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# Elevated cleavage of neuregulin-1 by beta-secretase 1 in plasma of schizophrenia patients

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ABSTRACT

Neuregulin 1 (NRG1) is a key candidate susceptibility gene for schizophrenia. It is reported that the function of NRG1 can be regulated by cleavage *via* the β-Secretase (BACE1), particularly during early development. While current knowledge suggested that schizophrenia might have different phenotypes, it is unknown whether BACE1-cleaved-NRG1 (BACE1-NRG1) activity is related to clinical phenotypes of schizophrenia. In the current study, we used a newly developed enzymatic assay to detect BACE1-NRG1 activity in the human plasma and investigated the levels of cleavage of NRG1 by BACE1 in the plasma from schizophrenia patients. Our results are the first to demonstrate that the level of plasma BACE1-NRG1 activity was significantly increased in subjects affected with schizophrenia compared with healthy controls. Interestingly, the elevated BACE1-NRG1 activity was correlated with the disease severity and duration of schizophrenia, such as patients suffering from shorter-term course and worse disease status expressed higher BACE1-NRG1 activity levels compared to whom with longer duration and less severity of the disease. Furthermore, this is also the first report that the alternation of BACE1-NRG1 activity was a substrate –specific event in schizophrenia. Together, our findings suggested that the plasma BACE1-NRG1 activity can be a potential biomarker for the early diagnosis of schizophrenia.

## 1. Introduction

Schizophrenia is a chronic psychiatric disorder in which stage and status of illness appear to influence the course, outcome, prognosis, and treatment response (Insel, 2010). Due to strong causative genetic mutations and lack of objective diagnostic biomarkers, the treatments for schizophrenic symptoms still have great limitations. Identification of prognostic biomarkers in schizophrenia will provide great benefits in predication of the disease.

During the past decades, neuregulin 1 (*NRG1*) has been listed as one of schizophrenia candidate genes and shed light on the underlying mechanism of etiology (Allen et al., 2008; Mulle, 2012; Schizophrenia Working Group of the Psychiatric Genomics, 2014; Stefansson et al., 2003; Stefansson et al., 2002). The genetic association between NRG1 and schizophrenia was first reported in an Icelandic study, confirmed in other populations such as Chinese (Yang et al., 2003), Japanese (Fukui et al., 2006), Finnish (Turunen et al., 2007) and Italian (Squassina et al., 2010). Several variants of *NRG1* are linked to specific neurophysiological endophenotypes including alterations in pre-pulse inhibition (PPI) (Roussos et al., 2011), evoked response potential (ERP) (Bramon et al., 2008), eye movement (EMT) (Smyrnis et al., 2011) and neuroimaging (Knickmeyer et al., 2014; Thirunavukkarasu et al., 2014) suggesting that NRG1 has potential effects on biological aspects of brain function.

Neuregulin-1 is highly expressed in various brain regions (frontal cortex and cerebellum) (Chen et al., 2010; Rieff et al., 1999) as well as in peripheral nervous system (Ronchi et al., 2016; Wang et al., 2017). NRG1 plays prominent roles in aspects of neurodevelopment, including regulation of neuronal migration, neuronal survival, synaptic plasticity and myelination formation. There are six types of NRG1 (I, to VI) are encoded by a single *NRG1* gene, only type I and III can be cleaved by BACE1 and produce soluble N-terminal fragments (NRG1-NTF) (Luo et al., 2011). The NRG1-NTF fragments contain an epidermal growth

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factor (EGF)-like domain, which binds to tyrosine kinase receptor *ErbB4*, and activates its downstream extracellular signal-regulated kinase (ERK) and serine/threonine kinase (AKT) signal pathways (Mei and Xiong, 2008; Nave and Salzer, 2006; Yarden and Sliwkowski, 2001). Lines of evidence show that mice with altered *NRG1* activity exhibit disrupted synaptic plasticity and abnormal myelination, and develop schizophrenia-like behaviors (Newell et al., 2013; Stefansson et al., 2002). Particularly, the significantly elevated expression of NRG1-NTF was reported in human brain area BA9, a region highly related to schizophrenia, indicating the involvement of altered NRG1 cleavage in schizophrenia (Marballi et al., 2012). Overexpression of NRG1-NTF is sufficient to cause schizophrenia-like phenotypes in animal models (Luo et al., 2014). Current evidence suggested that BACE-dependent NRG1 might play specific roles in the neuropathology of schizophrenia.

Although it is known that soluble NRG1 fragment is associated with schizophrenia disease, to our knowledge, there is no report on direct measurement of BACE1-dependent NRG1 cleavage activity in living people with schizophrenia. Here, we aim to explore dynamic changes of BACE1-dependent NRG1 cleavage process in plasma from psychiatric patients. First, we developed a valid enzymatic activity assay to detect human plasma activity of BACE1 in cleaving NRG1 as substrate (referring BACE1-NRG1 activity). Then, we compared plasma BACE1-NRG1 activity in schizophrenic patients and healthy controls and analyzed linkage between clinical characters and level of plasma BACE1-NRG1 activity in patients with schizophrenia. We further verified the disease-specific association between plasma BACE1-NRG1 activity and their protein expression level. Finally, we investigated a substrate-specificity of BACE1-NRG1 activity in schizophrenia.

