*].

Diagnostic Findings

The diagnostic findings are summarized in **Table 1**. Taken together, the immunological alterations were compatible with the previously known ulcerative colitis (11). The electrophysiological findings (**Figure 1**) would be compatible with primary (idiopathic) generalized epilepsy; however, the history for epileptic seizures including absences and myoclonic jerks was negative.

Differential Diagnosis

The schizophreniform symptoms fulfilled the criteria of paranoid-hallucinatory schizophrenia (see footnote 1). Therefore, the most important differential diagnoses would be schizophrenia plus coincidental epileptiform discharges. Although the findings of the investigation led us to our consideration of neuropsychiatric SLE, the American College of Rheumatology classification criteria for SLE were not fulfilled.[2]

Therapy and Outcome

External neuroleptic treatment with risperidone (4 mg), amisulpride (600 mg), perazine (100 mg), aripiprazole (15 mg), and clozapine (275 mg) did not lead to long-term stabilization. Additional treatment with fluoxetine (20 mg) and citalopram (20 mg) for affective, negative, and cognitive symptoms did not successfully improve these symptoms. During the first visit in our clinic, in 2013, we detected the abovementioned epileptiform discharges. Assuming a (para)epileptic pathomechanism, we added antiepileptic treatment with valproate (1,500 mg) to the neuroleptic medication with clozapine and aripiprazole. At this point, the cognitive deficits improved significantly. Also, the EEG improved except for the (F)IRDAs. Another paranoid-hallucinatory episode in 2014 was treated successfully with a dose increase of clozapine and valproate. Because of a strong weight gain, the therapy with valproate was changed to topiramate (200 mg) in 2014. Assuming a (para)epileptic pathomechanism, clozapine was reduced and stopped in 2014. Aripiprazole was reduced in January 2014 and stopped in 2015. Normal results were found in both the routine EEG and in the EEG after sleep deprivation (2014). In the further course, topiramate led to a severe loss of appetite and was therefore changed to levetiracetam (1,500 mg) in 2014. The mental condition stabilized with the short-term antiepileptic treatment with topiramate and the subsequent antiepileptic treatment with levetiracetam (since 2014, and since 2015 as monotherapy). There were no more paranoid-hallucinatory episodes, the negative symptoms declined, the patient became a mother (in the spring of 2016); she lived alone, took care of her daughter, and simultaneously finished her vocational training (in the summer of 2016). She was able to suspend the mesalazine therapy and therefore only took levetiracetam (1,500 mg) at the time of stabilization.

DISCUSSION

We present the case of a patient with a schizophrenia-like disorder and, following our judgment, a (para)epileptic pathomechanism, because of the distinct epileptiform discharges without seizures and remission under the anticonvulsive treatment with topiramate and subsequently levetiracetam monotherapy.

Reason and Potential Pathophysiology of Network Instability

The EEG alterations might be due to a polygenetic effect caused by different genes (12). The immunological mechanisms in the framework of ulcerative colitis and increased antinuclear antibody titers might have supported the network instability by mild inflammatory processes (7, 13–15). Medication might also disclose underlying polygenetic or immunological network instability (16). The local area network inhibition (LANI) hypothesis might explain the causal relationship between epileptiform EEG discharges and schizophrenia-like symptoms. Excitatory network activity, as represented by the 3 Hz polyspike wave complexes, might lead to consecutive inhibitory processes in a physiological attempt of the central nervous system to stabilize the excitatory–inhibitory equilibrium of local cerebral networks. The repetitive

[2] www.rheumatology.org/Portals/0/Files/1997%20Update%20of%201982%20Revised.pdf.

excitatory activity, as documented by consecutive EEGs in our patient, could have exceeded a critical threshold, leading to the successive hyperinhibition of cerebral networks. Following the LANI hypothesis, the symptoms are due to the secondary induced processes of hyperinhibition (e.g., temporal hyperinhibition might have led to auditory hallucinations or memory deficits) (2–5, 17).

