Increased Blood-Reelin-Levels in First Episode Schizophrenia

Tobias Hornig*, Lukas Sturm, Bernd Fiebich, Ludger Tebartz van Elst

Department of Psychiatry, Albert-Ludwigs-University, Hauptstr. 5, 79104 Freiburg, Germany

* tobias.hornig@uniklinik-freiburg.de

Abstract

Background
Reelin is an extracellular glycoprotein involved in several functions of brain development, synaptogenesis and dendritic proliferation. Numerous studies found perturbation in the reelin system and altered serum reelin levels in neuropsychiatric patients using the western blot procedure. In the international literature, this is the first study that made use of an enzyme-linked immunosorbent assay to analyze serum reelin protein concentration quantitatively.

Rationale
In order to study possible alterations in reelin blood levels in schizophrenia, we analyzed this signal in schizophrenic patients with a first episode hallucinatory and paranoid syndrome and control subjects in a pilot study design.

Results
We found increased blood reelin protein concentration in schizophrenic patients compared to healthy controls.

Discussion
Our findings point to a relevant role of reelin metabolism in the pathogenesis of schizophrenia. Reelin could be a biomarker for the course of disease or psychopharmacological treatment.

Conclusion
We conclude that the reelin protein blood concentration might be a relevant signal with respect to the pathophysiology of schizophrenia.

Introduction
Reelin (RELN) is a large extracellular glycoprotein involved in embryonic neuronal migration and in several functions of brain development [1–3]. It is expressed by CajalRetzius cells in...
layer I and GABAergic interneurons in all layers of the cortex and hippocampus as well as in the cerebellum by glutamatergic neurons [4,5]. At present five processed fragments in biopsy fluids (340-kDa, 270-kDa, 190-kDa, 180-kDa, 80-kDa) of this 450 kDa large protein are known [6,7]. Furthermore, RELN plays an important role in dendritic growth, synaptic plasticity and, as a modulatory element of the N-Methyl-D-Aspartate (NMDA) receptor [8,9], in long-term potentiation (LTP) and thus also in memory and learning [10]. In line with these findings Apo- E- and Very low-density lipoprotein (VLDL)- receptor deficient knockout mice respectively display an impairment in fear conditioned memory formation, spatial learning and defective LTP induction [11–13]. Disturbed RELN metabolism leads to deficient cortical lamina- tion for example in the Reeler Mouse phenotype[2,14–16]or to lissencephaly in humans [17,18]. Lissencephaly is a condition in which the cerebral and cerebellar gyration is reversed with very different characteristics like mental retardation, epilepsy or muscle spasticity [2,19–21]. Also, there is evidence that RELN could be involved in other neuropsychiatric disorders such as Alzheimers disease, frontotemporal dementia, autism spectrum disorders, bipolar affective disorders, some subtypes of epilepsy and also schizophrenia[22–27].

RELN-findings in schizophrenia

Table 1 summarized all RELN-related findings with respect to schizophrenia.

Post mortem analyses revealed reduced RELN mRNA in the brains of schizophrenic patients [28–31]. Especially in the prefrontal lobe and hippocampus as well as in the cerebel- lum, a smaller dendritic length and a reduced density of dendritic spines were found [1,30,32,33]. Fatemi et al. found increased blood RELN concentration in patients with schizo- phrenia and decreased RELN concentration in bipolar affective disorders [34]. Others show an increased cerebrospinal RELN concentration in patients with Alzheimers disease and Fronto- temporal Dementia [23,35]. Current studies indicate hypermethylation of the RELN- gene pro- motor in patients with schizophreniform and autism spectrum disorders while the DNA Methyltransferase 1 (DNMT1) mRNA concentration was increased in the same samples, whereby DNMT1 methylates the RELN promotor.[36–39]. These results point to a possible pathogenetic effect of RELN in different neuropsychiatric disorders. Some drugs, such as the anticonvulsant and mood stabilizer valproic acid, cause a demethylation of these regions and a acetylation of histone proteins and therefore increased transcription rates of the RELN gene [29,40,41].