## 2. Materials and methods

### 2.1. Human samples

The plasma samples were selected from Anding Hospital Bio-Bank, including 51 healthy controls matched to 51 patients with schizophrenia. The consensus diagnoses were made by at least two experienced psychiatrists according to Diagnosis and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV) criteria for schizophrenic. Healthy controls were free of psychiatric disorder or a first-degree relative with a psychiatric disorder. All subjects were free of pregnancy, recent electro convulsive therapy, substance abuse, severe cardiovascular, hepatic or renal diseases. All procedures were approved by the Ethical Committee (IRB equivalent) of Beijing Anding Hospital, Capital Medical University. The demographics and clinical information of all samples were given in Table 1. For patients with schizophrenia, the disease severity was recorded by the Clinical Global Impression (CGI) score for Schizophrenia (CGI-SCH) (Haro et al., 2003). Psychiatric symptoms were recorded by the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987). To validate the BACE and NRG1 expression in human brain and plasma, we also included a cerebral cortex sample from postmortem brain of an aged female health individual (#BB038) from the brain bank at University of Science and Technology of China (USTC).

**Table 1**
General Demographics of subjects and clinical categories.

| | CON ($n$ = 51) | SCZ (n = 51) |
|---|---|---|
| Sex, Male/Female, n | 27/24 | 21/30 |
| Age (years) | 38.05 ± 1.85 | 36.86 ± 1.93 |
| Onset Age (years) | NA | 28.10 ± 1.39 |
| Duration (months) | NA | 110.43 ± 16.08 |
| PPANSS | NA | 17.72 ± 1.20 |
| NPANSS | NA | 15.60 ± 1.00 |
| GPANSS | NA | 31.85 ± 1.70 |
| PANSS | NA | 64.96 ± 3.47 |
| Medicine (Olan convert/mg) | NA | 11.07 ± 1.00 |

All values was Mean ± SEM.
*CON* healthy individuals, *SCZ* schizophrenic patients, *PANSS* Positive and Negative Syndrome Scale, *PPANSS* Positive symptoms in PANSS, *NPANSS* Negative symptoms in PANSS, *GPANSS* General symptoms in PANSS.

### 2.2. BACE1 enzymatic activity assay

BACE1 enzymatic activity assays were performed as previous described with modifications (He et al., 2007; Shen et al., 2018). In brief, a synthetic peptide substrate containing BACE1 cleavage site [NRG1 (Abz)-GIEFMEAEK-(Dnp); GL Biochem, Shanghai] at 40 μM concentration in reaction buffer (50 mmol/L acetic acid buffer, 100 mmol/L sodium chloride). Ten microliters of plasma were mixed with 100 L of buffer with the final pH of approximately 4.5, an optimized pH for the BACE1 activity assay. Fluorescence intensity was measured with microplate reader (Biotek, Synergy H1) at 320 nm (excitation wavelength) and 420 nm (emission wavelength), for one hour. BACE1 activity was corrected with total protein content and calculated through two indictors, $V_{max}$ and $V_{mean}$ as previously described (Zhong et al., 2007) and expressed in fluorescence units/μg protein. Total protein was measured by Bicinchoninic acid (BCA) kit (Thermo Scientific #23225). To confirm the specificity, BACE1 activity was tested in plasma mixed with different BACE1 inhibitors AZD3839, LY2886721, LY2811376 (Selleck) and BACE Inhibitor C3 (Merck #565788). As a positive control, HEK293T cells were cultured in DMEM containing 10% fetal bovine serum and were transfected with 0.5 μg of BACE1 expression plasmid by Lipofectamine 2000 (Invitrogen) in a 35 mm dish. The cells were harvested 2 days after transfection. After centrifugation, the supernatant was collected and proceed to the BACE1-NRG1 activity experiment.

### 2.3. Western blot and enzyme-linked immunosorbent assays (ELISA)

Cytoplasm and membrane protein of human brain tissues were isolated using the MinuteTM plasma membrane protein isolation kit (Invent sm-005). Proteins from brain or plasma were lysed in RIPA buffer (Sigma-Aldrich) with protease inhibitor mixture (Roche Applied Biosciences). Proteins were detected by anti-BACE1 ectodomain monoclonal antibody (MAB931; R&D Systems) or anti-NRG1 antibody (ab53104; Abcam). The protein expression were detected by Bio-Rad Imager system, analyzed by NIH Image J. BACE1 and NRG1 protein sandwich ELISAs were performed using the human Beta-secretase1 Elisa Kit (EHBACE1, Thermo) and human NRG1-β1 (NRG1) Elisa Kit (EHNRG1, Thermo), according to the manufacturer's instructions.

### 2.4. Statistical analysis

Comparisons between two groups were made using Student's unpaired two-tailed *t*-test. Comparisons among three or more groups were made using one-way ANOVA analyses followed by Tukey multiple-comparisons test. The two effect on enzyme activity was calculated by univariate analysis (Two-way ANOVA test). The Spearman rank correlation was analyzed between clinical information and BACE1-NRG1 activity. All analyses were performed using a software program (SPSS version 18).