Treatment Considerations

Following the LANI hypothesis, the cognitive improvement after the addition of valproate to the neuroleptic treatment would be explained by the reduced epileptic activity and therefore the subsequent amelioration of inhibitory processes. In line with this assumption, comprehensive long-term stabilization was not achieved by several attempts of neuroleptic medication alone in spite of clear and very convincing effects of the treatment with clozapine in particular on positive symptoms. However, such a comprehensive improvement and even full remission was achieved with topiramate and later levetiracetam monotherapy. Thus, clozapine with its well-known proconvulsive properties might well have counteracted inhibitory processes, while it is at the same time most likely unable to improve causative excitatory neuronal activity. By contrast, by reducing the epileptiform activity, topiramate and levitiracetam monotherapy might have resulted in a more causal and therefore more comprehensive improvement of relevant pathophysiology. Earlier, we published a case of a (para) epileptic schizophrenia-like disorder successfully treated with valproate (4). Valproate, and likewise lamotrigine, is already established as an augmentative treatment strategy in schizophrenia (18). One might hypothesize that patients with (para) epileptic pathomechanisms will benefit significantly more from antiepileptic treatment than other subgroups. To our knowledge, this is the first published case study that describes a patient with a schizophrenia-like disorder who was successfully treated with levetiracetam. Levetiracetam is rarely used off-label in psychiatry probably because of its potential side effects, such as agitation, aggression, fear, and psychosis (2). The advantage of levetiracetam is that it can be rapidly dosed up to effective concentrations. Therefore, on a single case basis, the working hypothesis of a (para)epileptic pathomechanism could be tested quickly. In comparison, valproate effects could be due to combined γ-aminobutyric acid (GABAergic) and antiglutamatergic effects, and lamotrigine effects might be due to potential antiglutamatergic effects. However, the mechanism of levetiracetam cannot be explained by such direct transmitter effects. The effects of levetiracetam seem to be associated with the binding of the synaptic vesicle glycoprotein 2A (SV2A) (2). SV2A can be found in presynaptic membranes; it controls the calcium-dependent exocytosis of different neurotransmitters into the synaptic gap (19, 20). Therefore, it might also influence GABAergic and glutamatergic transmission (21).

Limitations

Epileptiform discharges are found in less than 1% of healthy adults (1, 22–24) and as a rare consequence of clozapine treatment (16). Therefore, the EEG alterations could be interpreted either as an incidental finding in a patient with schizophrenia or as a clozapine side effect. However, the clinical course—with improvement under antiepileptic treatment in parallel with EEG normalization—speaks against the assumption. The pathophysiological processes might be explained by the LANI hypothesis; however, this is only an unproven, theoretical framework that needs further investigation.

Conclusion

This case report illustrates the idea of a possible (para)epileptic pathomechanism in a patient with a schizophrenia-like disorder. Regarding diagnostic procedure, our case shows the importance of EEG examinations in typical schizophrenia-like disorders. Regarding pathophysiology, the case illustrates a potential pathogenetic role of epileptiform discharges in a subgroup of patients with schizophrenia-like symptoms. Regarding treatment, the case demonstrates that anticonvulsive medication with levetiracetam and also topiramate or valproate could be a successful treatment alternative in schizophrenia with EEG alterations.

ETHICS STATEMENT

The patient has given her informed and written consent for this case report, including the presented images, to be published.

AUTHOR CONTRIBUTIONS

LTvE treated the patient. DE wrote the paper and performed the data collection. DE and LTvE performed the interpretation of the diagnostic findings and therapy effects. BF performed and interpreted the EEG analysis. NV performed and interpreted the immunological analyses. EP and D-MA reviewed the diagnostic results and contributed to the manuscript preparation. All the authors were significantly involved in the theoretical discussion and the preparation of the manuscript, and they read and approved the final version of the manuscript.

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CASE REPORT
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Primary Sjogren's Syndrome Associated With Treatment-Resistant Obsessive–Compulsive Disorder

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There is an increasing awareness that autoimmune diseases can present with neuropsychiatric manifestations. We present the case of a 17-year-old female requiring psychiatric hospitalization for obsessive–compulsive disorder and major depressive disorder with mixed affective features, who was subsequently diagnosed with primary Sjogren's syndrome. Treatment with potent immunosuppression resulted in remission of psychiatric illness. Due to a lack of awareness and/or the lack of specific biomarkers, clinicians may not associate psychiatric symptoms with autoimmune disease, including primary Sjogren's syndrome. This case demonstrates that Sjogren's syndrome may be a causative or aggravating factor in mental disorders and that autoimmune diseases should be carefully considered in the differential diagnosis of psychiatric illness especially in cases of concurrent physical symptomatology and severity or treatment resistance of psychiatric disease.

Keywords: Sjogren's syndrome, obsessive–compulsive disorder, major depressive disorder, treatment resistance, autoimmune serology

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Although there is increasing awareness that a subset of psychiatric presentations may result from underlying autoimmune disease, the evidence for an association between autoimmune disease and obsessive–compulsive disorder (OCD) specifically, appears modest, and is perhaps strongest in conditions such as rheumatic fever (1). Here, we present a case of OCD, which appears to be associated with underlying autoimmune disease, most likely primary Sjogren's syndrome (pSS) and discuss the need for a high degree of suspicion in diagnosis and timely management of such presentations.

