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Research report

Peripheral DISC1 protein levels as a trait marker for schizophrenia and modulating effects of nicotine

Svenja V. Trossbach[a], Karin Fehsel[c], Uwe Henning[c], Georg Winterer[c,1], Christian Luckhaus[c], Sandra Schäble[b,2], M. Angelica de Souza Silva[b], Carsten Korth[a,*]

[a] Department Neuropathology, Medical Faculty, Heinrich Heine University Düsseldorf, Germany
[b] Center for Behavioral Neuroscience, Heinrich Heine University Düsseldorf, Germany
[c] Department of Psychiatry and Psychotherapy, Medical Faculty, Heinrich Heine University Düsseldorf, Germany

HIGHLIGHTS

- Detection of a protein from a major mental illness gene, Disrupted-in-Schizophrenia 1 (DISC1) in lymphocytes of humans.
- Demonstration of decreased levels of lymphocytic DISC1 in patients with schizophrenia suggesting its potential for a schizophrenia biomarker.
- Demonstration that peripheral DISC1 levels are modulated by nicotine but not to the degree of interfering with schizophrenia diagnosis establishing its possible peripheral readout for psychotropic drug effects.
- Demonstration that administration of nicotine leads to decreased aggregated DISC1 in the rat mPFC.

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ABSTRACT

The Disrupted-in-Schizophrenia 1 (DISC1) protein plays a key role in behavioral control and vulnerability for mental illnesses, including schizophrenia. In this study we asked whether peripheral DISC1 protein levels in lymphocytes of patients diagnosed with schizophrenia can serve as a trait marker for the disease. Since a prominent comorbidity of schizophrenia patients is nicotine abuse or addiction, we also examined modulation of lymphocyte DISC1 protein levels in smokers, as well as the relationship between nicotine and DISC1 solubility status. We show decreased DISC1 levels in patients diagnosed with schizophrenia independent of smoking, indicating its potential use as a trait marker of this disease. In addition, lymphocytic DISC1 protein levels were decreased in smoking, mentally healthy individuals but not to the degree of overriding the trait level. Since DISC1 protein has been reported to exist in different solubility states in the brain, we also investigated DISC1 protein solubility in brains of rats treated with nicotine. Subchronic treatment with progressively increasing doses of nicotine from 0.25 mg/kg to 1 mg/kg for 15 days led to a decrease of insoluble DISC1 in the medial prefrontal cortex. Our results demonstrate that DISC1 protein levels in human lymphocytes are correlated with the diagnosis of schizophrenia independent of smoking and thus present a potential biomarker. Reduced DISC1 protein levels in lymphocytes of healthy individuals exposed to nicotine suggest that peripheral DISC1 could have potential for monitoring the effects of psychoactive substances.

1. Introduction

Schizophrenia is a mental illness exclusively diagnosed clinically by the occurrence of positive, negative, and cognitive symptoms. Positive symptoms are, for example, hallucinations or delusions, negative symptoms include affective flattening, lack of motivation, whereas cognitive impairments comprise attention deficits and impaired working memory [1].

One comorbid behavioral characteristic of patients diagnosed with schizophrenia is the high prevalence for nicotine abuse by excessive smoking. Among patients with schizophrenia, 70–80%

* Corresponding author at: Neurodegeneration Unit, Department Neuropathology, University of Düsseldorf Medical School, Moorenstrasse 5, 40225 Düsseldorf, Germany. Tel.: +49 211 8116153; fax: +49 211 8117804.
E-mail address: ckorth@hhu.de (C. Korth).
[1] Present address: Experimental and Clinical Research Center (ECRC), Charité – University Medicine Berlin, Berlin, Germany.
[2] Present address: Comparative Psychology, Heinrich Heine University Düsseldorf, Düsseldorf, Germany.

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smoke in contrast to 20–30% of the general population and 30–50% in other psychiatric illnesses [2,3]. One attempt to explain this phenomenon has been the self-medication hypothesis of schizophrenia, stating that smoking and thereby nicotine administration serves to reduce particularly negative symptoms [4]. In support of this hypothesis it was shown that nicotine reversed sensory gating deficits, i.e. the inability to process sensory information in patients diagnosed with schizophrenia. This deficit, measured, e.g. by the diminished gating of the P50 auditory-evoked response to repeated stimuli, could be rescued by treatment with nicotine in patients and their non-affected family members [5,6] and thus presents an endophenotype. Nicotine administration or smoking also improved sensorimotor gating in rats [7] and in non-psychiatric human control subjects [8,9]. The sensory gating deficit in schizophrenia is genetically linked to chromosome 15q14 [10], the gene locus of the nicotinic α7 receptor subunit, interrelating schizophrenia and nicotine.