## 3. Results

### 3.1. Development of a valid BACE1-dependent cleavage NRG1 enzymatic activity assay

To develop an enzymatic assay for BACE1 cleavage of NRG1 in plasma, we constructed serial fluorescent transfer peptides bearing the same BACE1 cleavage site from type I and III NRG1 as shown in Fig. 1A. First, we tested cleavage of NRG1 substrate by extraction of 293 T cells

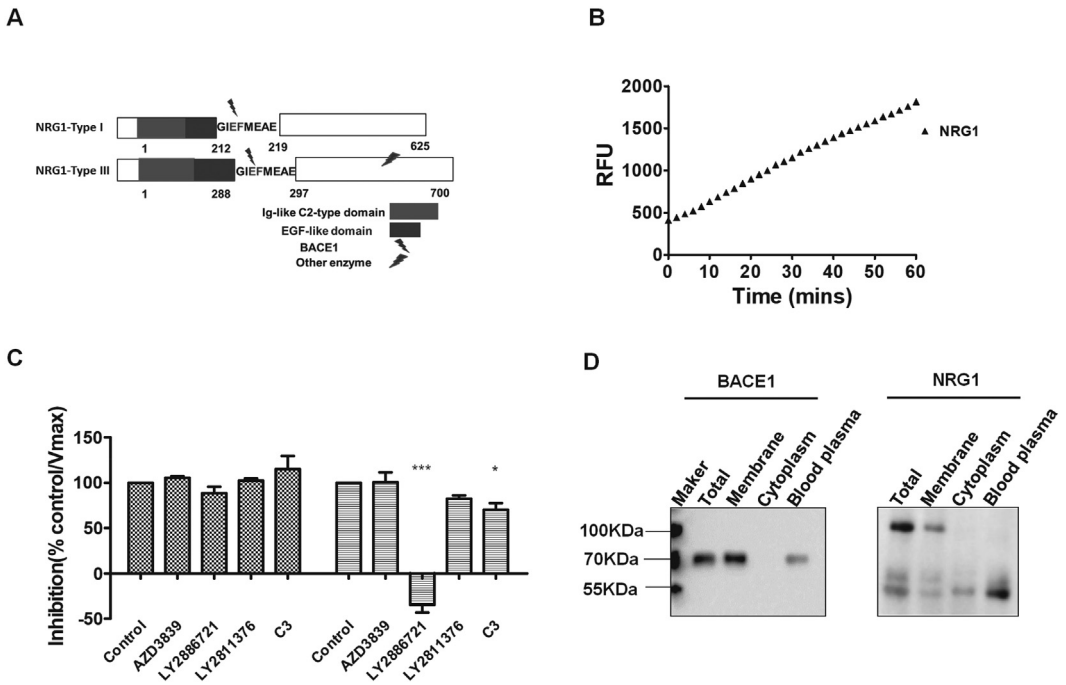



**Fig. 1.** Plasma BACE1-NRG1 activity assay and BACE1 and NRG1 protein expression in brain and plasma.
A) The schematic illustration of the experiment designed for dependent BACE1 cleavage NRG1 enzymatic activity assay. EF and ME residues were key sequences cleaved by BACE1. B) The enzymatic reaction curve of overexpressed BACE1 protein cleavage its substrate NRG1 by time-dependent. C) The effect of dose-response of plasma BACE1 cleavage NRG1 enzymatic activity treated with four different inhibitors (AZD3839, LY2886721, LY2811376 and C3). The inhibition percentage was shown as $V_{max}$. D) Immunoblotting of BACE1 and NRG1 in human brain fractions and blood plasma. All the data was repeated at least three times by independent experiments. One way ANOVA test, *$P < .05$, *** $P < .001$, Bars represent mean ± SEM.

that overexpressed BACE1 plasmid DNA *in vitro*. We observed a time-dependent cleavage curve of NRG1 peptide containing 11 amino acids that existed in both type I and III isoform (Fig. 1B). To confirm BACE1-NRG1 enzymatic specificity, we also carried out dose-dependent inhibitory experiments in plasma with four different BACE1 inhibitors, such as AZD3839, LY2886721, LY2811376 and BACE Inhibitor C3. Interestingly, only LY2886721 showed obvious dose-dependent inhibitory effect on plasma BACE1-NRG1 activity, C3 just had mild effect as shown in Fig. 1C. No effects of other BACE1 inhibitors on BACE1-NRG1 activity were identified. To investigate NRG1 and BACE1 protein distribution in human brain homogenates and expression in human plasma, we divided human postmortem brain protein into three fractions including total, membrane, and cytoplasm. We found plasma BACE1 and NRG1 protein (NRG1-NTF) at approximately 70 kDa and 55 kDa, respectively, similarly to their sizes in the brain fractions by immunoblotting (Fig. 1D). These findings indicated the plasma BACE1 enzymatic activity could be effectively measured using NRG1 as a substrate.

### 3.2. Elevation of plasma BACE1-NRG1 activity in patients with schizophrenia compared to healthy controls

To examine the relationship between BACE1 cleaving NRG1 and schizophrenia, we detected BACE1-NRG1 activity in samples from schizophrenia patients and age- and sex-matched healthy individuals. Samples from schizophrenia expressed higher level of BACE1-NRG1 activity compared with controls, particularly the $V_{max}$ value (37.56 ± 1.38 *vs* 32.83 ± 1.05, $P = .008$) in Fig. 2A. It was noted that BACE1-NRG1 activity was not differed between females and males in either patients or healthy controls (Main effect of sex $P = .124$) as shown in Fig. 2B, but an obviously increased enzymatic activity was observed in female patients compared with females controls ($P = .018$).

