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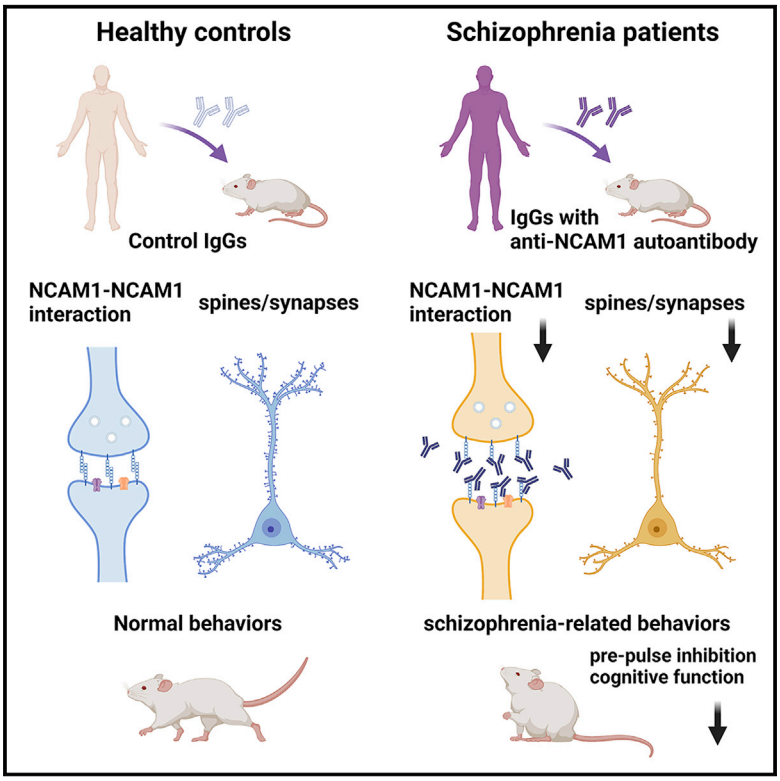
Autoantibodies against NCAM1 from patients with schizophrenia cause schizophrenia-related behavior and changes in synapses in mice

Graphical abstract



Highlights

- Some patients with schizophrenia are positive for anti-NCAM1 autoantibodies
- Anti-NCAM1 antibody from schizophrenia patients inhibits NCAM1-NCAM1 interactions
- Anti-NCAM1 antibody from schizophrenia patients reduces spines and synapses in mice
- Anti-NCAM1 antibody from patients induces schizophrenia-related behavior in mice

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In brief

Shiwaku et al. show that autoantibodies against NCAM1 from schizophrenia patients cause schizophrenia-related behavior and changes in spines and synapses in mice. These autoantibodies may cause symptoms of schizophrenia and can therefore be regarded as a therapeutic target for patients who are positive for anti-NCAM1 autoantibodies.

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Autoantibodies against NCAM1 from patients with schizophrenia cause schizophrenia-related behavior and changes in synapses in mice

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SUMMARY

From genetic and etiological studies, autoimmune mechanisms underlying schizophrenia are suspected; however, the details remain unclear. In this study, we describe autoantibodies against neural cell adhesion molecule (NCAM1) in patients with schizophrenia (5.4%, cell-based assay; 6.7%, ELISA) in a Japanese cohort (n = 223). Anti-NCAM1 autoantibody disrupts both NCAM1-NCAM1 and NCAM1-glial cell line-derived neurotrophic factor (GDNF) interactions. Furthermore, the anti-NCAM1 antibody purified from patients with schizophrenia interrupts NCAM1-Fyn interaction and inhibits phosphorylation of FAK, MEK1, and ERK1 when introduced into the cerebrospinal fluid of mice and also reduces the number of spines and synapses in frontal cortex. In addition, it induces schizophrenia-related behavior in mice, including deficient pre-pulse inhibition and cognitive impairment. In conclusion, anti-NCAM1 autoantibodies in patients with schizophrenia cause schizophrenia-related behavior and changes in synapses in mice. These antibodies may be a potential therapeutic target and serve as a biomarker to distinguish a small but treatable subgroup in heterogeneous patients with schizophrenia.

INTRODUCTION

Patients with schizophrenia are symptomatically and genetically heterogeneous, and it is assumed that there are various underlying pathological mechanisms.[1] However, there are no biomarkers for these heterozygous subgroups, and elucidation of pathological mechanisms underlying treatment resistance in patients and/or symptoms is insufficient.

Genetic analysis of schizophrenia has revealed risk genes related to synapse, chromatin modification, and the immune system.[2–4] In particular, mutations in the major histocompatibility complex (MHC) region carry the highest risk of schizophrenia.[2] The MHC region also plays a role in autoimmunity. In fact, there is an epidemiological relationship between schizophrenia and autoimmunity;[5] however, the role of autoimmune responses in schizophrenia remains unclear.

Autoantibodies are a primary driver of autoimmunity. Indeed, autoantibodies specific for synaptic membrane molecules have been found in patients with encephalitis.[6,7] Some autoantibodies specific for these molecules cause psychotic symptoms in those with encephalitis. One of the most extensively studied autoantibody-mediated forms of encephalitis is anti-N-methyl-*d*-aspartate (NMDA) receptor antibody encephalitis, which is associated with schizophrenia-related symptoms.[8] In addition, anti-$GABA_AR\alpha1$ receptor antibody encephalitis causes psychotic symptoms.[9] Although these autoantibodies are also present in patients with schizophrenia, it is unclear whether they play a role in the symptoms of schizophrenia.[10,11]

When searching for autoantibodies related to the pathology of schizophrenia, there are several prerequisites: the target antigen must be (1) a membrane molecule expressed in the nervous system (autoantibodies do not usually enter cells *in vivo*) and (2) a molecule implicated in schizophrenia and whose function would be affected by autoantibodies or at least be involved in synaptic function. From this point of view, we used a cell-based assay and ELISA to identify novel autoantibodies that may contribute to schizophrenia

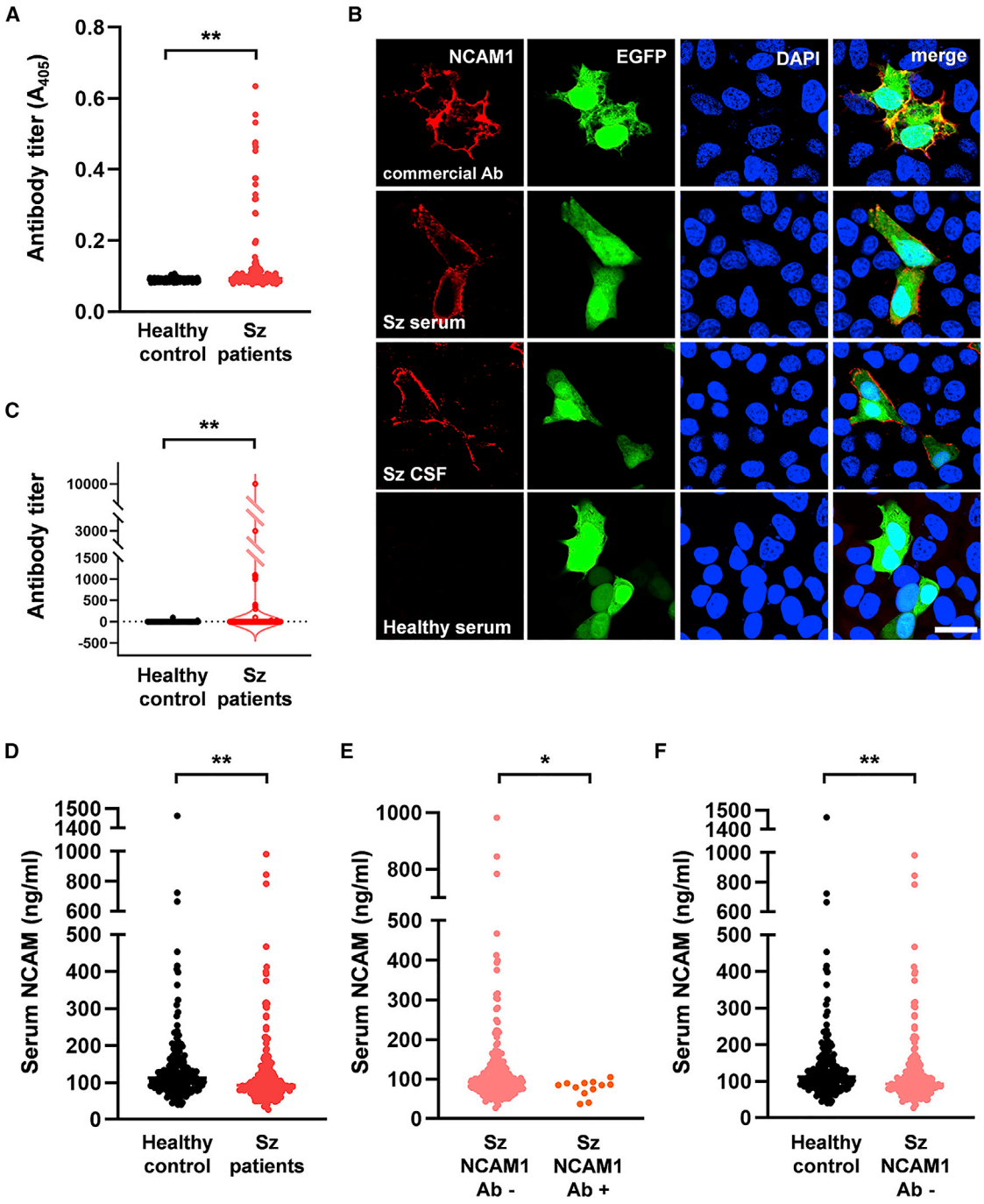

Figure 1. Identification of anti-NCAM1 autoantibodies

(A) Titers of anti-NCAM1 autoantibodies in serum by ELISA. **p < 0.01 (n = 201, healthy controls; n = 223, patients with schizophrenia; Mann-Whitney U test).