In the past, schizophrenia was thought to be a degenerative development disorder [42,43]. In the international literature, however, there are neither clues for cortical dyslamination such as in the reeler mouse or in patients with lissencephaly nor convincing evidence for morpho- logical neurodegenerative aspects [44,45].Nevertheless there are a lot of hints for synaptic dys- function and lack of neuronal plasticity [46–48].

As aforementioned, a lack of higher cognitive functions and neuronal plasticity is assumed to be caused by defective RELN homoeostasis. Numerous previous studies found respective deficits in schizophrenic patients especially in verbal and working memory, spatial learning as well as executive functions [49–53]. On the basis of this findings and considering the preceding studies we hypothesized altered blood RELN concentration in schizophrenia without prediction the direction of alteration.

To test this hypothesis and to get quantitative figures of blood reelin protein concentration in schizophrenic and healthy subjects, we used an Enzyme-linked immunosorbent assay (ELISA) of serum RELN-protein. This approach is unprecedented so far and for that reason no standard values were defined until now.
Materials and Methods

2.1 Patients and control subjects

Twenty medicated patients with Diagnostic and Statistical Manual of Mental Disease (DSM)-IV-defined schizophrenia (10 male and 10 female), hospitalized for acute exacerbation were identified among the in-patients of the Department of Psychiatry and Psychotherapy of the University Hospital of Freiburg which were treated unsolicited and voluntarily. The clinical diagnosis of schizophrenia was made by experienced senior consultant psychiatrists based on a structured interview according to DSM-IV criteria, the ability of consent based on the psychopathological interview according to the Arbeitsgemeinschaft für Methodik und Dokumentation in der Psychiatrie (AMDP)-system and refers to the common principles of decision-making [54]. After having given informed written consent to participate on a voluntary basis, the patients were included into this study. We did not include patients that where housed judicial or where situated in legal care. All patients received a full neurological and medical history and examination as well as routine blood tests and EEG examinations in order to exclude any other medical or neurological disorder. Patients were excluded if they had any other DSM-IV axis I diagnosis or met criteria for substance abuse within the previous 6 months. In order to keep the sample homogenous by avoiding an overlap of psychogeriatric problems, the study
group was restricted to patients with a first episode diagnosis of schizophrenia under the age of 45 years and with a normal IQ range. The control subjects were recruited in our neurological ambulance where subjects were assessed by experienced senior consultants. Based on a comprehensive neuropsychiatric assessment including a thorough medical and family possible all neuropsychiatric disorders were excluded.

2.1.1 Ethics Statement. Approval from the Albert-Ludwigs University ethics committee Freiburg was obtained before onset of the study (no.: 335/13).

2.2 Enzyme-Linked Immunosorbent Assay
We used an ELISA kit by Cusabio, performing a quantitative sandwich enzyme immunoassay that detects human reelin-protein in serum, plasma and tissue homogenates. Its detection range is 0.156 ng/μl–10 ng/μl, the sensitivity is typically <0.039 ng/μl. Sample signals below the detectable concentration limit were therefore approximated 0 ng/μl. The assay is highly specific for human reelin. Repeated testing of reference samples accounted for a recovery rate of 94.59% in human serum. No significant cross reactivities have been observed. Intra- and inter-assay coefficients of variation, determined by testing three reference samples twenty times on one plate respectively on twenty different plates, are <8% respectively <10%. Thus retest reliability of this method is very good. Epitope mapping was not done.

The standard series was obtained by performing six 1:2 dilution steps starting from a high standard with an antigen concentration of 10 ng/ml (thus resulting in concentrations of 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.312 ng/ml, 0.156 ng/ml). A solution with a concentration of 0 ng/ml served as zero standard.