To understand whether the changes of plasma BACE1-NRG1 activity found in schizophrenia patients were attributed to alteration in protein expression of BACE1 and NRG1, we measured expression of BACE1 and soluble NRG1 in schizophrenic samples by ELISA. No significant change of NRG1 and BACE1 was observed in plasma of schizophrenia compared with health controls (Fig. 2C, E). Plasma BACE1 and NRG1 appear to no statistically difference between females and males in patients or controls (Fig. 2D, F). However, we do find a positive correlation between the enzymatic activity and protein expression of BACE1 and NRG1 in the schizophrenia only (BACE1, Correlation Coefficient = 0.306, $P = .029$; NRG1, Correlation Coefficient = 0.290, $P = .039$) (Fig. 2G). Our results suggested that the elevation of BACE1-NRG1 enzymatic activity in schizophrenic patients may be partially related to the increased BACE1 protein levels which in turn to cleave more NRG1 substrate and produced soluble NRG1 in the plasma.

### 3.3. BACE1-NRG1 activity associated with clinical features

We investigated the association between the level of BACE1-NRG1 activity and clinical features of schizophrenia, such as disease onset, duration, severity, clinical symptoms and medication. Firstly, we found an inverse correlation between plasma BACE1-NRG1 activity and illness duration (Spearman Correlation Coefficient = −0.326, $P = .020$) (Fig. 3A). The patients having less than eight-year illness showed significantly higher level of BACE-NRG1 activity than patients having longer- term disease duration (40.35 ± 1.814 *vs* 32.88 ± 1.637, $P = .007$) (Fig. 3B). This findings was further supported that plasma average enzymatic activity for BACE1-NRG1 ($V_{mean}$) was also associated with shorter-term duration (Spearman Correlation Coefficient = −0.400, $P = .004$), with similar evaluation tendency compared with longer-term course (9.49 ± 0.718 *vs* 5.23 ± 0.4212, $P = .0001$).

In addition, we found a positive correlation between diseases severity and plasma BACE1-NRG1 activity in schizophrenia patients. As shown in Fig. 3C, compared to schizophrenia patients with low Clinical Global Impression (CGI) scores, patients with high CGI had higher level of BACE1-NRG1 activity, in the $V_{max}$ value (40.70 ± 2.04 *vs*