Written, informed consent was obtained for the publication of the following case report. A 17-year-old female was admitted to a private psychiatric unit with features consistent with severe OCD. The patient suffered from recurrent obsessions regarding contamination, hoarding and symmetry, and compulsions including hand washing, showering, cleaning, and checking. Her symptoms had caused significant distress and interference with academic and social functioning, to the extent that she had become isolated from her peers and was unable to attend school. There was no drug use or diagnosed medical illnesses to account for her symptoms. Therefore, her symptomatology satisfied DSM-IV criteria for diagnosis of OCD. A score of 36 out of 40 on the Yale-Brown Obsessive–Compulsiveness score (YBOCS) indicated that symptoms were extreme in severity.

In addition to satisfying DSM-IV criteria for OCD, she also satisfied criteria for major depressive disorder, with melancholic features—in particular psychomotor agitation. She had been referred from a local general hospital after presenting there with suicidal ideation. A psychologist in the community had been managing her with psychotherapy for a number of months but her mental

state had continued to deteriorate. She had been commenced on amitriptyline 10 mg daily by her family physician 2 weeks prior to admission, which was replaced fluoxetine 20 mg daily 1 week prior to admission.

There was no history of previously diagnosed mental disorder, although frequent nocturnal awakenings had been a problem for most of her life. Obsessive–compulsive symptoms were noted during primary school years, including checking the alignment of objects on a wall and checking that power points had been switched off. As a child, the patient demonstrated age-appropriate developmental milestones. The patient's academic performance was considered to be superior throughout her school career. This was, however, interposed with a history of bullying and later depressive symptoms following a relationship breakup.

Her past medical history included facial lacerations from a dog attack in early childhood, subsequently requiring plastic surgery, tonsillectomy, adenoidectomy, and grommet insertion. There was a family history of anorexia nervosa, systemic lupus erythematosus (without cerebral lupus), but not OCD.

The patient was hospitalized and fluoxetine was increased to 80 mg daily (for her OCD and depressive features). In addition, quetiapine 350 mg daily (for her agitation) and prazosin 2 mg daily (for her nightmares) were commenced, in conjunction with group therapy and individual psychotherapy with her psychiatrist. There was a modest partial improvement in her condition.

Interestingly, the patient also complained of fleeting, nonspecific and non-reproducible sensations in her head and upper body, fatigue and intermittent perceptual disturbances, both simple auditory hallucinations in the form of tinnitus (such as a cicada) and fleeting simple visual hallucinations (such as "a man dressed in black walking past"), which she had experienced every few weeks over the year previous to her current admission. There was an awareness by the inpatient treating team of the potential for autoimmune disease to present with psychiatric symptoms in young adults, so she was referred to an immunologist who was considered a member of the multidisciplinary team. An assessment for evidence of underlying autoimmune disease was conducted.

Autoimmune serology showed a positive antinuclear antibody with a speckled pattern (ANA titer >1:640). Extractable nuclear antigen antibody testing was also positive for SSA (Ro60 and Ro52) and SSB. Serum electrophoresis showed a large polyclonal increase in gammaglobulins (22 g/L, normal 4–12 g/L) and rheumatoid factor was also elevated at 64 IU/mL. Other markers associated with systemic lupus erythematosus such as anti Sm, anti dsDNA antibodies were not elevated and complement levels were normal. Thyroid function tests, ASOT and DNAseB were normal, while syphilis serology was negative and vitamin B12 and folate levels were replete.

Further history was sought regarding clinical manifestations of Sjogren's syndrome (SS). There was a history of sicca a year prior to admission, which appeared to resolve spontaneously. Around the time of admission, a purpuric rash was noted on the legs, which was not biopsied, but demonstrated features suggestive of cutaneous vasculitis. There were no associated arthralgias or other extra-articular manifestations of SS.

Brain imaging in the form of a cerebral single photon emission computed tomography did not show evidence of cerebral hypoperfusion. Magnetic resonance imaging (MRI) showed no intracranial structural abnormalities in the cerebral hemispheres, cerebellum, brain stem, or intracranial arteries. Magnetic resonance spectroscopy, however, showed reduction in *N*-acetylaspartate (NAA) levels in both hippocampi, greater on the right side, which was suggestive of neuronal dysfunction. There were no abnormal myo-inositol or choline levels seen and no lipid or lactate peak with normal FA values throughout the cerebral hemispheres.

Further evidence of cerebral inflammation was sought through cerebrospinal fluid (CSF) analysis, which showed a pleocytosis with a white cell count of 10×10^6/L (predominantly mononuclear cells), red cell count of 2×10^6/L, elevated protein (429 mg/L, normal <400 mg/L), neopterin (24 nmol/L, normal <13 nmol/L), CSF IgG (111 mg/L, normal 1–40 mg/L), CSF IgG to albumin ratio (55%, normal 1–14%) and the presence of CSF-restricted oligoclonal bands. Antibodies to neuronal cell surface antigens including voltage gated potassium channel antibodies (LGI and CASPR2) and *N*-methyl-D-aspartate receptor antibodies were not detected in the CSF.