100 μl of sample respectively standard at a time were pipetted into the wells of a 12 x 8 polystyrene microtiter plate precoated with a human reelin-specific monoclonal mouse antibody and incubated for two hours at 37°C. After removal of supernatant 100 μl of a solution containing biotinylated polyclonal mouse antibody specific for human reelin was added to each well and incubated for one hour at 37°C. Unbound detection antibody was thereafter removed by applying 200 μl of wash buffer and aspirating three times per well. Subsequently 100 μl of avidin-bound HRP (horseradish peroxidase) solution were added to each well and incubated for one hour at 37°C. Afterwards aspiration/washing was repeated five times. Then 90 μl of TMB substrate (3.3',5.5'-tetramethylebenzidine) were added and incubated at 37°C. The probes were protected from light. After 25 minutes 50 μl of stop solution (1N sulfuric acid) at a time were added. Within a period of five minutes the optical densities were determined using a Dynex MRX microplate reader set to a wavelength of 450 nm. Samples and standards were then evaluated with the Dynex Revelations software.

2.2.1 Western Blot. For electrophoresis, equal amounts of serum (3 μl) were loaded per gel lane, size-fractionated on a 3–8% Tris-acetate-polyacrylamide gel (Invitrogen, Karlsruhe, Germany), and blotted onto a polyvinylidenedifluoride (PVDF) membrane (Roche, Mannheim, Germany). For immunodetection, PVDF membranes were pretreated with 1-Block buffer (Tropix, Bedford, MA, USA) followed by incubation with the following antibodies: mouse monoclonal anti-human Reelin (1:500; 142; Chemicon) or rabbit polyclonal anti-Ceruloplasmin (1:5000; Dako, Glostrup, Denmark) for 1 h at room temperature. After several washing steps, the membranes were incubated with the respective alkaline-phosphatase-conjugated secondary antibody (1:10.000; Tropix) for 1 h at room temperature and CDP Star (Tropix) was used as substrate for chemiluminescent detection by the Chemismart System (Peqlab Biotechnologies, Erlangen, Germany).

Quantification of Western blot signals was performed by optical density (OD) measurement using the Bio-1D software (Peqlab). OD values were determined for each of the three Reelin
isoforms (400, 320 and 180 kDa) detected by the Reelin antibody. Data were normalized to Ceruloplasmin signals as loading control.

### 2.3 Statistical Analysis

We analyzed the socio-demographically comparability of our patient and control group using a T-test for metric variables like age and a Chi-square-test for categorical variables like gender or education. Between group effects in our primary outcome measure was analyzed using a standard t-test procedure with group as main factor and RELN concentration as depending variable. In order to analyze a possible confounding effect of the factor medication on RELN signal we compared RELN levels between the different main antipsychotic groups (risperdone: n = 12; olanzapine: n = 5; aripiprazole: n = 1). In order to analyze a possible interaction between factors like school education, or family history for psychosis or any other psychiatric disorder we additionally calculated an univariate ANOVA in a general linear model with these factors. A p-value of .05 was chosen as the criterion of significance.

### Results

#### 3.1 Demographic details

Table 2 summarizes the demographic and psychosocial data of our patients and group matched healthy controls. Two probands had to be excluded from both groups because the samples’ reelin concentrations were outside the detection range:

- Both groups were matched in terms of age, gender and school education.

#### 3.2 Serum reelin concentration

We found a mean serum RELN concentration of 1.32 ng/μl on the side of the healthy controls as can be seen in Table 2. The range was from under 0.156 ng/μl until 3.17 ng/μl. In comparison the mean serum RELN concentration of patients was 2.59 ng/μl and thus nearly twice as large as the concentration of controls with a range from under 0.156 ng/μl until 5.29 ng/μl (see Fig 1). Thus, five patients were out of the range of the healthy controls.