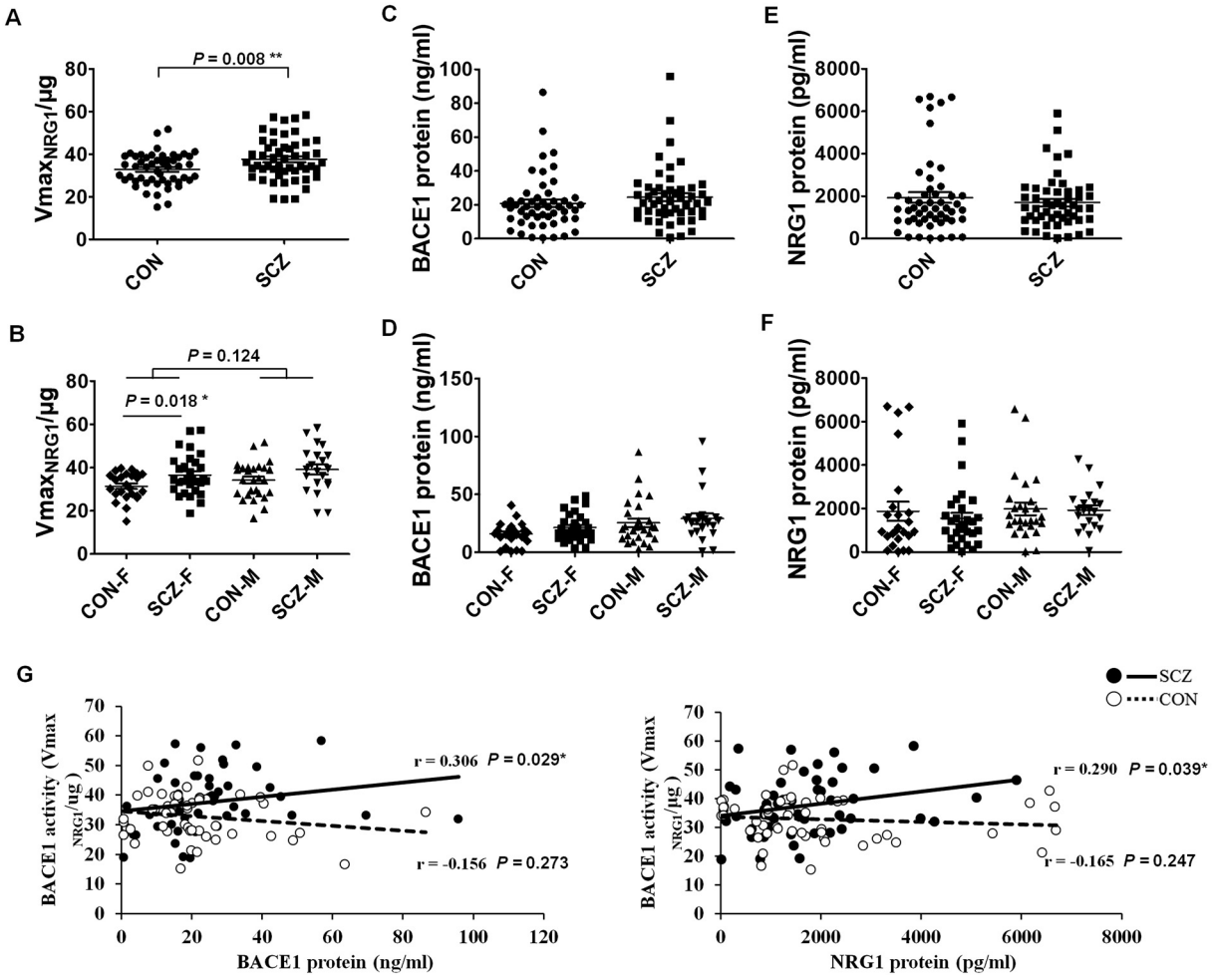



**Fig. 2.** Plasma BACE1-NRG1 activity and their protein level in schizophrenia.
A) Plasma BACE1-NRG1 enzymatic activity from fifty-one schizophrenia patients (SCZ) and fifth-one sex, aged-matched healthy control (CON) expressed as $V_{max}$. B) The level of BACE1-NRG1 activity in males (CON = 27, SCZ = 21) and females (CON = 24, SCZ = 30). Protein expression of BACE1 (C) and NRG1 (E) by ELISA in plasma of schizophrenic patients and healthy controls. The comparison of plasma BACE1 (D) and NRG1 (F) protein levels between males and females with schizophrenia or controls. G) Association between plasma BACE1 protein expression (left panel) and plasma NRG1 protein expression (right panel) and plasma BACE1-NRG1 activity ($V_{max}$) from patients and controls, respectively. Student's *t*-test, Two way ANOVA test, Spearman rank correlation test; * $P < .05$, ** $P < .01$, Bars represent mean ± SEM.

32.74 ± 1.84, $P = .025$). This is to say that the patients with shorter-term disease course and more severity status has higher BACE1-NRG1 activity in plasma (Fig. 3D). Our data showed no significant correlation between BACE1-NRG1 activity and Positive and Negative Syndrome Scale (PANSS) scores and antipsychotic treatment (expressed in equivalent dosage of olanzapine (Leucht et al., 2016)) as shown in Table 2. However, we analyzed BACE1-NRG1 activity in three groups depending on level of disease severity defined by PANSS: mild (PANSS score 58–75), moderate (PANSS score 75–95), or severe (more than PANSS score 95) (Ortiz et al., 2014). The patients with severe disease condition (> 95) had higher level of plasma BACE1-NRG1 activity than the moderate and mild groups (43.02 ± 2.20 *vs* 36.45 ± 2.34 and 36.89 ± 2.19), but no statically difference. Together, our data suggested that alternations of BACE1-NRG1 activity might play more important roles in the illness duration and global symptomatic status and could be considered a potential risk factor for schizophrenia disorder.

### *3.4. Validation of substrate specificity of plasma BACE1-NRG1 activity in schizophrenia*

There are several substrates for BACE1 cleavage, while amyloid precursor protein (APP) is the one most studied and related to Alzheimer's disease (Das and Yan, 2017; Wang et al., 2013; Zetterberg et al., 2010). To validate substrate specificity of BACE1-NRG1 in schizophrenia, we compared BACE1 cleavage of APP (BACE1-APP) (Shen et al., 2018) with BACE1-NRG1 activity in schizophrenia and control samples. Our data showed similar levels of plasma BACE1-APP activity between schizophrenia and controls with no sex difference, and no correlation between BACE1-APP activity and schizophrenic clinical symptoms and disease duration (Fig. 4). These findings indicated that the changes of plasma BACE1-NRG1 activity in schizophrenia were substrate NRG1 specific.

By operating characteristic (ROC) curve analysis, the performance of BACE1-NRG1 activity was moderate with an area under curve (AUC) of 0.728 in early period of schizophrenia (Fig. 5). We found that discriminant scores of plasma BACE1-NRG1 cleavage activity are much more sensitive and specific than other indicators, suggesting plasma BACE1-NRG1 activity as a more effective factor to the diagnosis of schizophrenia in early period.

## 4. Discussion

Both human and mouse genetic studies have demonstrated NRG1 as a risk gene for schizophrenia. It is also known that BACE1 can cleave