Three months following the initial admission, the patient developed a rash typical of urticarial vasculitis. She was commenced on prednisolone 25 mg daily and methotrexate 10 mg weekly. This was escalated to 15 mg weekly, 2 weeks subsequently. On review 2 months later, there had been a significant improvement in residual symptoms of OCD and mood disorder. The vasculitic rash was still present, but had improved in extent and frequency.

Five months following the initial admission, a worsening of depressive symptoms and suicidal ideation resulted in a further but brief hospitalization. Pulsed methylprednisolone was administered intravenously over 3 days with a significant improvement in mental state after her second dose. Psychotropic medications had been maintained continuously from the initial hospitalization, with the exception of a slight increase in quetiapine to 400 mg daily during the admission for elevated anxiety and agitation.

Given the initial improvement with immunomodulatory therapies, consideration was given to further intensifying treatment. Thus, 7 months following her initial admission, a course of plasmapheresis was given with five exchanges over a 10-day period. This was followed by intravenous immunoglobulin at 0.4 g/kg/day for 5 days and monthly maintenance doses at 0.4 g/kg on each occasion. Psychotropic medications were maintained continuously from the initial hospitalization.

A marked improvement was noted following plasmapheresis, such that her OCD symptomatology had completely resolved. There were, however, residual depressive symptoms noted in the week prior to each intravenous immunoglobulin infusion. The vasculitic rash had resolved completely. Ten months following her initial admission, the patient had recovered to the extent that she was able to enroll in a course in commercial bakery and participate in part time employment. Psychiatric treatment had remained essentially unchanged following the second psychiatric admission.

DISCUSSION

Primary Sjogren's syndrome is a chronic autoimmune disease, which predominantly manifests with symptoms of sicca, but can have many and varied extraglandular manifestations including arthralgias, peripheral nervous system (PNS), and central nervous system (CNS) manifestations. Although recognized as potentially complicating the illness, PNS and CNS symptoms are not incorporated in the currently accepted classification criteria (2), which instead rely on a combination of sicca symptoms, glandular dysfunction, serology, and histopathology to classify patients with pSS.

The suspicion of associated autoimmune disease in this case was triggered by non-specific physical and neurological symptoms as well as the severity and treatment refractory nature of the psychiatric illness. Subsequent history, examination, and serology was most consistent with SS, and significant derangements in CSF markers (pleocytosis, elevated neopterin, CSF-restricted oligoclonal bands) indicated the presence of a neuroinflammatory process.

The association between pSS and OCD was underscored by the response to immunomodulatory therapy. Initial treatment with SSRI, atypical antipsychotic, and alpha antagonist resulted in mild improvement in symptoms within 2–4 weeks. Initial immunomodulatory therapy in the form of plaquenil, methotrexate, and prednisone was commenced approximately 2.5 months later due to persistent, partially treated symptoms. This resulted in rapid improvement as would be expected with glucocorticoid treatment of autoimmune disease. Prednisone was decreased 2 weeks later due to mood related side effects; however, by this time, checking behaviors had already ceased. Probably as a result of this and the slow onset of action of methotrexate, the patient was readmitted 1 month subsequently with increased agitation, decline in mood and suicidal ideation, at which point she was treated with IV methylprednisolone. Plasmapheresis was commenced approximately 2 months after this admission. This produced the most definitive change in mental state, with very rapid improvements within days of treatment, in the context of psychotropic medication being unchanged. On the basis of this, there appears to have been an association between pSS and OCD in this patient, although we cannot be conclusive about its nature. It is possible that pSS directly gave rise to OCD symptomatology, however, the patient's premorbid vulnerability and isolated obsessive–compulsive characteristics might suggest that underlying OCD was exacerbated pSS.

Although the details presented in this case report do not completely fulfill the classification criteria for pSS, we contend that the serological evidence and response to immunomodulatory therapy makes the diagnosis of pSS most likely. The lack of lupus associated serological markers makes a diagnosis of SLE less likely, and pediatric autoimmune neuropsychiatric disorder associated with group A streptococci is also unlikely due to the age of onset, lack of history of streptococcal infections, and normal ASOT and DNAseB. Nevertheless, this cannot be completely excluded. Another weakness of this case report is the absence of objective markers of psychiatric improvement, which could have been addressed with serial measurements on the YBOCS or other psychiatric evaluation tool.