**3.2.1 Group comparison concerning serum reelin levels.** Table 2 also shows that the mean value of the serum RELN concentration in patients and healthy controls differed significantly in that patients displayed significantly increased signals (p = 0.007, df = 34, T = 2.8). Fig 1 illustrates the scatterplot of this main finding of our pilot study. There was no significant interaction between RELN signals and school education (p = 0.314, F = 1.2), medication

### Table 2. Demographic data and RELN levels of our patients and group matched healthy controls.

|                | Schiz. (n = 18) | Contr. (n = 18) | Statistics |
|----------------|-----------------|-----------------|------------|
| Age            | 26.22 [5.07]*   | 26.33 [3.94]*   | T = -0.073, df = 34, p = .94 |
| Gender         | F:M / 8:10      | F:M / 9:9       | Chi² = 0.22, df = 1, p = 0.896 |
| School education | L:M:H / 3:7:8 | L:M:H / 4:5:9 | Chi² = 0.11, df = 1, p = 0.5 |
| RELN conc. 1   | 2.59 ng/μl [1.5]* | 1.32 ng/μl [1.1]* | T = 2.8, df = 34, p = .007 |
| RELN conc. 2   | 0.587 ng/μl [0.582]* | 0.387 ng/μl [0.287]* | T = 1.43, df = 34, p = .16 |

| [Age/RELN conc.:*mean [SD]; Gender: F = female, M = male; School education: L = low, M = middle, H = high; conc. = concentration, conc. 1 with ELISA, conc. 2 with Western Blot.]

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In this pilot study we measured the blood RELN protein concentration of schizophrenic patients and healthy controls with an Enzyme-linked immunosorbent assay. Our main finding was an increased blood RELN protein level in schizophrenic patients. This finding relates well to the only other study that analyzed RELN blood levels i.e. the paper by Fatemi et al. who also found increased blood RELN concentrations in schizophrenic patients compared to healthy controls [34]. Apart from that there are a number of studies analyzing RELN and DNA Methyltransferase 1 (DNMT1) mRNA concentration in the cerebrum and cerebellum (Table 1).

To compare our finding obtained with the ELISA method to findings of other authors who used the western blot procedure in an post-hoc approach we also measured the serum RELN protein concentration with the Western Blot technique following Tinnes S, Ringwald J and Haas CA [55]. For that purpose we only included those samples with signals above the predefined ELISA cut off threshold of 0.156 ng/μl. Doing this we found a significant correlation between the ELISA and Western Blot signals for the 340 k-Da RELN fragment (r = 0.4, p = 0.02). Comparing Western Blot based RELN signals between groups we again identified increased average RELN levels in schizophrenic patients (0.58 Optical Density) vs. controls (0.36 Optical Density), which just failed to reach level of significance (p = 0.16, T = 1.4) as you can see in Fig 1. This is no surprise given the small sample size and corresponds well to the findings of Fatemi et al. in Caucasian schizophrenics [34].

When also including those patients with signals below the official ELISA detection threshold, there was no significant correlation between the two signals. This of course reflects the fact that including measurement outside the accepted measurement range of a method nessecarily

**Fig 1. RELN serum concentration for schizophrenic patients and healthy controls.** Long bars correspond to the mean values, short bars show the 95% CI.

(p = 0.581, F = 0.56), family history of mental illness (p = 0.477, F = 0.51), age (p = 0.179, F = 1.89) or gender (p = 0.834, F = 0.04) in the two groups.

**Discussion**

In this pilot study we measured the blood RELN protein concentration of schizophrenic patients and healthy controls with an Enzyme-linked immunosorbert assay.

Our main finding was an increased blood RELN protein level in schizophrenic patients. This finding relates well to the only other study that analyzed RELN blood levels i.e. the paper by Fatemi et al. who also found increased blood RELN concentrations in schizophrenic patients compared to healthy controls [34]. Apart from that there are a number of studies analyzing RELN and DNA Methyltransferase 1 (DNMT1) mRNA concentration in the cerebrum and cerebellum (Table 1).

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When also including those patients with signals below the official ELISA detection threshold, there was no significant correlation between the two signals. This of course reflects the fact that including measurement outside the accepted measurement range of a method nessecarily
induces noise in each experiment. Obviously the discriminating power of ELISA-based RELN signal detection below the recommended threshold should be regarded with caution.