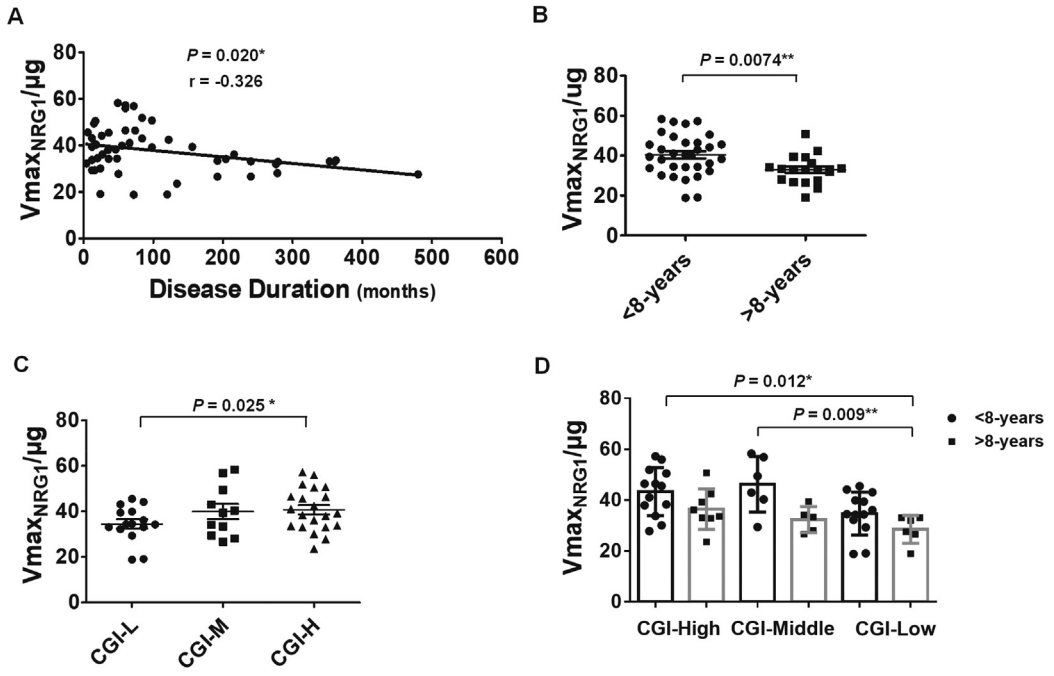



**Fig. 3.** The relationship between plasma BACE1-NRG1 activity and clinical symptoms in schizophrenia.
A) A correlation between plasma BACE1 activity and disease duration. B) The enzymatic activity from patients suffering less 8 years ($N = 32$) are higher level than those with more years ($N = 19$) at $V_{max}$. C) Distributions of plasma BACE1 activity among CGI scores (low ≤ 3 N = 19, middle = 4 $N = 11$, high ≥ 5 $N = 21$). D) Plasma BACE1-NRG1 activity in schizophrenia with different illness course and symptomatic status, low ($N_{< 8\ years} = 13$ and $N_{> 8\ years} = 8$), middle ($N_{< 8\ years} = 6$ and $N_{> 8\ years} = 5$), high ($N_{< 8\ years} = 13$ and $N_{> 8\ years} = 6$). Student's t-test, One/Two way ANOVA test, Spearman rank correlation test; * $P < .05$, ** $P < .01$, Bars represent mean ± SEM.

**Table 2**
The relationship between plasma BACE1-NRG1 activity and clinical symptoms in schizophrenia.

| Schizophrenic patients | | Age | Onset age | Disease duration | BACE1 protein | NRG1 protein | Positive | Negative | General | Total-PANSS | Medicine |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $Vmax/_{NRG1}$ | Coefficient | −0.195 | −0.010 | −0.326 | 0.306 | 0.290 | 0.207 | 0.169 | 0.144 | 0.194 | 0.030 |
| | Significance | 0.171 | 0.945 | 0.020* | 0.029* | 0.039* | 0.163 | 0.257 | 0.334 | 0.191 | 0.837 |

*PANSS* Positive and Negative Syndrome Scale.
* Significance.

NRG1 and produce soluble NRG1 fragments, which activate a cascade of signals. We developed a sensitive and specific assay to measure BACE1 dependent enzymatic activity in cleaving NRG1 in human plasma samples. We found elevated BACE1-NRG1 activity in plasma of schizophrenia compared with sex- and age-matched healthy subjects. Furthermore, BACE1-NRG1 activity was attributed to higher levels of the BACE1 and NRG1 protein in plasma of schizophrenia patients. To further understand the role of BACE1-NRG1 activity in the schizophrenic disease process, we investigated the relationship between the level of BACE1-NRG1 activity and clinical features of schizophrenia. The BACE1-NRG1 activity was highly associated with disease severity as well as disease duration, with a substrate-specific event. Our data suggested that plasma BACE1-NRG1 activity assay could be detected in plasma of individuals and this indicator has a potential role in early diagnosis for patients with severe schizophrenia, as a candidate mechanistic marker.

Recently, the difficulty in schizophrenia diagnosis may attribute to its etiological heterogeneity, and the complex interactions between genetic and environmental factors. Therefore, it is necessary to be classified into subgroups using biological markers to improve validity and reliability of schizophrenia diagnosis. Numerous genes have been reported to be associated with schizophrenia, and thought to increase the risk for the disorder (Farrell et al., 2015; Schizophrenia Working Group of the Psychiatric Genomics, 2014). Within them, NRG1 is an important risk gene. NRGI plays a crucial role in mediation of several neurodevelopmental processes and has been shown to be linked to schizophrenia in different populations (Hu et al., 2016; Zhang et al., 2017). At this stage, three major splicing isoforms of NRG1, including Type I (acetylcholine receptor), Type II (glial growth factor), and Type III (sensory and motor neuron-derived factor), are extensively studies, each of them appears to have distinct effects on human brain development and maturation (Falls, 2003; Mei and Xiong, 2008). The extracellular EGF-domain of NRG1 can be released into extracellular matrix by enzymatic cleavage, thus binds with tyrosine kinase ErbB4 receptor and activates downstream signal pathway (Schmid et al., 2003). It is thought that the NRG1/ErbB signal pathway participates in regulating development of the central nervous system, particularly mediation of neuronal migration, myelination, and glutamatergic networks (Kwon et al., 2005; Miyamoto et al., 2017). Many studies have implicated that brain development plays an etiologic role in schizophrenia (Corfas et al., 2004). For example, NRG1, at both mRNA and protein levels, is elevated in schizophrenic postmortem brain relative to healthy individuals (Chong et al., 2008). Other evidence that abnormal NRG1/ErbB signaling in schizophrenia from comparisons of gene expression in dorsolateral prefrontal cortex is related to antipsychotic medication dosage-dependent elevation of NRG1 type I level (Hashimoto et al., 2004). Hence, it is crucial to explore how BACE1, a key secretase, regulates NRG1 cleavage process in living schizophrenia.