(B) Immunocytochemistry using a commercial anti-NCAM1 antibody, serum and CSF from schizophrenia patient 1, and serum from healthy controls. NCAM1 and EGFP were expressed from a plasmid. Confocal images show antibodies bound to the membrane of EGFP-positive HeLa cells. Similar results were obtained for all anti-NCAM1 antibody-positive patients with schizophrenia (Figure S1B). Antibodies in serum did not react with EGFP because (1) they did not react with EGFP in the nucleus and (2) they did not react with cells transfected with an empty plasmid expressing only EGFP (data not shown). Scale bar: 10 μm. Ab, antibody; Sz, schizophrenia.

(C) Titers of anti-NCAM1 autoantibodies in serum by cell-based assay. **p < 0.01 (n = 201, healthy controls; n = 223, patients with schizophrenia; Mann-Whitney U test).

(legend continued on next page)

pathophysiology. One candidate antigen was neural cell adhesion molecule (NCAM1), a synaptic adhesion molecule that supports synaptic connections by *trans*-homophilic binding.[12] NCAM1 also binds to glial-cell-line-derived neurotrophic factor (GDNF) and contributes to synapse formation.[12,13] Various studies have been conducted on NCAM1 and schizophrenia; changes in mRNA expression levels and the cleaved soluble form of NCAM1 are associated with schizophrenia.[14–19] Furthermore, NCAM1 knockout and transgenic mice with a dominant-negative form of NCAM1 show schizophrenia-related behavioral changes.[20–23] Furthermore, single-nucleotide polymorphisms (SNPs) of *NCAM1* are associated with schizophrenia.[24–26]

In this study, we identified anti-NCAM1 autoantibodies in some patients with schizophrenia. Using a disease model in which mice were administered immunoglobulin G (IgG) purified from patients with schizophrenia, we show that anti-NCAM1 autoantibodies inhibit spine and synapse formation in the frontal cortex and induce schizophrenia-related behavior.

RESULTS

Identification of anti-NCAM1 autoantibodies in patients with schizophrenia

Serum samples were obtained from 201 healthy controls (125 males and 76 females; age, 22–90 years; median, 48 years) and 223 patients with schizophrenia (112 males and 111 females; age, 16–84 years; median, 52 years). Schizophrenia was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). There were no significant differences between the groups with respect to age. All samples were tested using the ELISA and the cell-based assay. The ELISA analysis detected 15 patients with schizophrenia as positive for anti-NCAM1 autoantibodies, where two standard deviations above the mean of absorbance was defined as positive for this autoantibody (Figure 1A). None of the healthy control subjects was positive for anti-NCAM1 autoantibodies in the ELISA. The antibody titers of patients with schizophrenia were significantly high compared with those in healthy controls (Figure 1A). In the cell-based assay using HeLa cells, human NCAM1 and EGFP were expressed from a plasmid, and all transfected cells expressing EGFP showed exogenous expression of NCAM1 (Figures 1B and S1A). Twelve patients with schizophrenia (5.4%) were positive for anti-NCAM1 autoantibodies (Figures 1B, 1C, and S1B; Table 1). To test whether these patients also have other autoantibodies against other synaptic molecules, we induced the expression of NLGN1, NLGN2, NLGN3, NLGN4, NRXN1, NRXN3, ephrin B1–B3, ERBB4, NRG1, NR1, NR2, and $GABA_AR\alpha1$ in the same cell-based assay approach. However, we found no autoantibodies against these molecules in these 12 schizophrenia patients with anti-NCAM1 autoantibodies (data not shown). Among these 12 patients, 11 patients with schizophrenia were detected as being positive for anti-NCAM1 autoantibody by both ELISA and cell-based assay. Two healthy controls (a male; age, 26; antibody titer, 1:30; and a female with a past medical history of breast cancer; age, 46; antibody titer, 1:100) were positive for anti-NCAM1 antibodies (1.0%) in the cell-based assay. The antibody titers of patients with schizophrenia were significantly high compared with those in healthy controls (i.e., 1:1,000–10,000; Figure 1C). Anti-NCAM1 autoantibodies were also present in the cerebrospinal fluid (CSF) in anti-NCAM1 autoantibody-positive patients with schizophrenia (Figures 1B and S1C; Table 1). Protein concentration and leukocyte numbers of CSF in these patients were normal.

NCAM1 is highly expressed in the nervous system.[18] We performed western blot analysis to verify the expression of NCAM1 in the mouse brain and found that NCAM1 was indeed expressed at very high levels compared with peripheral organs (Figure S1D). NCAM1 is a cell adhesion molecule in the synapse with a transmembrane region.[12,18] The extracellular region of NCAM1 is cleaved by ADAM10 and ADAM17,[27,28] and a small amount of its soluble form has been found in serum. Because changes in soluble NCAM1 have been reported in patients with schizophrenia,[16,17,19] we tested whether autoantibodies against NCAM1 affect the soluble form of NCAM1 in serum. We performed ELISAs to analyze soluble NCAM1 in serum and found that soluble NCAM1 is significantly reduced in patients with schizophrenia (Figure 1D). Furthermore, soluble NCAM1 in serum was significantly reduced in patients with schizophrenia with anti-NCAM1 autoantibodies detected by the cell-based assay compared with patients without anti-NCAM1 autoantibodies (Figure 1E). It is also noted that the concentration of serum-soluble NCAM in anti-NCAM1 autoantibody-negative patients with schizophrenia was still significantly lower than those of healthy controls (Figure 1F). Although these results indicate an association between anti-NCAM1 autoantibodies and soluble NCAM, scatterplot analysis of NCAM1 titers by ELISA and concentrations of soluble NCAM did not show a significant relationship (Figure S1E). Therefore, the consequences of the immune complex may be more complicated than anticipated.

The clinical features of the 12 patients with anti-NCAM1 autoantibodies detected by cell-based assay are described in Table 1. There were no distinct psychiatric or neurological symptoms, including delirium and encephalitis, in these patients compared with other patients. Furthermore, there was no common past medical history, such as cancer or autoimmune disease, shared among the patients. However, psychiatric symptoms, including hallucinations and delusions in these patients, were refractory to antipsychotics.

(D) ELISA analysis of serum-soluble NCAM in healthy controls (n = 201) and patients with schizophrenia (n = 223). **$p < 0.01$ (Mann-Whitney U test).
(E) ELISA analysis of serum-soluble NCAM in schizophrenia patients with (n = 211) or without (n = 12) anti-NCAM1 autoantibodies defined by cell-based assay. *$p < 0.05$ (Mann-Whitney U test).
(F) ELISA analysis of serum-soluble NCAM in healthy controls (n = 201) and schizophrenia patients without anti-NCAM1 autoantibodies (n = 211) defined by cell-based assay. **$p < 0.01$ (Mann-Whitney U test).

Table 1. Clinical characteristics of antibody-positive patients

Case no./sex/age	Illness duration (years)	Antibody titer (CBA) (serum/CSF)	EEG	Neuroimaging	PANSS score, comorbidity
1/F/56	23	10,000/10	generalized slowing (basic rhythm 7 to 8 Hz)	MRI, no special notes	total, 102 P, 27; N, 22; G, 53
2/F/81	45	1,000/5	NA	MRI, no special notes	total, 88 P, 21; N, 20; G, 47
3/M/48	18	1,000/5	normal	MRI, no special notes	total, 86 P, 22; N, 20; G, 44 chronic hepatitis C
4/F/50	40	100/1	normal	MRI, no special notes	total, 83 P, 22; N, 18; G, 43
5/F/44	24	30/NA	NA	MRI, no special notes	total, 87 P, 22; N, 20; G, 45
6/F/26	10	300/2	normal	MRI, no special notes	total, 75 P, 25; N, 11; G, 39
7/M/58	10	30/NA	normal	MRI, no special notes	total, 98 P, 27; N, 22; G, 49
8/F/16	1	1,000/5	normal	MRI, no special notes	Total, 88 P, 22; N, 21; G, 45
9/F/70	33	300/2	normal	MRI, no special notes	total, 82 P, 21; N, 20; G, 41
10/M/41	25	300/2	normal	MRI, no special notes	total, 86 P, 25; N, 22; G, 49
11/F/64	43	1,000/5	normal	MRI, no special notes	total, 84 P, 22; N, 21; G, 41
12/F/57	30	3,000/5	normal	MRI, no special notes	total, 90 P, 23; N, 22; G, 45

CBA, cell based assay; CSF, cerebrospinal fluid; EEG, electroencephalogram; F, female; G, global score; M, male; MRI, magnetic resonance imaging; N, negative symptom score; NA, not available; P, positive symptom score; PANSS, positive and negative syndrome scale.

The medications administered to these patients are listed in Table S1.

The main epitope recognized by anti-NCAM1 antibodies in schizophrenia resides within the Ig1 domain

To identify the epitope recognized by the anti-NCAM1 autoantibody, we constructed truncated forms of NCAM1 (Figure 2A). The extracellular region of NCAM1 comprises five N-terminal immunoglobulin domains (Ig1–Ig5) and two fibronectin type III domains (FN3). The cell-based assay revealed that serum from 12 patients with schizophrenia reacted with the truncated forms of ΔIg2 and ΔIg2–5, which lack the Ig2 domain and Ig2–5 domains, respectively; however, they did not react with those of ΔIg1 and ΔIg1–5, which lack the Ig1 domain and Ig1–5 domains, respectively (Figures 2B and 2C). These binding patterns were confirmed by western blotting (Figure 2D). These data indicate that the main epitope region resides within the Ig1 domain.

The polysialylated form of NCAM1 (PSA-NCAM) is abundant during developmental stages in the nervous system and is associated with cell migration and axonal growth.[29] Polysialylation occurs on the Ig5 domain of NCAM1.[30,31] Thus, we hypothesized that anti-NCAM1 autoantibodies also detect PSA-NCAM. Western blot analysis using the cortex of postnatal day 0 mice revealed that anti-NCAM1 autoantibodies detect both NCAM1 and PSA-NCAM (Figure S2A).

There are about 500 molecules that contain Ig domains.[32] Therefore, we analyze whether anti-NCAM1 autoantibodies cross-react with other molecules that contain Ig domains, such as NCAM2, L1CAM, and TAG1. The cell-based assay revealed that none of the anti-NCAM1 autoantibodies in 12 patients identified by the cell-based assay react with these molecules (Figures S3B–S3D). These results suggest that anti-NCAM1 autoantibodies react with an NCAM1-specific sequence.