The neuroimaging is also worthy of discussion in this case. CNS disease in pSS can either be focal or diffuse, and although MRI is able to detect focal disease, it may not always detect diffuse disease (3) as in our patient. The MR spectroscopy results are also interesting as hippocampal changes are not typical of functional neuroimaging studies, which implicate the cortico-striatal-thalamo-cortical circuit in the pathophysiology of OCD (4). Despite this, hippocampal MR spectroscopic abnormalities have been previously found in OCD patients (5) and it is possible that the changes seen in our patient reflect this. Alternatively, low hippocampal NAA levels have been found previously in depressed adolescents (6). The hippocampal abnormalities may have correlated clinically with subjective cognitive dysfunction (the patient was unable to complete her higher school leaving examinations), although this was not formally tested. MR spectroscopic changes have also been found in patients with pSS, most notably decreased NAA levels or NAA/Cr ratios in the subcortical frontal and basal ganglia white matter (7). Although the subject of this report did not demonstrate these changes, a large proportion of patients in the aforementioned study also failed to show these MRS changes. More studies are required to characterize the range of MR spectroscopic abnormalities in pSS and their associated disease manifestations.

The published literature reports significant variability in estimates of CNS involvement in pSS with manifestations such as spinal cord dysfunction, seizures, migraines, and movement disorders occurring in 0–60% of patients (3). Estimates of the prevalence of neuropsychiatric disease in pSS are even more varied, ranging from 7 to 100% of individuals with pSS (3). This probably relates to difficulties in attributing psychiatric disorders to pSS as they may (1) arise concurrently, but independently of pSS, (2) arise in response to pSS, or (3) arise directly as a result of the pSS disease process. Although more research and clarification is required regarding the true incidence of neuropsychiatric disease due to pSS, data suggest that it may not be as rare as first thought, and may be missed due to subclinical disease, change in acuity over time or a low index of suspicion.

Attributing psychiatric disease to pSS is also complicated by variability in clinical presentation. Women with pSS rated themselves higher on symptoms of somatization, depression, anxiety, and paranoid ideation than healthy controls (8). Other studies have reported cognitive impairment (9), psychosis (9, 10), mood disorders (11–13), anxiety disorders (12, 13), somatization disorders (12), and dissociative states (12) or a combination of these in pSS patients. Despite this, very few published studies comprehensively phenotype the neuropsychiatric manifestations of pSS, disease course, and response to treatment. This information might better allow clinicians to differentiate pSS-related neuropsychiatric disease from primary psychiatric disease.

One final point of discussion is the recognition that CNS injury secondary to pSS may lead to permanent and irreversible damage. Some of the suggested mechanisms include direct CNS infiltration by mononuclear cells (14), vascular injury due to anti-Ro antibodies (15) and small vessel vasculitis (16). Because

of this, it is particularly important that CNS manifestations of pSS be recognized and treated promptly.

In conclusion, we have presented the case of a patient with OCD and major depressive disorder that was associated with primary Sjogren's syndrome. Although the attribution of mental disorder to pSS may be difficult, the potential for persistent, undertreated psychiatric morbidity, or permanent neurological damage should prompt timely referral of suspected cases to an immunologist or rheumatologist skilled in this field for further assessment and consideration of immunomodulatory therapy.

ETHICS STATEMENT

Informed patient consent was obtained for purposes of publishing this case report.

AUTHOR CONTRIBUTIONS

The manuscript was prepared by LO and revised by GG and DB, both of whom also cared for the patient who was the subject of this case report.

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CASE REPORT
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The Role of Infection and Immune Responsiveness in a Case of Treatment-Resistant Pediatric Bipolar Disorder

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A case of psychotropic-resistant pediatric bipolar disorder is presented. Both awareness and proper treatment of previously unrecognized infections and their effects on the immune system were very important in stabilizing the patient's psychiatric symptoms.

Keywords: pediatric bipolar disorder, treatment resistance, inflammation, infections and psychiatric illness, molecular mimicry

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INTRODUCTION

There is a growing recognition that the immune system plays an important role in the development and perpetuation of certain neuropsychiatric disorders (1, 2). Increased awareness of factors such as inflammation and autoimmunity or immune hyperresponsiveness may facilitate new understanding of mental disorders and potentially provide new insight into treatment-resistant patients. This case report provides an example of a possible link between pediatric bipolar disorder and immune-mediated processes. The presentation is intended to inform future discussions of novel treatment interventions targeted to neuron inflammatory pathways.

BACKGROUND

Identifying Information

P, a 16-year-old high school student who resides with his parents and two younger siblings, ages 13 and 11 years, has been in treatment with his present psychiatrist since the age of 10 years. Upon initial presentation, history was positive for longstanding difficulty with mood shifts, oppositional behavior, and verbal and physical aggressive outbursts that have been refractory to multiple medication trials.

History of Present Illness

Despite the product of an unremarkable pregnancy and delivery, his parents felt he was somewhat different from birth. He did not like being held and was hard to soothe. Once ambulatory, P appeared very curious, overactive, and frequently into "mischief." In toddlerhood, he also began to exhibit difficulty with moodiness and oppositional defiant behaviors with peers as well as authority figures. He could be extra silly, but more often easily unhappy and angry. In nursery school, he would hit and bite peers when frustrated and was defiant with teachers. Behavioral interventions were successful.