In keeping with the hypothesis, abundant BACE1 in plasma was observed as the same size as human brain protein in membrane.

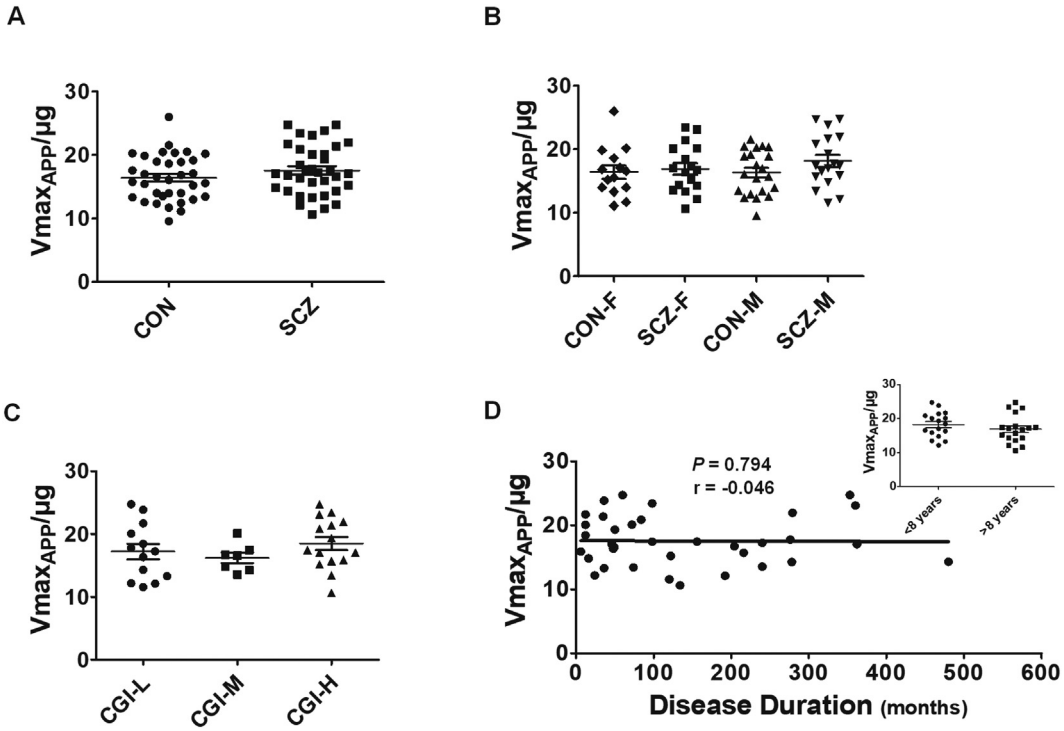
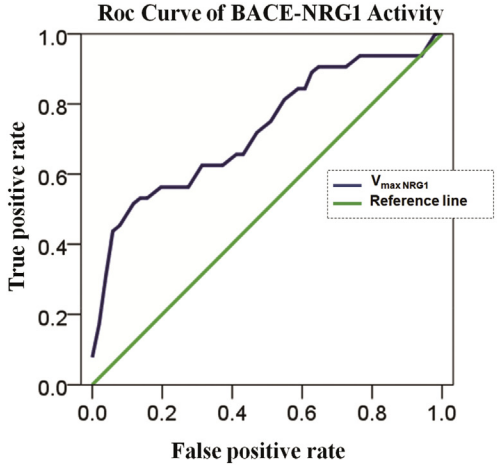



**Fig. 4.** Normal plasma BACE1-APP activity in schizophrenic patients compared with healthy controls.
A) Plasma BACE1-APP activity was no obvious difference between schizophrenia and controls expressed as $V_{max}$ (CON = 35, SCZ = 35). B) No sex difference between schizophrenia and controls was found in plasma BACE1 cleavage APP, males (CON = 21, SCZ = 18) and females (CON = 14, SCZ = 17). C) Plasma BACE1 activity is not related with disease severity assessed as CGI scores (low ≤ 3 $N$ = 13, middle = 4 $N$ = 7, high ≥ 5 $N$ = 15). D) No statistically significant associations between BACE1-APP activity and disease duration, $N_{<100months}$ = 17 and $N_{>100months}$ = 18. Student's t-test, Mann Whitney test, One/Two way ANOVA test, Spearman rank correlation test; Bars represent mean ± SEM.

Besides, we detected three obvious fragments in total, among which membrane fractions of NRG1 was ~120 kDa and ~70 kDa size and soluble NRG1 in cytoplasm was ~55 kDa. The fragment at the expected size of ~55 kDa was detected in plasma, which was a soluble cleavage product by secretases (Fledrich et al., 2014). Due to ADAM17 or ADAM10 involving in successive release of EGF-like domain of NRG1, plasma NRG1 protein level may not completely represent ability of BACE1 cleavage activity (Fleck et al., 2013). However, BACE1 dependent cleavage is essential for proteolytic processing and has a crucial role in regulation NRG1 function (Horiuchi et al., 2005). We have developed a valid and novel assay of BACE1 activity from plasma of Alzheimer patient (Li et al., 2004; Wu et al., 2012). Consistent with previous reports, BACE1 maximizes its enzymatic ability in acid environments. Similarly, plasma BACE1-NRG1 activity was detected in lower pH condition. Hence, we developed and optimized a plasma-based assay to detect enzymatic activity of BACE1 cleavage schizophrenia-related substrate NRG1 in human plasma samples.