Anti-NCAM1 autoantibodies disrupt NCAM1-NCAM1 and NCAM1-GDNF interactions

NCAM1 forms synapses through homophilic binding via immunoglobulin domains, including the Ig1 domain.[12,33] In addition, GDNF promotes development of spines through binding to NCAM1.[13,34] Thus, we hypothesized that anti-NCAM1 autoantibodies would inhibit NCAM1-NCAM1 and NCAM1-GDNF interactions. A pull-down assay showed that IgG purified from schizophrenia patient 1 inhibited NCAM1-NCAM1 and NCAM1-GDNF interactions, whereas IgG purified from healthy controls did not (Figures 3A and 3B). A pull-down assay showed that IgG purified from schizophrenia patients 2 and 3 also inhibited NCAM1-NCAM1 and NCAM1-GDNF interactions (Figures S3A and S3B).

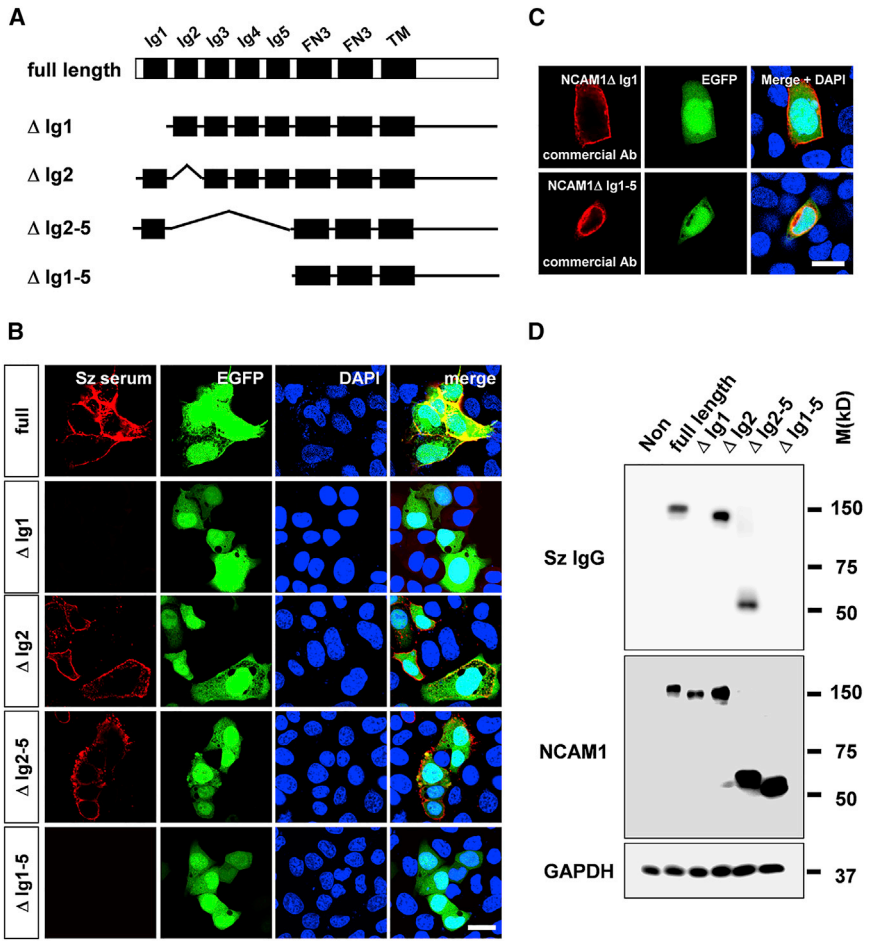

Figure 2. The main epitope recognized by anti-NCAM1 antibodies in schizophrenia resides within the Ig1 domain

(A) NCAM1 deletion constructs.

(B) Immunocytochemistry using serum from patient 1 with schizophrenia, who was positive for anti-NCAM1 autoantibodies. NCAM1 deletion constructs and EGFP were expressed from a plasmid. Similar results were obtained from all anti-NCAM1 antibody-positive patients with schizophrenia. Scale bar: 10 μm.

(C) Immunocytochemical confirmation of the expression of NCAM1ΔIg1 and NCAM1ΔIg1–5 using a commercial anti-NCAM1 antibody. Scale bar: 10 μm.

(D) Western blot analysis of deletion constructs of NCAM1 transfected into HeLa cells revealed that the main epitope recognized by anti-NCAM1 autoantibodies is in the Ig1 domain.

Anti-NCAM1 autoantibodies from a patient with schizophrenia inhibited NCAM1-Fyn-FAK-MEK1-ERK1 pathway in mice

If anti-NCAM1 antibodies found in patients with schizophrenia inhibit NCAM1-NCAM1 and NCAM1-GDNF interactions, we assumed that they would also cause abnormal molecular signaling, abnormal spine and synapse formation, and schizophrenia-related behavior in mice. To test this, IgG was purified from a patient with schizophrenia (patient 1) and an age- and sex-matched healthy subject and then injected into the CSF of mice (8 weeks of age). We then analyzed molecular signaling, spine and synapse formation, and behavior of mice at 9 weeks of age (Figure 4A). We confirmed that anti-NCAM1 autoantibodies from patients with schizophrenia reacted with NCAM1 expressed by primary cultured neurons (Figure S4A) and in the frontal cortex of mice (Figure S4B). Previous studies report that antibodies administered to mice against specific antigens within the nervous system stay in the brain for more than a week.[35,36] However, non-specific IgGs injected into the CSF transferred to serum within a day.[37] Consistent with these reports, our immunohistochemical analysis confirmed that intrathecal administration of anti-NCAM1 autoantibodies from patients with schizophrenia were still present in mice at 9 weeks of age (Figure S8B). There was no evidence of microglial activation or encephalitis (Figures S5A and S5B).

NCAM1-NCAM1 interactions as well as GDNF-NCAM interactions induce contact between the cytoplasmic domain of NCAM1 and Fyn.[34,38] This interaction activates Fyn, which in turn phosphorylates FAK on Tyr-397.[34,38] This signaling leads to phosphorylation of MEK and ERK1 and, subsequently, spine formation.[13,18] Thus, we tested whether IgG purified from patient 1 administered to mice interrupt these signalings; NCAM1-Fyn interaction and phosphorylation of FAK, MEK1, and ERK1. Using mice administered with IgG purified from patient 1 and immunoprecipitation assay, we analyzed the amount of Fyn coimmunoprecipitated with NCAM1. This revealed NCAM1-Fyn interaction is reduced in mice administered with IgG purified from patient 1 (Figure 4B). Furthermore, IgG purified from patient 1 with schizophrenia inhibited phosphorylation of FAK, MEK1, and ERK1, whereas IgG purified from a healthy subject did not (Figures 4C and 4D). These results indicate that inhibition of NCAM1-NCAM1 and NCAM1-GDNF interactions by anti-NCAM1 autoantibodies impairs the interaction of NCAM1-Fyn and reduces phosphorylation of FAK, MEK1, and ERK1.

Anti-NCAM1 autoantibodies from a patient with schizophrenia reduced the number of spines and synapses in frontal cortex in mice

Inhibiting phosphorylation of FAK, ERK1, and MEK1 and interrupting *trans*-homophilic NCAM1 interactions between the pre- and post-synapse, a process that maintains synapses,[12] indicates that anti-NCAM1 autoantibodies cause changes in spines and synapses. To examine this, we performed two-photon analysis of mice that received patient IgG intrathecally. Neurites and spines were visualized using adeno-associated virus 1 (AAV1)-EGFP, driven by the synapsin I promoter

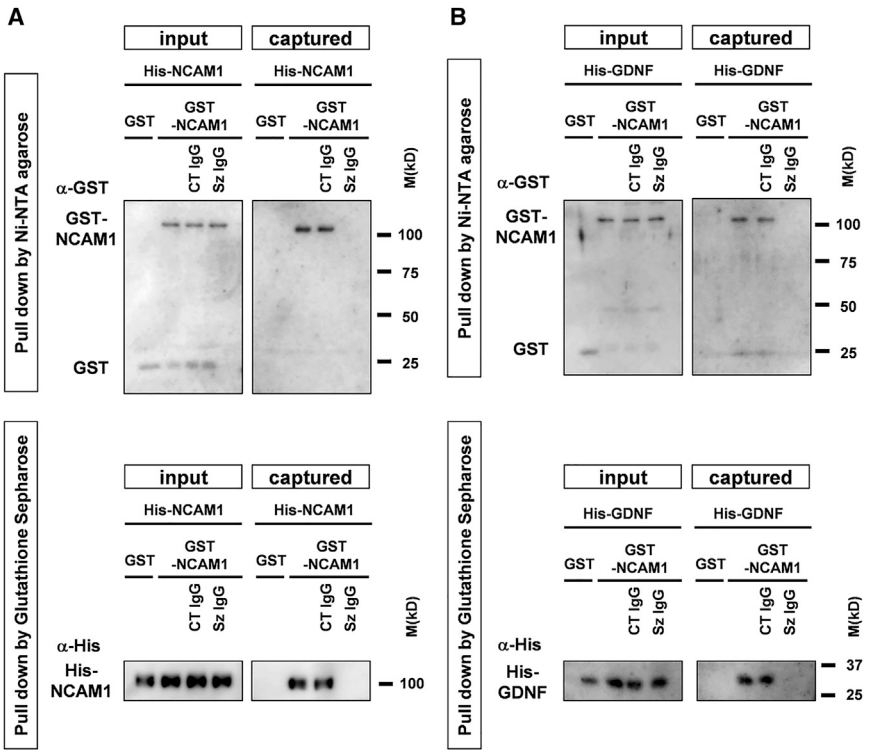

Figure 3. Anti-NCAM1 autoantibodies disrupt NCAM1-NCAM1 and NCAM1-GDNF interactions

(A) Pull-down assay confirming that IgG purified from a patient with schizophrenia who was positive for anti-NCAM1 autoantibodies disrupts NCAM1-NCAM1 interactions. His-tagged proteins were pulled down by Ni-NTA-agarose, and GST-tagged proteins were pulled down by Glutathione Sepharose.