By the time he was in the first grade, since his interactions were fraught with conflict, following evaluation, he was deemed eligible for special services in the academic setting. P was given a 1:1

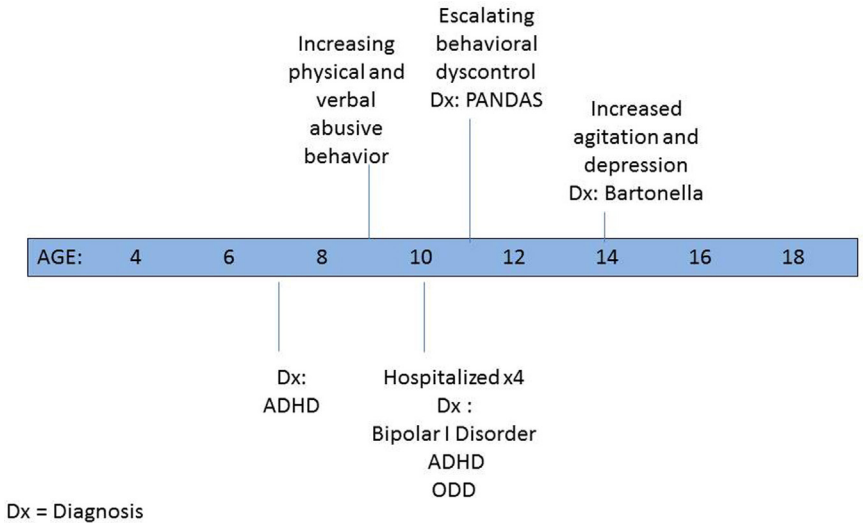

FIGURE 1 | **Psychiatric timeline (in years)**.

aide both in school to help keep him on task and on the bus to prevent behavioral outbursts. Psychological counseling was initiated at the age of 7 years and soon thereafter a psychiatrist placed him on medication for a newly diagnosed attention-deficit hyperactivity disorder (ADHD) (3) (see **Figure 1**). By the age of 10 years, despite multiple medication trials and family and individual psychotherapy, P's behavior escalated to the point that he was verbally and physically violent and uncontrollable at home. Between ages 10 and 11 years, he was psychiatrically hospitalized for more than a week on four occasions because of his aggressive threats and actions. He repeatedly stated that he wanted to kill his family and himself. Discharge diagnoses included bipolar I disorder—mixed, ADHD—combined type, and oppositional defiant disorder.

P's behavior appeared treatment resistant with minimal improvement despite multiple medication trials prior to and during hospitalizations. These included dexmethylphenidate hydrochloride extended-release tablets, methylphenidate hydrochloride extended-release tablets in a variety of preparations, mixed dextroamphetamine salts in extended release, risperidone, fluoxetine, and aripiprazole. Parents noted that he became more angry and agitated when he was on the stimulants. In an attempt to reevaluate his situation, soon after hospital discharge all psychotropic medication was stopped.

Over a 6-week period, post medication discontinuation, he started to become less volatile and violent, but exhibited rapid cycling behaviors such as laughing a lot, talking fast, exhibited increased energy, suddenly became very interested in cleaning his room, had problems falling asleep, and became preoccupied with and talked about Star Wars incessantly. He would then shift to periods of anger and destructive behavior and verbalize that he felt everything was wrong. Initiation of carbamazepine and low-dose olanzapine decreased the rapid cycling and eliminated the violent physical behavior, but his negative and critical speech at home continued. He did minimal schoolwork, was socially withdrawn, and described by mother as joyless, rarely smiling, and verbalized being unhappy. His appetite was poor. He said he hated most food, and although he grew taller he had not gained weight in 2 years.

Past Medical History

He was hospitalized for 48 h at 4 days old due to a low-grade fever (99°) of unknown origin.

Family History

Psychiatric History

Maternal side: bipolar disorder in at least three generations, postpartum depression, alcoholism, obsessive-compulsive disorder, anger management issues.

Paternal side: strongly positive for alcoholism.

Immune Dysfunction

Maternal side: mother diagnosed with chronic fatigue syndrome; Hashimoto's thyroiditis in a first cousin; thyroid dysfunction of unclear etiology in another relative.

Paternal side: a paternal grandfather with aplastic anemia.

Both parents had relatives with adult-onset diabetes mellitus.

COURSE OF TREATMENT

A change in antipsychotic to risperidone and titration of his carbamazepine dose helped decrease the frequency of his aggressive outbursts temporarily. Of note, intermittently over the years in treatment, P experienced transient episodes of urinary frequency and urgency despite a negative urine culture. In addition, he occasionally exhibited transient motor tics (eye blinking, mouth opening, and a shoulder shrug).