This assay was applied to detect plasma BACE1-NRG1 activity from chronic schizophrenia and age, sex- matched controls. Our results showed significantly higher BACE1-NRG1 activity in schizophrenia than healthy controls. Given heterogeneity and complex of schizophrenic pathology, we used statistic methods to operate clinical




| | AUC | Cut-off value | Sensibility | Specificity | *P* |
|---|---|---|---|---|---|
| $Vmax_{NRG1}$ | 0.728 | 39.845 | 0.531 | 0.882 | 0.001 |
| $Vmean_{NRG1}$ | 0.670 | 7.100 | 0.688 | 0.588 | 0.009 |
| NRG1(Elisa) | 0.475 | 950.000 | 0.938 | 0.118 | 0.708 |
| BACE1(Eisa) | 0.613 | 15.295 | 0.844 | 0.392 | 0.083 |
| $Vmax_{App}$ | 0.648 | 19.240 | 0.500 | 0.778 | 0.092 |

**Fig. 5.** Roc curve assessing different indicators for early schizophrenia.
ROC curve was drawn for BACE-NRG1 activity for schizophrenia. The area under the curve was 0.728. Different threshold values ($Vmax_{NRG1}$, $Vmean_{NRG1}$, NRG1-Elisa, BACE1-Elisa and $Vmax_{APP}$) was listed for schizophrenia and healthy controls based on clinical diagnosis in early period.

characteristic analyses for BACE1-NRG1 activity. We found the patients with less eight-year term course have greater elevations in plasma BACE1-NRG1 activity than those who are affected with a longer disease course, suggesting that abnormal enzymatic activity existed in those suffering from severe symptoms in early stage. Time course of illness was regarded as an important pathognomonic feature of the disorder (An Der Heiden and Hafner, 2015). There is evidence suggesting the existence of apparent brain abnormalities at the onset psychosis, which is progressively altered over the course of illness (Arango et al., 2012; Pantelis et al., 2003). Identifying a biomarker of progression at a particular period in the course of illness could promote guided treatment and assist in predicting outcome of disorders. In addition, we examined the relationship of BACE1-NRG1 activity and severity of illness using two measures. We found no direct association between BACE1-NRG1 and PANSS scores, but it is an indicator that higher BACE1-NRG1 activity existed in patients with higher PANSS scores. Moreover, there was a significant relationship with severity as measured with CGI. Thus, the clinical character study offered important insights into evaluated BACE-NRG1 activity in more narrowly specified populations suffering from severe schizophrenic condition in an acute period.

The activity of BACE1 in plasma to cleave APP, an Alzheimer's disease related substrate, shows no significant difference between schizophrenia and controls, suggesting a substrate-specific BACE1 from schizophrenic plasma, which is more sensitive in schizophrenia-related protein NRG1 cleavage. BACE1 has at least 15 well-known physiological substrates that are involved in neurite outgrowth, neurotransmitter and synapse formation, implicating it in the pathophysiology of psychiatric disorders (Kuhn et al., 2012; Yan et al., 2014).

Although there was no statistic difference between schizophrenia and controls in NRG1 and BACE1 protein expression, schizophrenia patients with plasma higher NRG1 and BACE1 expression level have much more increased BACE1-NRG1 enzymatic activity. As single measurement of plasma protein expression was not sufficient, combination of other available methods such as analysis of BACE1-NRG1 activity is needed to achieve more precise diagnosis. This is partly consistent with previous results obtained by Ketan Marballi et al. (Marballi et al., 2012), who found abnormal BACE1 dependent NRG1 cleavage processing in BA9 region of schizophrenia. Moreover, overexpression of NRG1-NTF into mouse brains was sufficient to cause schizophrenia-like behaviors (Luo et al., 2014). Therefore, dysfunction of BACE1-dependent proteolytic activity may affect its substrate NRG1, which regulates the downstream NRG1/ErbB signaling pathway, and therefore increases risk factors for schizophrenia. In future, we will conduct more extensive studies including patients with chronic but stable schizophrenia. Moreover, results from this study would be important to justify the study of subjects at the onset of their illness, or individuals with high risk. We will also further investigate underlying mechanisms of why BACE1 levels are elevated in certain groups of schizophrenia.

Together, our findings indicated that BACE1-NRG1 activity was significantly increased in patients suffering from more severe schizophrenia early in their course of illness, using an accurate plasma enzymatic activity assay. We propose that this may represent a reliable biomarker for schizophrenia, which can improve the early diagnosis of disorder and facilitate the initiation of disease therapy.

## Author contributions

ZZ and JC performed most of experiments as well as manuscript writing. FG and YL provide technical support for the BACE-NRG1 assay. GZ and ML provided patients samples from Anding Hospital BioBank. RY and YS provided consultation on the project design and discussion of the manuscript. RL was responsible for the experimental design, results discussion as well as helped manuscript preparation for publication.

## Ethical statement

All participants in this study were provide with written information and written consent was sought from all eligible individuals prior to participation. These samples from individuals were collected in Anding Hospital Bio-Bank (project number D131100005313011). This study was approved by the Ethical Committee (IRB equivalent) of Beijing Anding Hospital, Capital Medical University.

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## Conflict of interest statement

The authors declare no biomedical financial interests or potential conflicts of interest.

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