(B) Pull-down assay showing that IgG purified from a patient with schizophrenia who was anti-NCAM1 autoantibody-positive disrupts the NCAM1-GDNF interaction. His-tagged proteins were pulled down by Ni-NTA-agarose, and GST-tagged proteins were pulled down by Glutathione Sepharose.

(AAV1-SYN-EGFP); axon terminals in contact with a spine were visualized using AAV2-VAMP2-mCherry (Figure 4A). As expected, mice treated with IgG from patient 1 with schizophrenia showed reduced spines and synapses in the frontal cortex; these changes were not seen in mice receiving IgG from a healthy subject (Figures 4E, 4F, and S6).

Anti-NCAM1 autoantibodies from a patient with schizophrenia cause schizophrenia-related behavior in mice

To test whether anti-NCAM1 autoantibodies trigger symptoms of schizophrenia, we performed behavioral analysis of autoantibody-treated mice. Administration of IgG purified from patient 1 with schizophrenia reduced cognitive function in the Y maze test (Figure 4G; Videos S1 and S2). Moreover, mice treated with IgG from a patient with schizophrenia were deficient in pre-pulse inhibition (Figure 4H), which is an established endophenotype of schizophrenia.[39–42] Mice treated with IgG from a healthy subject showed normal pre-pulse inhibition. Mice receiving IgG from patient 1 with schizophrenia showed no abnormal locomotor activity, anxiety behavior, or social interaction in an open-field test, an elevated plus maze test, or a three-chamber test, respectively (Figures S7A–S7H).

Absorption and removal of anti-NCAM1 antibodies improved the molecular, spinal, and behavioral changes

To confirm that it was the anti-NCAM1 antibodies among IgGs purified from patient 1 that inhibited phosphorylation, reduced the number of spines and synapses, and induced schizophrenia-related behavior in mice, we performed an absorption experiment in which anti-NCAM1 antibodies were removed from IgGs purified from patient 1 with schizophrenia prior to administration to mice (absorbed Sz IgG). Adsorption and removal of anti-NCAM1 antibodies by glutathione S-transferase (GST) pull-down were confirmed in a cell-based assay and by immunohistochemistry (Figures S8A and S8B). Phosphorylation, spines and synapses, and behavior improved after the absorption experiment (Figures 4B–4H). These results confirm that anti-NCAM1 antibodies from patient 1 alter phosphorylation and induce synaptic changes and schizophrenia-related behavior in mice.

Anti-NCAM1 autoantibodies from patients with schizophrenia cause schizophrenia-related behavior and changes in synapses in mice

Finally, to confirm that the results from schizophrenia patient 1 could be observed in other patients who are positive for anti-NCAM1 autoantibodies, we conducted two-photon analysis and behavioral analysis using IgG purified from schizophrenia patients 2 and 3 (Figure 5A). IgG from these patients also reduced spine and synapse numbers in the frontal cortex, as well as inducing cognitive impairment and a deficiency in pre-pulse inhibition (Figures 5B–5E).

DISCUSSION

In this study, we identified anti-NCAM1 autoantibodies in patients with schizophrenia. Administration of IgG antibodies isolated from patients with schizophrenia interrupted NCAM1-NCAM1 and NCAM1-GDNF interactions. These interruptions affect the NCAM1-Fyn-FAK-MEK1-ERK1 signaling pathway and inhibited NCAM1-Fyn interactions as well as the phosphorylation of FAK, MEK1, and ERK1. Furthermore, direct administration of anti-NCAM1 antibodies into the CSF of mice reduced the number of spines and synapses in the frontal cortex and induced cognitive impairment and a deficiency in pre-pulse inhibition, which is an established endophenotype of schizophrenia in humans and mice.[41–43] Indeed, cognitive impairment and

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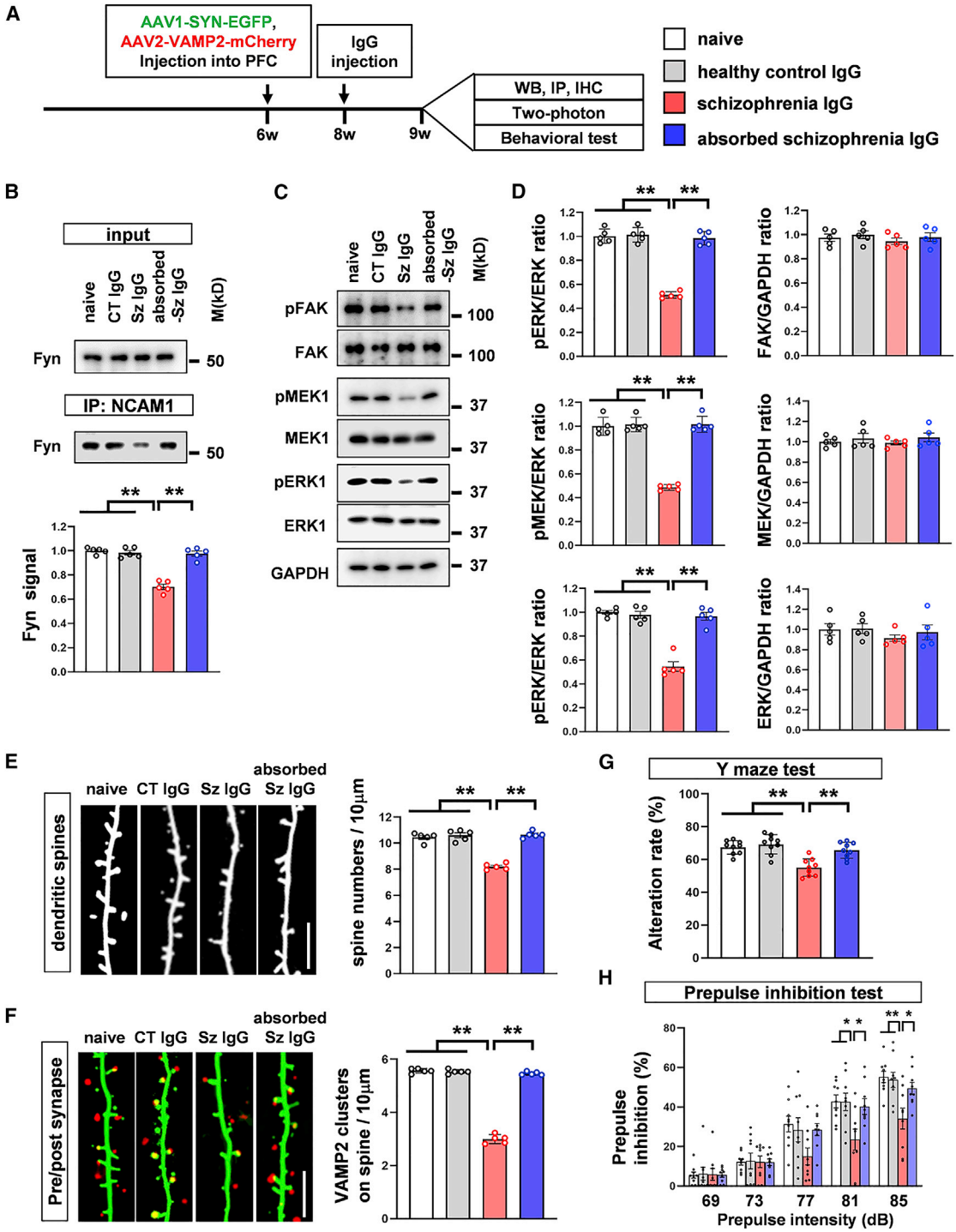

Figure 4. Injection of anti-NCAM1 autoantibodies from a patient with schizophrenia into mice

(A) Experimental protocol for IgG injection. AAV1-SYN1-EGFP and AAV2-VAMP2-mCherry were injected into the frontal cortex of mice aged 6 weeks, and purified IgG was injected into the CSF of mice aged 8 weeks. Molecular, histological, two-photon microscopy, and behavioral analyses were performed in 9-week-old mice. IHC, immunohistochemistry; IP, immunoprecipitation; WB, western blot.

(B) Immunoprecipitation analysis of tissue from the frontal cortex of mice revealed that the NCAM1-Fyn interaction was inhibited by anti-NCAM1 autoantibodies acquired from patients with schizophrenia. CT, computed tomography.

(C) Effect of IgG purified from patient 1 with schizophrenia on FAK, MEK1, and ERK1 phosphorylation in the frontal cortex. Removal of anti-NCAM1 antibodies from purified IgG reversed the decrease in pFAK, pMEK, and pERK1.