Presently, we do not know how increased serum RELN signals translate into cerebral RELN concentrations. Up to date only little is known about the mutual interaction between peripheral and brain RELN homeostasis. Apart from the RELN expression in cerebral cells, there are clues for its expression in liver-, plasma-, adrenal- and pituitary cells with a different glycosylation in plasma and cerebral fluid [35,56,57]. Moreover, there are several different influences such as post translational effects, loss of origin cells (e.g. GABAergic interneurons) or medication that could have variable or even opposite effects on the concentration of RELN in cerebral spinal fluid (CSF) or blood respectively[58]. Therefore conclusions about the interaction between peripheral and central RELN levels should be handled carefully.

Until now, only three chronically schizophrenic patients were measured with regard to the RELN concentration of cerebral fluid in vivo [59]. At present, there is no empirical information about the dynamics of RELN levels in the brain or cerebral fluid over the course of disease or in relation to healthy controls.

However, there is evidence to suggest that reelin (N-terminal 180 kDa fragment) regulates dendritic maturation in vitro [60]. Blocked serotonin receptors (5-HT 3A subtype) in CajalRetzius cells caused a reduced reelin release and a dysregulated dendritic proliferation. A similarly altered dendritic complexity was found in 5-HT 3A receptor lacking mice [61,62]. On the other hand the expression of 5-HT 3A receptors in peripheral blood mononuclear cells (lymphocytes) was up-regulated after medication with antipsychotic drugs [63] and studies with serotonin transporter antagonists indicate higher serum RELN levels in adults [64]. Therefore we have to consider that our finding might be modulated by medication effects. If this was the case RELN concentration could serve as an objective marker of therapeutic response to neuroleptic drugs. Supporting such assumptions there is preliminary evidence that some psychopharmacological drugs are involved in epigenetic mechanisms of the RELN promoter region and enhance the transcription rate of RELN[59]. All of these findings are in line with previous findings and indicate, that a psychopharmacological therapy could affect the peripheral RELN homeostasis [29,38,40,41,65]. However, further research is necessary to prove such propositions.

The possible meaning of our finding: Taking together all available data and our findings there is ample evidence to support the notion that the RELN system might well play an important role in the pathophysiology of schizophrenia. It might even turn out to be a possible biomarker for the course of disease. Thus, in relationship to other findings, RELN could indicate the response of psychopharmacological medication [56]. At present it is unknown how the altered expression of RELN in brain or peripheral tissue is regulated in the course of disease irrespective of psychopharmacological medication. This should be subject of further studies.

Methodological limitation: The sample size of our study is small but comparable to similar studies. Furthermore we did not measure unmedicated patients and there was no parallel assessment of peripheral and central RELN. Altogether we do not know actually how far we measured a medical effect or how far cerebral or peripheral mechanisms are responsible for blood or CSF RELN levels in the course of disease.

Furthermore no standard values in relation to the serum RELN concentration were defined. So we cannot validate our results or compare it with results in the international literature. However, this is not a critical point at this stage of research, where case-control studies are the method of choice to increase our knowledge of this interesting signal.

Summary: We found an increased level of blood RELN concentration in patients with schizophrenia in comparison to a healthy control population. Our finding is in line with the only other study analyzing blood RELN signal in schizophrenia. Further studies with larger
sample size will have to answer the question in which way the RELN concentration is related to the dynamics of a psychopharmacological therapy respectively the blood levels of the psychopharmacological agents and in which way the cerebral and peripheral RELN levels are linked to the schizophreniform disease processes.

Supporting Information
S1 Dataset. (PDF)

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Author Contributions
Conceived and designed the experiments: TH LTvE. Performed the experiments: TH LS. Analyzed the data: TH BF. Contributed reagents/materials/analysis tools: LTvE BF. Wrote the paper: TH.

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