After 8 months, his mood and behavior worsened and residential treatment was considered. Laboratory testing looking for evidence of a possible infectious trigger was done. Streptococcal and mycoplasma titers were drawn even though his illness was chronic, not acute in onset as seen in pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) or pediatric acute-onset neuropsychiatric syndrome (PANS) (4). Antistreptolysin O and anti-DNAase B titers were markedly elevated at 790 (normal 0–200) and 1,340 (normal 0–170). Throat culture was negative. His pediatrician prescribed a 20-day course of amoxicillin and clavulanate potassium. Psychiatric medication remained unchanged. Within a few days, P's agitation lessened and he became happier, more affectionate, and more engaged in his schoolwork. Upon antibiotic discontinuance, his irritability returned, and resumption of treatment helped him regain control. After 6 weeks of treatment, repeat ASO was 791 with an anti-DNAase B titer of 1,090, i.e., basically unchanged. A Cunningham panel to check for evidence of immune dysfunction and antineural antibodies was unremarkable (5). A lithium trial was unsuccessful. Given his resumption of his behavioral problems soon after the completion of the second antibiotic trial, an immunology consult with a PANDAS/PANS specialist was obtained.

Consultation resulted in the diagnosis of autoimmune encephalopathy as well as an active sinus infection. P was placed on amoxicillin and clavulanate potassium, azithromycin, and a brief course of prednisone starting at 30 mg and tapered over 1 week. When next seen here, 3–4 weeks after initiating treatment, there was a dramatic positive change in P's functioning.

P's bossy and irritable behavior would intermittently return when a family member or a peer became ill. The addition of ibuprofen 300 mg bid often helped him become less aggressive but still annoying. If these symptoms continued and escalated, a change in his antibiotics was often efficacious. Occasional use of a brief steroid trial also appeared beneficial.

In the middle of eighth grade, P became severely angry and depressed and developed hyperacusis. Evidence of a *Bartonella* infection on fluorescent–*in situ* hybridization (FISH) testing was found and subsequently treated (6). Psychiatric symptoms lessened, and his noise sensitivity dissipated. Maintenance on risperidone and carbamazepine continued throughout treatment.

Currently, P is functioning fairly well outside the home and his bossiness and negativity with his family are nowhere near where they were at initial presentation. He remains on his psychiatric medication, along with amoxicillin and clavulanate potassium for streptococcal prophylaxis.

DISCUSSION

Although this case of childhood bipolar disorder has a strong genetic component, it also provides some support for the hypothesis that the various psychiatric symptoms (that are consistent with bipolar disease) may have been exacerbated by abnormal neuro-immune responses initiated by systemic infections. In this article, the relevant infections being group A beta-hemolytic *Streptococcus* and *Bartonella*. Despite intense antibiotic treatment, it took approximately 3 months for the ASO titers to begin to decrease. This ongoing evidence of immune activation could be seen as consistent with Bechter and Mueller's hypothesis that psychiatric symptoms may be the result of a low-level "smoldering" inflammatory process in the central nervous system (CNS) or what could be viewed as "a chronic mild encephalitis or encephalitic process" (7). Younger and Bouboulis speculated that the sustained high streptococcal titers as occurred in this case could to be the result of reinfection, or slower rates of the decline in antibody rise as well as possibly a more potent immune response (8).

Currently, inflammation in mood disorders is an active area of exploration (9–11). Anti-inflammatory agents such as acetylsalicylic acid, nonsteroidal anti-inflammatory agents (e.g., ibuprofen), minocycline, and cox-2 inhibitors have been studied as adjuvants to bolster the effects of treatment in adults with depression (12). Some caution has been raised about the combination of ibuprofen and selective serotonin reuptake inhibitors as there is some evidence of increased risk of gastrointestinal adverse reactions (13). The use of ibuprofen in P's case appeared to be very helpful especially when he was exposed to other children with illness. The recrudescence of his symptoms can be postulated to be the result of a hyperimmune response to stimulation (other children illness) that was modified and somewhat contained by ibuprofen's anti-inflammatory action. Care was taken to monitor for any evidence of possible increased bleeding, i.e., increased bruising, nosebleeds, and hematuria. Use of ibuprofen was also time limited (i.e., generally not more than a few days or weeks at a time), and complete blood counts, bleeding parameters, and renal and hepatic function tests were monitored if there was any indication of a problem.

The recurrent positive response of psychiatric symptomatology to brief steroid trials lends support to the hypothesis that their anti-inflammatory actions were helpful in intervening in his extreme agitation. In this case, it helped P to control his violent and destructive behavior as opposed to more common use of steroids for out-of-control physical symptoms.