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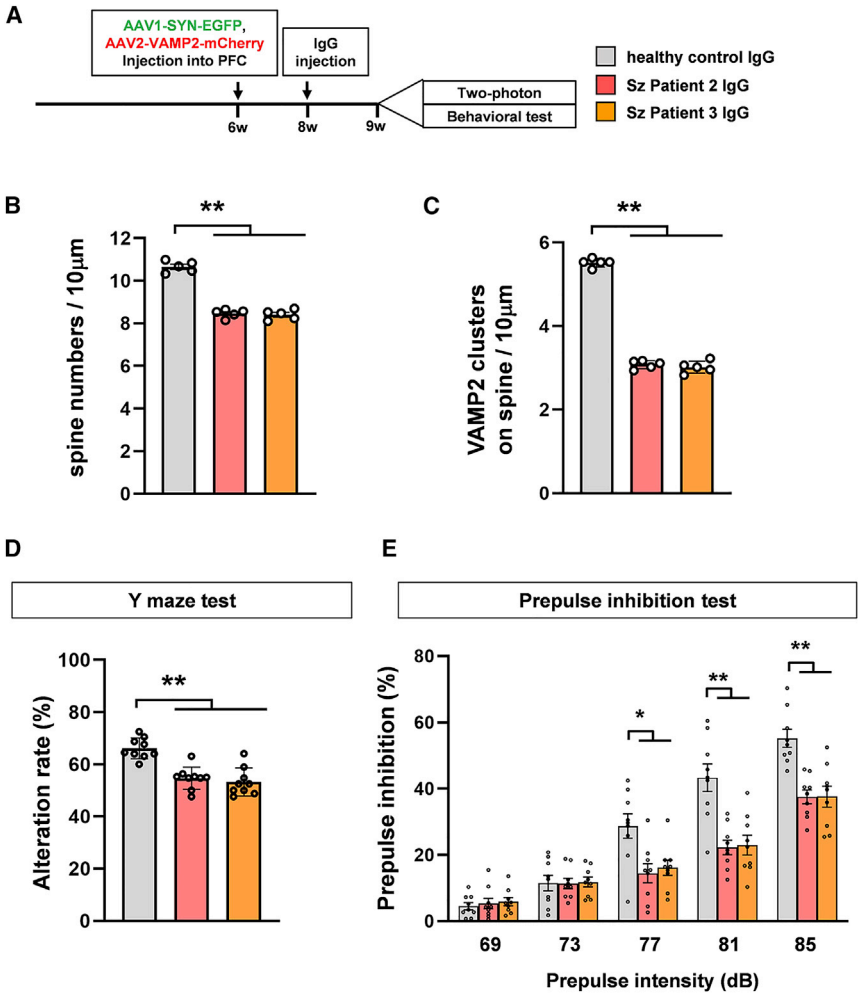

Figure 5. Anti-NCAM1 autoantibodies from patients with schizophrenia cause schizophrenia-related behavior and changes in synapse numbers in mice

(A) Experimental protocol for IgG injection. AAV1-SYN1-EGFP and AAV2-VAMP2-mCherry were injected into the frontal cortex of mice aged 6 weeks, and purified IgG was injected into the CSF of mice aged 8 weeks. Two-photon microscopy and behavioral analyses were performed in 9-week-old mice.

(B) Two-photon microscopic analysis of dendritic spines in the first layer of the frontal cortex of mice injected with AAV1-SYN1-EGFP and IgG purified from a healthy control and patient 2 and patient 3 with schizophrenia. **$p < 0.01$ ($n = 5$ mice per group; 50 dendrites/mouse, 500 spines/mouse; Tukey's HSD test). Data are expressed as the mean ± SEM.

(C) Two-photon microscopic analysis of axon terminals merged with spines in the first layer of the frontal cortex of mice injected with AAV2-VAMP2-mCherry, AAV1-SYN1-EGFP, and IgG purified from a healthy control and patient 2 and patient 3 with schizophrenia. **$p < 0.01$ ($n = 5$ mice per group; 50 dendrites/mouse, 500 spines/mouse; Tukey's HSD test). Data are expressed as the mean ± SEM. Scale bar: 5 μm.

(D) Alteration ratios in the Y maze test after injection of IgG purified from a healthy control and patient 2 and patient 3 with schizophrenia. **$p < 0.01$ ($n = 9$ mice per group; Tukey's HSD test). Data are expressed as the mean ± SEM.

(E) Pre-pulse inhibition rates of mice injected with IgG purified from a healthy control and patient 2 and patient 3 with schizophrenia. *$p < 0.05$ and **$p < 0.01$ ($n = 9$ mice per group; Tukey's HSD test). Data are expressed as the mean ± SEM.

deficiency in pre-pulse inhibition are reported in multiple mice models of schizophrenia, such as the 22q11.2 deletion syndrome mouse model, DISC1-knockout (KO) mice, and Setd1a-KO mice.[44–46] A reduction in the number of spines and synapses has also been reported in patients with schizophrenia as well as mice models of schizophrenia.[47–50]

These behavioral and synaptic changes are also found in NCAM1 KO mice and transgenic mice with a dominant negative form of NCAM1.[20–23] Furthermore, SNPs of NCAM1 and schizophrenia are repeatedly reported.[24–26] The reports that SNPs of NCAM1 or the expression level of soluble NCAM1 are associated with cognitive impairment of patients with schizophrenia

(D) Quantitative analyses of western blots with five mice per group. **$p < 0.01$ ($n = 5$, Tukey's honest significant difference [HSD] test). Data are expressed as the mean ± SEM.

(E) Two-photon microscopic images of dendritic spines in the first layer of the frontal cortex of mice injected with AAV1-SYN1-EGFP and IgG purified from patient 1 with schizophrenia or IgG purified from a healthy control. Removal of anti-NCAM1 antibodies from the purified IgG reversed the decrease in the number of spines. The graph on the right shows quantitative analysis of spine number. **$p < 0.01$ ($n = 5$ mice per group; 50 dendrites/mouse, 500 spines/mouse; Tukey's HSD test). Data are expressed as the mean ± SEM. Scale bar: 5 μm.

(F) Two-photon microscopic images showing contact between axon terminals and dendritic spines in the first layer of the frontal cortex of mice injected with AAV2-VAMP2-mCherry, AAV1-SYN1-EGFP, IgG purified from patient 1 with schizophrenia, or IgG from a healthy control. The graph on the right shows quantitative analysis of axon terminals merged with spines. **$p < 0.01$ ($n = 5$ mice per group; 50 dendrites/mouse, 500 spines/mouse; Tukey's HSD test). Data are expressed as the mean ± SEM. Scale bar: 5 μm.

(G) Alteration ratios in the Y maze test after injection of purified IgG from patient 1 with schizophrenia or from a healthy control. Removal of anti-NCAM1 antibodies from purified IgG reversed the decrease in the alteration ratios. **$p < 0.01$ ($n = 9$ mice per group; Tukey's HSD test). Data are expressed as the mean ± SEM.

(H) Pre-pulse inhibition rates of mice injected with IgG purified from patient 1 with schizophrenia or a healthy control. Removal of anti-NCAM1 antibodies from purified IgG reversed the deficiency in pre-pulse inhibition. *$p < 0.05$ and **$p < 0.01$ ($n = 9$ mice per group; Tukey's HSD test). Data are expressed as the mean ± SEM.

may relate to our analysis of cognitive impairment in mice administered with anti-NCAM1 autoantibodies from patients with schizophrenia.[16,26] NCAM1-Fyn-FAK pathway, which is disturbed both in NCAM1 mutant mice and by anti-NCAM1 autoantibodies, regulates the development of not only excitatory synapses but also GABAergic synapses.[51] Transgenic mice with a dominant negative form of NCAM1 show a reduction of PV and GAD65/67, markers for GABAergic neurons.[52] These reductions of GABAergic neurons and disturbances of the GABA system are observed in patients with schizophrenia.[53–56] The data presented herein, together with previous reports, indicate that dysfunction of NCAM1 by genetic or anti-NCAM1 autoantibodies is associated with schizophrenia.

However, if anti-NCAM1 antibodies are generated postnatally, while genetic mutation of NCAM1 affects neural development from the embryonic stage, it is uncertain whether postnatal inhibition of NCAM1 by anti-NCAM1 autoantibodies can induce changes comparable to those in NCAM mutant mice. Furthermore, there is a possibility that a single type of NCAM1 dysfunction will not trigger the onset of schizophrenia, as is often the case for polygenic disorders.

One of the explanations for this is that anti-NCAM1 antibodies may have pathogenic properties other than inducing dysfunction in NCAM1. For example, targeting of synapses by autoantibodies may result in microglial synaptic pruning, even if the autoantibodies do not provoke an inflammatory response in the microglia. Such synaptic pruning has been reported during development, although it is not known how such targeted pruning is regulated.[57,58] During normal development, synaptic pruning usually stops during adolescence, whereas progression of synaptic pruning after adolescence is hypothesized for schizophrenia.[59] Autoantibodies against synaptic molecules, including NCAM1, may be involved in such progressive synaptic punning by acting as a target marker for microglia. These themes will be tested in the future to examine hypotheses that autoantibodies specific for synaptic molecules, such as NCAM1, have an impact beyond dysfunction of an antigen molecule.

In addition, anti-NCAM1 autoantibodies may cross-react with other molecules. Such cross-reactivity is observed in other autoantibodies, such as anti-NMDA receptor autoantibodies and anti-GABA receptor autoantibodies.[60,61] In our analysis, we could not find the cross-reactivity to other representative molecules containing Ig domains and other synaptic molecules. However, identifying such other cross-reactive antigens may also explain the broad pathogenic features of anti-NCAM1 autoantibody. Moreover, there is also a possibility that patients with schizophrenia with anti-NCAM1 autoantibodies have other autoantibodies against synaptic molecules. Although our patients with anti-NCAM1 autoantibodies were negative for autoantibodies against other tested representative synaptic molecules and absorption experiments with recombinant NCAM1 restored the phenotypes observed in mice administered with IgGs purified from patients with schizophrenia, this does not entirely dismiss the existence of other autoantibodies. Therefore, future experiments with highly purified anti-NCAM1 autoantibodies from patients with schizophrenia would be ideal to reveal the specific effect of this autoantibody. Identifying cross-reactive antigens and co-existing autoantibodies is an effective way to reveal complete pictures of autoantibody pathologies in autoantibody-positive patients.

Although expression of NCAM1 protein was very high in the brain, there was very weak systemic expression in the heart and spleen (Figure S1C). However, there was no obvious systemic inflammation or damage in these organs in patients who were positive for anti-NCAM1 antibodies. One reason for the absence of inflammation may be differences in NCAM1 expression levels in these tissues. Furthermore, there are no reports of phenotypes other than that in the brain of KO mice.[23] This is also consistent with the absence of damage or dysfunction in organs other than the brain in patients who are positive for anti-NCAM1 antibodies. However, it should be borne in mind that systemic inflammation in these organs is theoretically possible.

Similarly, we found that patients with anti-NCAM1 antibodies in the CSF did not develop encephalitis in our study; however, encephalitis is possible theoretically when antibody titers in the CSF increase further. In fact, there are clinical and literature cases in which autoantibodies cause psychiatric symptoms or epilepsy without causing encephalitis; these conditions are improved by plasma exchange.[9,62] Conversely, it is possible that some idiopathic cases of encephalitis are caused by anti-NCAM1 autoantibodies. In our study, the administration of purified and diluted IgGs from patients 2 and 3 into the CSF of mice was sufficient to induce a similar abnormality of spines, synapses, and behaviors to those that manifested from the administration of IgG from patient 1. This may indicate that this concentration is sufficient to induce psychiatric symptoms. Conversely, we also observed generalized slowing of the basic rhythm on electroencephalogram (EEG) with theta activity in patient 1 (Table 1). This supports the hypothesis that an increase of anti-NCAM1 autoantibodies in the CSF can affect an EEG and further increase can contribute to encephalitis. These theories will be explored in future studies.

We screened anti-NCAM1 autoantibodies with both an ELISA and cell-based assay. ELISA is highly quantitative, while the cell-based assay has the advantage of analyzing more physiological molecular structures. Since NCAM1 is a cell membrane molecule, positive signal by anti-NCAM1 autoantibody is easy to distinguish from non-specific staining within the cell-based assay. Therefore, this assay seems to have high specificity. Differences in the results between the ELISA and cell-based assay may indicate a variation of anti-NCAM1 autoantibodies. Determining the significance of each property of autoantibodies will be necessary for the clinical utility of these analyses.

Soluble NCAM is cleaved from extracellular NCAM and presents an epitope for the anti-NCAM1 autoantibody.[18] The immune complex of anti-NCAM1 autoantibody-soluble NCAM may be a target for degradation. In fact, the concentration of soluble NCAM in the blood was significantly decreased in patients with schizophrenia with anti-NCAM1 autoantibody detected by the cell-based assay (Figure 1E). Although anti-NCAM1 autoantibodies did not cause apparent systemic or neuronal inflammation in our patients, chronic low-grade inflammation has been reported in schizophrenia.[63,64] Interaction between anti-NCAM autoantibody and serum-soluble NCAM

or peripheral NCAM1 can theoretically cause such chronic inflammation. However, our results suggest that there are factors other than anti-NCAM1 autoantibodies that contribute to the decrease in serum-soluble NCAM in patients with schizophrenia. This is due to the finding that the concentration of serum-soluble NCAM in schizophrenia patients without anti-NCAM1 autoantibodies was lower than that in healthy subjects (Figure 1F). Although these data suggest a relationship between anti-NCAM1 autoantibodies and the concentration of serum-soluble NCAM, the consequences of the immune complex may not be that simple. Indeed, a scatterplot analysis of NCAM1 titer by ELISA and concentration of soluble NCAM did not show a significant relationship. The consequences of the immune complex may be widespread. For example, the double-stranded DNA (dsDNA)-autoantibody complex inhibits the degradation of dsDNA in serum, and dsDNA increases when anti-dsDNA autoantibodies are increased.[65] Thus, interpretation of the relationship between anti-NCAM1 autoantibodies and serum-soluble NCAM may be intricate, owing to the possible variations of anti-NCAM1 autoantibodies and other unknown factors that affect serum-soluble NCAM.

In our study, there were two healthy subjects with low titers of anti-NCAM1 antibody. There are several interpretations regarding autoantibodies in healthy individuals. Various autoantibodies have been reported in healthy subjects, such as anti-NMDA receptor antibody, anti-CASPR2 antibody, and anti-amphiphysin antibody, which have all been established as causes of encephalitis.[66,67] Whether these autoantibodies lead to the onset of neurological or psychiatric symptoms depends on their antibody titer and whether the autoantibodies reach the nervous system through the blood-brain barrier (BBB). Autoantibody penetration across the BBB is a complex matter involving genetic and other factors.[68,69] In fact, BBB vulnerability is reported in patients with schizophrenia.[68,70,71] Thus, antibody titer or BBB vulnerability may be insufficient for onset of psychotic symptoms in these two healthy subjects. At the same time, they may be at a risk of developing psychotic symptoms or encephalitis if their antibody titer and BBB vulnerability increase in the future.

To more accurately determine the prevalence of anti-NCAM1 antibody in patients with schizophrenia, the analysis of a larger cohort involving several thousands of patients is necessary, although many autoantibodies have been discovered in cohorts of approximately 100 patients.[72–75] Examples of such autoantibodies include autoantibodies against LGI1, CASPR2, $GABA_AR\alpha1$, and NRXN3, which have been established as causes of encephalitis.[72–75] Similarly, we discovered anti-NCAM1 autoantibodies in a cohort of 223 patients with schizophrenia. The main theme of this study is the discovery of anti-NCAM1 autoantibodies and the elucidation of their pathological significance. An analysis of cohorts of thousands of patients, which has been conducted for some other autoantibodies after their discoveries,[66] is anticipated in the future. Nevertheless, the analysis involving 100–200 patients and healthy subjects is also valuable, and analyses of autoantibodies in smaller cohorts of such a size have been published.[67,76] Meta-analysis of these data will also establish a more accurate prevalence rate in the future.

An identification of anti-NCAM1 autoantibodies is important, because these autoantibodies can serve as biomarkers that distinguish a subgroup of patients with schizophrenia; also, immunological interventions such as plasma exchange and immunoglobulin therapy, which are usually used to treat autoantibody encephalitis, may work in this subgroup. A clinical study testing whether anti-NCAM1 autoantibodies play a role in schizophrenia would show that interventions improve the symptoms of schizophrenia.

Why and how anti-NCAM1 antibodies are produced in some patients with schizophrenia is a topic for future research. The anti-NCAM1-antibodies-positive patients examined herein had no common past medical history, such as cancer or autoimmune disease. Analysis of the MHC gene region may be one possible approach to clarifying the mechanism underlying generation of anti-NCAM1 antibodies.

In conclusion, we identified anti-NCAM1 antibodies in a small subgroup of patients with schizophrenia. These antibodies cause schizophrenia-related behavior and changes in synapses in mice. Further clinical studies are necessary to confirm the pathological significance of these antibodies in schizophrenia.

Limitations of the study

Despite the elucidation that anti-NCAM1 autoantibodies from patients with schizophrenia cause synaptic changes and schizophrenia-related behaviors in mice, this study has not shown whether anti-NCAM1 autoantibodies relate to symptoms in patients with schizophrenia. To examine this question, it is necessary to conduct clinical studies to remove anti-NCAM1 autoantibodies from patients with schizophrenia by plasma exchange or immunological interventions and then assess the improvement of symptoms. Moreover, another limitation is that the mouse model used in this study is a relatively acute model involving single injection of anti-NCAM1 autoantibodies. To clarify the effects of exposure to autoantibodies on the nervous system over a long period of time, which better reflects the case of schizophrenia, a model involving chronic injection is needed. Furthermore, this study does not highlight the duration of NCAM1 antibodies in patients with schizophrenia, which relates to whether NCAM1 antibodies are involved in the onset of schizophrenia. Thus, it is critical to epidemiologically analyze whether NCAM1 antibodies are present before the onset of schizophrenia and whether NCAM1-antibody-positive adolescents are at higher risk of developing schizophrenia.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100597.

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AUTHOR CONTRIBUTIONS

H.S. designed and supervised the study, performed the experiments, analyzed the data, and wrote the manuscript. H.S., Y. Nakano, and S.K. performed and analyzed the immunocytochemical and immunohistochemical experiments and the behavior experiments. Y. Nakano, S.K., and Y.Y. cloned the plasmids. H.S., S.K., Y. Nakano, H. Tanaka, K.F., and K.K. performed two-photon experiments. H.S., H. Tamaki, H. Takebayashi, O.T., Y. Nagase, T.N., K.I., and H. Takahashi collected human samples. H. Tanaka and K.F. performed immunostaining and cell culture. T.K., H.O., and H. Takahashi supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD56 Monoclonal Antibody (56C04) (anti-NCAM1)	Thermo Fisher Scientific	Cat# MA5-11563
Anti-NCAM1 antibody [CAL53]	Abcam	Cat# ab237708
Anti-Polysialic Acid-NCAM Antibody, clone 2-2B	Merck Millipore	Cat# MAB5324
Anti-Fyn Antibody	Santa Cruz Biotechnology	Cat# sc-434
Anti-Fyn Antibody	Cell Signaling Technology	Cat# 4023
Anti-FAK Antibody	Cell Signaling Technology	Cat# 3285
Anti-Phospho-FAK (Tyr397) Antibody	Cell Signaling Technology	Cat# 3283
Anti-MEK1 antibody	Abcam	Cat# ab32088
Anti-phospho-MEK1 (Thr386)	Millipore	Cat# 07-853
Anti-p44/42 MAPK (Erk1/2) Antibody	Cell Signaling Technology	Cat# 9102
Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb	Cell Signaling Technology	Cat# 4370
Anti-GST antibody	Santa Cruz Biotechnology	Cat# sc-458
Anti-6x-His Tag Monoclonal Antibody (3D5)	Thermo Fisher Scientific	Cat# R930-25
Anti-RGS-His Antibody	QIAGEN	Cat# 34650
Anti-GAPDH Antibody, clone 6C5	Merck Millipore	Cat# MAB374
Anti-NFκB p65	Santa Cruz Biotechnology	Cat# sc-372
Anti-Iba1 antibody	Fujifilm WAKO chemicals	Cat# 019-19741
Anti-CD171 (L1CAM) Antibody (UJ127)	Thermo Fisher Scientific	Cat# MA514140
Anti-NCAM2 Antibody (2H2L19)	Thermo Fisher Scientific	Cat# 703642
Anti-Contactin 2 (TAG1) Antibody	Thermo Fisher Scientific	Cat# PA5-101541
Cy3 AffiniPure Donkey Anti-Human IgG	Jackson ImmunoResearch Laboratories	Cat# 709-165-149
Donkey anti-Rabbit IgG Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-21206
Goat Anti-Mouse IgM HRP Conjugate	TGI	Cat# G0417
Anti-Rabbit IgG, HRP-Linked Whole Ab	Cytiva	Cat# NA934
Anti-Mouse IgG, HRP-Linked Whole Ab	Cytiva	Cat# NA931
Anti-Human IgG Antibody, HRP conjugate	Merck Millipore	Cat# AP112P
Anti-human IgG-alkaline phosphatase	Sigma-Aldrich	Cat# 2064
Bacterial and virus strains		
AAV1-SYN1-EGFP	Dr. Shin-ichi Muramatsu (Jichi medical university)	N/A
AAV2-VAMP2-mCherry	Vector BioLabs	Build-to-Order
Biological samples		
Human serum	This study	N/A
Human cerebrospinal fluid	This study	N/A
Chemicals, peptides, and recombinant proteins		
NCAM Protein, Human, Recombinant (ECD, His Tag)	Sino Biological	Cat# 10673-H08H
Recombinant Human NCAM1	enQuire Bio	Cat# QP8663-ec
GDNF Human Recombinant Protein	Origene	Cat# TP760516
GST	This study	N/A
Ni-NTA Agarose	QIAGEN	Cat# 30210
Glutathione Sepharose 4B	Cytiva	Cat# 17513201