Although these infections may seem temporally related to the patients' difficulties that does not mean causality. On a few occasions when antibiotics were stopped, P's functioning quickly deteriorated with responsiveness upon resumption of the antibiotic medications. This raises the questions of whether or not there was reinfection, or perhaps a remnant of the previously treated infection perpetuating ongoing antibody production? Current maintenance on antibiotic treatment is used to minimize future exposure to infectious triggers that could potentially reactivate the immune system and cause an augmented autoimmune response. For P, this immune hyperstimulation could result in another psychiatric symptom flare-up.

An important question in the interconnections of infections, immune dysfunction, autoimmunity, and psychiatric illness is how does the peripheral immune system penetrate or communicate with the CNS which is an area protected by the blood–brain barrier (BBB). Of note, P was first diagnosed with a sinus infection when he was initially seen by the immunologist at the age of 11 years and he experienced intermittent recurrences. The existence of any sinusitis prior to the first

visit is unknown. The high preponderance of sinus infections in bipolar youth has been previously noted by this author in a previous text (14).

There is some evidence that nasal lymphoid tissue may play a role in communication between the peripheral immune system and immune activity in the CNS. Dileepan et al. found that reinoculating mouse nasal lymphoid tissue with group A *Streptococcus* (GAS) resulted in "GAS-specific Th17 cells" migrating "into the brain through the cribriform plate along olfactory sensory axons and induce BBB breakdown and IgG extravasation" (15). This is a significant finding as the investigators were able to find GAS-specific TH17 cells in tonsils of individuals naturally exposed to GAS. Whether or not the human nasal lymphoid tissue is part of the path to the brain in the development of some autoimmune illnesses (especially those with neuropsychiatric manifestations) needs more investigation.

P did not have PANDAS or PANS due to the chronic nature of his illness. The overlap of several features seen in youth with PANDAS/PANS and pediatric bipolar disorder has been reported previously (16). P exhibited some symptoms that are seen in youngsters with the former named disorders including intermittent urinary symptoms, transient motor tics, and sudden onset of hyperacusis. These symptoms may be related to the immune dysfunction and part of a mild encephalitic process. Further exploration of the mechanism of initiation and perpetuation of these symptoms is needed. An additional commonality between the two groupings of disorders is the significant number of individuals with immune disturbances in P's pedigree and the heightened number of individuals with immune disturbances in the family histories of PANDAS patients (17).

The concept of molecular mimicry in which the mimicry of host antigens by infectious agents may cause cross-reactive autoimmune responses to epitopes within host proteins has been used to explain autoimmune dysfunction in some illnesses (e.g., PANDAS, post-viral myocarditis) (18). The Cunningham Panel whose purpose is to measure "the level of circulating antibodies directed against antigens concentrated in the brain, and measures the ability of these and other autoantibodies to increase the activity of an enzyme (CaMKII) that upregulates neurotransmitter in the brain" was unremarkable in this case (5). This test focuses on four possible autoantibodies directed against specific neuronal antigens, including: dopamine D1 receptor, dopamine D2L receptor, lysoganglioside GM1, and tubulin. An unremarkable result may be because other significant neural autoantibodies are not checked in this testing panel.

There are a number of different species of the Gram-negative bacteria *Bartonella* that can cause illness in animals and human populations, but it has been considered more of an opportunistic type of infection (19). Three types are responsible for most of the *Bartonella* infections seen in people: *Bartonella henselae* (Cat Scratch Fever), *Bartonella quintana* (Trench fever), and *Bartonella* Bacilliformi (Carrion's disease). In P's case, FISH testing identified the pathogen as *Bartonella* but it is not set up to identify the individual species (6).

In humans, *Bartonella* infections are associated with a variety of neuropsychiatric manifestations and can affect small blood vessels in the CNS. Symptoms attributed to Bartonellosis can include: mild cognitive impairment; white matter "subcortical disconnection"; impaired executive functioning; working memory impairment; processing speed delay; mood lability; severe agitation; panic disorder and treatment-resistant depression (20, 21). A number of autoimmune diseases have been known to be associated with Bartonellosis including autoimmune thyroiditis, Systemic Juvenile Rheumatoid Arthritis, vasculitis, glomerulonephritis, autoimmune hemolytic anemia, transverse myelitis, Henoch Schonlein Purpura, and Guillain–Barré Syndrome (22, 23).

CONCLUSION

This case illustrates how the interplay of infections and subsequent aberrant immune responses contributed to the manifestations of childhood-onset bipolar disorder in one youngster. It also raises the question of what is the meaning treatment resistance? Could it be that we are not looking at the correct causalities or all the relevant factors involved in the individual case? Although genetic, infectious, and immunologic influences are important factors in the development of psychiatric illness, the significance of epigenetic and psychosocial factors (peer groups, education attained, family functioning) should not be underestimated. Future controlled research may be warranted to elucidate the nature of the interrelationship of pediatric bipolar symptomatology, infectious illness, and immune/autoimmune responses.

ETHICS STATEMENT

Case report contains disguised identifying data and has been written up with approval from the family.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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