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lipofectamine 2000	Invitrogen	Cat# 11668-019
B27	GIBCO	Cat# 17504044
Protein G HP SpinTrap	Cytiva	Cat# 28903134
SIGMAFAST *p*-nitrophenyl phosphate tablets	Sigma-Aldrich	Cat# N1891
DMEM	Sigma-Aldrich	Cat# D5796
Neurobasal Medium	Thermo Fisher Scientific	Cat# 21103049
Protein G Sepharose	Cytiva	Cat# 17061801
ECL Prime	Cytiva	Cat# RPN2232
DAPI	DOJINDO	Cat# 342-07431
Zenon Alexa Fluor 568 Rabbit IgG Labeling Kit	Thermo Fisher Scientific	Cat# Z25306
THUNDERBIRD SYBR qPCR Mix	TOYOBO	Cat# QPS-201
SuperScript VILO cDNA Synthesis kit	Invitrogen	Cat# 11754050
RNeasy Mini kit	QIAGEN	Cat# 74104
Critical commercial assays		
Human N-CAM1 ELISA	RayBiotech	Cat# ELH-NCAM1-1
Experimental models: Cell lines		
HeLa cell	ATCC	Cat# 300194/p772_HeLa, RRID:CVCL_0030
Primary cortical neurons	This paper	N/A
Experimental models: Organisms/strains		
C57BL/6J mice	CLEA Japan	RRID:IMSR_JCL:JCL:mIN-0003
Oligonucleotides		
Oligonucleotides are described in Table S2	N/A	N/A
Recombinant DNA		
pRP[Exp]-EGFP-CMV>hNCAM1	VectorBuilder	Build-to-Order
pRP[Exp]-EGFP-CMV>hNCAM1ΔIg1	This paper	N/A
pRP[Exp]-EGFP-CMV>hNCAM1ΔIg2	This paper	N/A
pRP[Exp]-EGFP-CMV>hNCAM1ΔIg2-5	This paper	N/A
pRP[Exp]-EGFP-CMV>hNCAM1ΔIg1-5	This paper	N/A
pRP[Exp]-EGFP-CMV>hNCAM2	VectorBuilder	Build-to-Order
pRP[Exp]-EGFP-CMV>hL1CAM	VectorBuilder	Build-to-Order
pRP[Exp]-EGFP-CMV>hCNTN2(TAG1)	VectorBuilder	Build-to-Order
Software and algorithms		
GraphPad Prism (version 8.4.3)	GraphPad Software Inc.	RRID:SCR_002798
Imaris	Bitplane	RRID:SCR_007370
Packwin software V2.0	Panlab	https://www.panlab.com/en/products/packwin-software-panlab
ImageJ	National Institute of Health	RRID: SCR_003070
SMART video-tracking system	Panlab	RRID: SCR_002852
Fluoview FV10-ASW	Olympus	RRID:SCR_014215

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hiroki Shiwaku (shiwaku.npat@mri.tmd.ac.jp).

Materials availability

Plasmids generated in this study is available from the lead contact upon request following completion of a Material Transfer Agreement.

Data and code availability

All data for this study are available from the lead contact upon request.

There was no new code developed as a part of this study.

Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subjects

Serum was obtained from 223 patients with schizophrenia, and from 201 healthy controls. Patients were inpatients at Tokyo Medical and Dental University Medical Hospital, Kurita Hospital, and Takatsuki Hospital between April 1, 2016, and December 31, 2021. All were diagnosed with schizophrenia according to DSM5 criteria. None of the healthy controls had a previous history of psychiatric disorders. Healthy control sera were obtained from healthy volunteers and BioBank at Bioresource Research Center, Tokyo Medical and Dental University. All participants were Japanese.

Ethics

This study was performed in strict accordance with the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan, the Helsinki Declaration, and Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan. It was approved by the Committees on Gene Recombination Experiments, Human Ethics, and Animal Experiments of the Tokyo Medical and Dental University (G2020-002A, M2000-1866, and A2020-113A). All participants provided written informed consent.

Cell culture and transfection

HeLa cells were maintained at 37°C/5% CO_2 in DMEM (SIGMA, MI, USA) supplemented with 10% FBS, Cells were transfected with plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Mouse primary cerebral neurons were prepared from embryonic Day 15 C57BL/6J mice embryos. Cerebral cortexes (n = 4 to 6) were dissected, incubated with 0.05% trypsin in 4 mL of phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 15 min, and dissociated by pipetting. The cells were passed through a 70 μm cell strainer (Thermo Fisher Scientific, Waltham, MA, USA), collected by centrifugation, and cultured in neurobasal medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 2% B27, 0.5 mM L-glutamine, and 1% Penicillin/Streptomycin in the presence of 0.5 μM AraC. Immunohistochemistry of primary neurons was performed at 14 days (DIV 14).

Mice

C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). Mice were housed in standard cages in a temperature and humidity-controlled room with a 12 h light/dark cycle (lights on at 08:00). Purified human IgGs were injected into the subarachnoid space of the frontal cortex of 8-week-old mice. Molecular, histological, two-photon microscopy, and behavioral analyses were performed in 9-week-old mice. Investigators were blind to the treatment of mice when performing experiments and analyzing the data.

METHOD DETAILS

Serum samples and CSF samples

Serum samples were collected in the morning following an overnight fast or more than 2 h after eating. CSF samples were also collected from patients with schizophrenia with anti-NCAM1 autoantibodies in the morning within a month after serum samples were collected. The serum and CSF were aliquoted and stored in a −80 °C freezer. Cell-based assay and ELISA were performed with newly thawed samples and avoided freeze and thaw.

Construction of DNA vectors

NCAM1 and enhanced green fluorescent protein (EGFP) were cloned into a pRP vector (VectorBuilder, TX, USA) and expression was driven by the cytomegalovirus (CMV) promoter (Figure S1A). NCAM1 deletion constructs were generated using PrimeSTAR Max DNA Polymerase (Takara, Tokyo, Japan) and the primers (Table S2).

Anti-NCAM1 autoantibody ELISA

Polystyrene microtiter plates (3455, Thermo Scientific, Waltham, MA, USA) were coated with 100 μL (2 μg/mL) of NCAM1 recombinant protein (10673-H08H, Sino Biological) in TBS buffer and incubated overnight at 4°C. The plates were washed three times with TBS and then incubated for 1 h at 24°C with 100 μL/well TBS containing 1% BSA to block non-specific binding. They then incubated for 1h at 24°C with 100 μL of each dilution of serum and CSF samples (1:50 for serum, 1:1 for CSF in TBS containing 1% BSA). The plates were washed three times with TBS containing 0.1% Tween 20 and then incubated with anti-human IgG-alkaline phosphatase (1:50000; Cat# 2064, Sigma-Aldrich) in TBS containing 0.1% Tween 20 for 1 h at room temperature. After washing with TBS, 1 mg/mL

p-nitrophenyl phosphate in substrate buffer (N1891, Sigma-Aldrich) was added to each well. Absorbance at 405 nm was read on a microplate reader (Spark 10M, TECAN).

Serum-soluble NCAM ELISA

Serum-soluble NCAM ELISA was performed with ELISA kit (ELH-NCAM1-1, RayBiotech) according to the manufacturer's protocol. Briefly, recombinant NCAM for a standard curve and serum (1:10 dilution with the diluent provided in the kit) were added to provided ELISA plates. The plates were incubated overnight at 4°C. The plates were washed four times with the provided wash buffer and then incubated with biotinylated anti-Human N-CAM1 antibody provided in the kit for 1 h at 24 °C. After washing, HRP-conjugated streptavidin provided in the kit was added to each well and incubated for 45 min at 24°C. After washing, 3,3,5,5′-tetramethylbenzidine provided in the kit was added to each well and incubated for 30 min at 24°C. The reaction was terminated with 0.2 M sulfuric acid, and absorbance at 450 nm was read on a microplate reader (Spark 10M, TECAN). The coefficients of variation in the intra- and inter-assay were 4.7–6.0% and 4.0–9.1%, respectively. Each sample was assayed in triplicate. The operating range for the assay was 0.61–150 ng/mL.

Western blot analysis

Samples were lysed in 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 5% (v/v) glycerin, and 0.0025% (w/v) bromophenol blue. Samples were separated by SDS–PAGE, transferred onto Immobilon-P Transfer Membranes (Merck Millipore, Burlington, MA, USA) using a semi-dry method, and blocked with 5% milk in TBS/Tween 20 (TBST) (10 mM Tris/Cl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). The filters were incubated overnight at 4°C with each primary antibody. The following primary antibodies were diluted in Can Get Signal solution (Toyobo, Osaka, Japan): anti-NCAM1 (1:1000; MA5-11563, Thermo Scientific, Waltham, MA, USA); anti-NCAM (1:1000, ab237708, Abcam, Cambridge, UK); anti-phosphorylated-ERK1 (1:1000; #4370, Cell Signaling Technology, Danvers, MA, USA); anti-ERK1 (1:1000, #9102, Cell Signaling Technology, Danvers, MA, USA); anti-GST (1:1000; sc-458, Santa Cruz Biotechnology, Dallas, TX, USA); anti-His (1:1000; R930-25, Thermo Scientific, Waltham, MA, USA); anti-RGS-His Antibody 1:1000 (QIAGEN); anti-MEK1 (1:1000. ab32088, Abcam); anti-phospho-MEK1 (Thr386) (1:1000, 07-853, Millipore), anti-Polysialic Acid-NCAM antibody clone 2-2B (1:1000, MAB5324, Millipore), anti-Fyn antibody (1:1000, sc-434, Santa Cruz Biotechnology), anti-FAK antibody (1:1000, 3285, Cell Signaling Technology), anti-Phospho-FAK (Tyr397) antibody (1:1000, 3283, Cell Signaling Technology), and anti-GAPDH (1:1000–10,000; MAB374, Merck Millipore). Secondary antibodies were HRP-linked anti-rabbit IgG (1:3000; NA934, Cytiva, USA); HRP-linked anti-mouse IgG (1:3000; NA931, Cytiva, USA); and HRP-linked anti-human IgG (1:3000; AP112P, Merck Millipore, Burlington, MA, USA). Proteins were detected using ECL Prime Western Blotting Detection Reagent (RPN2232, Cytiva, USA) and a luminescence image analyzer (ImageQuant LAS 500, Cytiva, USA).

Immunoprecipitaion

Mouse cerebral cortex was lysed in a homogenizer with RIPA buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% DOC, 0.5% protease inhibitor cocktail (539134, Calbiochem, San Diego, CA, USA)). Lysates were rotated for 60 min at 4 °C, and then centrifuged (16,000 g × 10 min at 4°C). Lysates were incubated with 1 μg of anti-NCAM (1:200, ab237708, Abcam, Cambridge, UK) for 16 h at 4°C with rotation. Then lysates were incubated with protein G–Sepharose beads (17061801, Cytiva) for 2h, and then the beads were washed four times with lysis buffer. Bound proteins were eluted in sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% glycerol, 0.005% BPB, 5% 2-mercaptoethanol), separated on SDS–PAGE, and blotted with anti-Fyn (1:1000, sc-434, Santa Cruz Biotechnology, Dallas, TX, USA).

Immunocytochemistry, immunohistochemistry, and cell-based assays

HeLa cells and primary cortical neurons were fixed for 30 min at room temperature in 2% paraformaldehyde (prepared in phosphate buffer), treated for 10 min with 0.1% Triton X-100 in PBS, blocked for 30 min at room temperature with PBS containing 10% FBS or 1% BSA, and then incubated with either serum or primary antibody diluted in blocking buffer. For the cell based assay, serum with an autoantibody titer $\geq$1:30 was defined as autoantibody-positive. Previous studies show that NMDA receptor autoantibody and GABA receptor autoantibody titers are usually higher than 1:30.[9,10,77] Furthermore, diluting the serum has the advantage of preventing nonspecific staining. For immunohistochemistry, brain samples were fixed with 4% paraformaldehyde and embedded in paraffin. Sagittal or coronal sections (5 μm thick) were cut using a microtome (Microm HM 335 E, GMI, Ramsey, USA). Immunocytochemistry and Immunohistochemistry were performed using the following primary antibodies: anti-NCAM (1:200, MA5-11563, Thermo Scientific, Waltham, MA, USA); anti-NCAM (1:200, ab237708, Abcam, Cambridge, UK); anti-CD171 (L1CAM) (UJ127) (1:200, MA514140, Thermo Fisher Scientific), anti-NCAM2 (2H2L19) (1:200, 703642, Thermo Fisher Scientific), anti-Contactin 2 (TAG1) (1:200, PA5-101541, Thermo Fisher Scientific), anti-NFκB p65 (1:200, sc-372, Santa Cruz Biotechnology, Dallas, TX, USA); and anti-Iba1 (1:200, 019-19741, WAKO). These were detected using Cy3-conjugated anti-human IgG (1:500, 709-165-149, Jackson Laboratory, Bar Harbor, ME, USA); Alexa Fluor 488-conjugated anti-rabbit IgG (1:500, A21206, Thermo Scientific, Waltham, MA, USA), and a Zenon Alexa Fluor 568 Rabbit IgG Labeling Kit (Thermo Fisher Scientific, Z25306, Waltham, MA, USA). Nuclei were stained with DAPI (0.2 μg/mL in PBS; DOJINDO). Images were acquired under an Olympus FV1200 confocal microscope (Tokyo, Japan).

Behavioral tests

All behavioral tests were analyzed by a video-computerized tracking system (SMART, Panlab, Barcelona, Spain). All behavioral tests were performed with Male C57BL/6J mice.

Open field test

Mice were placed in an open field box (40 × 40 × 22 cm) and allowed to explore freely for 10 min. The total distance moved and the time spent in the central zone (20 × 20 cm) were measured.

Three-chamber sociability test

Mice were placed in a three-chambered box. Each chamber measured 40 cm by 20 cm by 22 cm (*L, W, H*). The dividing chamber walls contained openings allowing access into each chamber. The test comprised three sessions. During the first session (habituation), the mouse was allowed to explore three chambers for 5 min; then, the mouse was confined in the central chamber for another 5 min. In the following session (sociability), an unfamiliar mouse was placed in the wire cup in one of the side chambers and the test mouse was allowed to freely explore all three chambers for 10 min. In the last (social novelty preference) session, a new unfamiliar mouse was placed in the wire cup in the opposite side chamber and the test mouse was allowed to freely explore all three chambers for 10 min. The time spent in each chamber and actual interaction time was recorded. The interaction time in the figure is based on actual interaction time.

Elevated plus maze test

The elevated plus maze comprised two open arms and two closed arms (30 × 6 cm; 15 cm walls; apparatus suspended 50 cm above the floor). Mice were placed in the central square of the maze and activity was recorded for 5 min. Time spent in the open and closed arms was measured.

PPI test

The test was conducted using sound-attenuating startle boxes (Panlab, Barcelona, Spain). After acclimation for 5 min with background noise (65dB), the mouse was exposed to 10 blocks of six types of startle stimulus in pseudorandomized order. The trial types were as follows: startle-only; 40 ms, 120 dB sound burst; five pre-pulse trials; 120 dB startle stimulus preceded 100 ms earlier by 20 ms prepulses (69, 73, 77, 81, or 85 dB). The maximum startle response was recorded for each startle stimulus.

Y maze

Mice were placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The percentage of spontaneous alterations (indicated as an alteration rate) was calculated by dividing the number of entries into a new arm different from the previous one by the total number of transfers from one arm to another arm.

Pull-down assay

Purified IgGs (12 μg), His-NCAM1 (250 ng) (16067-H08H, SinoBiological) or His-GDNF (250 ng) (TP760516, Origene), and GST-NCAM1 (250 ng) (QP8663-EC, enQuire BioReagents) or GST (250 ng) were prepared as described previously[78], mixed in 400 μL of TBS, and incubated at 4°C for 12 h. After addition of 50 μL Ni-NTA agarose (30210, Qiagen, Hilden, Germany) or glutathione Sepharose (17513201, Cytiva, USA), the mixtures were incubated for a further 3 h at 4°C, centrifuged, and washed five times with TBS. Next, the beads were mixed with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, and 0.0025% (w/v) bromophenol blue) and boiled at 95°C for 10 min.

Purification of IgG from serum

IgG was purified from serum using Protein G HP SpinTrap columns (28903134, Cytiva, USA), according to the manufacturer's protocol.

Intrathecal injection of IgGs

Mice were anesthetized with 1% isoflurane using a small animal anesthetizer (TK-7, BioMachinery, Japan). Using an injection needle made with a micropipette puller (model P-1000, Sutter instrument, USA) and FemtoJet (Eppendorf, NY, USA), purified IgG (1 μg in 2 μL) from the serum of healthy controls or patients with schizophrenia was injected at the speed of 1 μL/min into the subarachnoid space of the frontal cortex of 8-week-old mice. Assuming a mouse CSF volume of 40 μL,[79] the dilution rate used for purification and injection corresponded to a 400- to 600-fold dilution from the serum. Because the antibody titer of anti-NCAM1 autoantibodies in serum are 1:1000-1:10000, we considered that the dilution rate used was appropriate for analyzing these autoantibodies (Table 1).

Two-photon microscopic analysis

This procedure has been described previously.[80,81] Briefly, Adeno-associated virus 1 (AAV1)-EGFP harboring the synapsin I promoter (titer: 1×10^{10} vector genomes/mL; 1 μL) and AAV2-VAMP2-mCherry harboring the CMV promoter were injected into adjacent positions in the frontal cortex (+1.0 mm from bregma (mediolateral 0.5 mm; depth, 1 mm; and +3.0 mm from bregma (mediolateral 0.5 mm; depth, 1 mm), respectively) under anesthesia with 1% isoflurane.

Two-photon imaging was performed using a laser-scanning microscope system FV1000MPE2 (Olympus, Tokyo, Japan) equipped with an upright microscope (BX61WI, Olympus, Japan), a water-immersion objective lens (XLPlanN25xW; numerical aperture, 1.05), and a pulsed laser (MaiTaiHP DeepSee, Spectra Physics, Santa Clara, CA, USA). EGFP and mCherry were excited at 920 nm and scanned at 495–540 nm and 575–630 nm, respectively. High-magnification imaging (101.28 μm × 101.28 μm; 1024 × 1024 pixels; 1 μm Z step) of cortical layer I was performed with a 5 × digital zoom through a thinned-skull window in the frontal cortex. Blinded

observers performed image acquisition and analysis. Image processing was performed with Imaris Interactive Microscopy Image Analysis software (Bitplane, Zurich, Switzerland).

Real-time qPCR

The total RNA was prepared from the frontal cortex of mice using an RNeasy Mini kit (74104, QIAGEN). Reverse transcription was performed using the SuperScript VILO cDNA Synthesis kit (11754-250, Invitrogen, USA). Real-time quantitative PCR (qRT-PCR) was performed using a LightCycler (Roche Diagnostics, Germany) and THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan), according to the manufacturer's protocol. Expression of individual genes was normalized to that of GAPDH. qRT-PCR was performed using the primers listed in Table S2.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, Inc, CA, USA). ELISA data and cell-based assay data were analyzed with a Mann–Whitney U test. Data groups were compared by Tukey's HSD test unless otherwise noted in the figure legends. Sample size was determined based on our previous studies.[81] All experiments were randomized. A p value < 0.05 was considered as statistically significant. Exact value of n, definition of center and dispersion and precision measures are described in the figure legends.