Disrupted-in-schizophrenia 1 (DISC1) is one of the best characterized vulnerability genes for psychiatric disorders. It was first discovered in a Scottish family in which carriers of a balanced chromosome t(1;11) (q42.1;q14.3) translocation are prone to mental illnesses of different clinical phenotypes ranging from schizophrenia and major depression to bipolar disorder, and anxiety disorders [11,12]. The translocation presumably leads to the expression of a C-terminally truncated protein named DISC1. Subsequent gene association studies corroborated the importance of the DISC1 locus in mental illness and substance abuse [13–15], however, importantly, not limited to one clinically defined disease entity, but as a risk factor to several mental illnesses [16–20]. Cell and molecular studies highlight its function as a scaffold protein [21] that interacts with a variety of proteins [22] for integrating cellular mechanisms like developmental control of neuronal migration, neuronal progenitor cell proliferation, signaling pathways, synaptic function, centrosome formation and neurite outgrowth, amongst others [23–26]. DISC1 transgenic animal models have clearly corroborated the key role of DISC1 in behavioral control, such as in working memory, locomotion and sensorimotor gating [27–33]. Our own previous findings in human *post mortem* brain material indicated that a subpopulation of mentally ill patients with schizophrenia or recurrent affective disorders was characterized by the presence of insoluble DISC1 protein in the brain, thus crossing current clinical diagnostic boundaries [34,35]. Subsequent *in vivo* and *in vitro* biochemical analyses investigating DISC1 protein assembly indicated that DISC1 builds oligomers and insoluble aggregates under specific physiological and pathological conditions [26,36,37].

A meta-analysis of several genome-wide association studies of smoking phenotypes indicated linkage of a single-nucleotide polymorphisms (SNP) in the 3′ region of the mental illness candidate gene DISC1 to earlier onset of smoking, although the analysis failed to reach significance due to multiple testing corrections [38].

In the present study we set out to test, whether the DISC1 protein may serve as a trait marker for schizophrenia by assessing DISC1 protein levels in lymphocytes derived from blood samples of patients diagnosed with schizophrenia and control subjects. We also investigated the role of DISC1 as a state marker by examining lymphocytic DISC1 protein levels in healthy smokers versus non-smokers. Plasma cotinine levels, the main metabolite of nicotine in the blood, of patients with schizophrenia and controls were measured in an attempt to correlate lymphocytic DISC1 levels and nicotine intake. Furthermore, to examine, whether nicotine can influence the solubility status of DISC1 in the brain, we measured levels of aggregated DISC1 in rats sub-chronically exposed for 15 days to up to daily 1 mg/kg nicotine.

2. Material and methods

2.1. Subjects and classification

Healthy subjects and patients diagnosed with schizophrenia were investigated as part of a clinical study comparing acute nicotine challenge with placebo in a neuroimaging setting (ClinicalTrial.gov Identifier: NCT00618280) [39]. The schizophrenia patients were clinically stable for more than six weeks. All patients and controls underwent a structured interview (SCID-1), and diagnosis was established according to DSM–IV criteria [40]. 78.4% of patients received antipsychotic monotherapy with either amisulpride, aripiprazole, fluphenazine, olanzapine, paliperidone, quetiapine, risperidone or ziprasidone. A small number of patients were treated with a combination of two antipsychotic drugs: flupentixol/quetiapine, haloperidol/quetiapine, olanzapine/ziprasidone or paliperidone/aripiprazole. Healthy controls were required to have no life-time diagnosis of schizophrenia, illegal drug or alcohol dependence. Exclusion criteria were: concomitant neurological diseases or any other medical condition that was considered as a potential confounder of the study, including a positive drug screen or a history of substance abuse during the last six months prior to participation in the study. In schizophrenia patients, assessment of current psychopathology was conducted with the Positive and Negative Syndrome Scale (PANSS; [41]). Nicotine dependency was verified using the Fagerstrom test (FTND [42]). Study participants were either habitual smokers as defined by a FTND score of >4, smoking 10–30 cigarettes per day or never-smokers who had smoked less than 20 cigarettes in their life-time. The study was approved by the Ethics committee of the Medical Faculty of the Heinrich-Heine-University Düsseldorf and the German regulatory agency for drug trials BfArM (Bundesarzneimittelbehörde) and under full consideration of the Declaration of Helsinki. Written informed consent was obtained from each study participant prior to the start of the study.

2.2. Animals

Animal experiments were performed in accordance with the German Animal Protection Law and were authorized by local authorities (LANUV NRW). Adult male Wistar rats were housed three animals per cage under standard laboratory conditions with a reversed day-night cycle and food and water access *ad libitum* (light from 6 am to 6 pm). After arrival in the animal facility, animals were allowed to habituate for two weeks before the injections started. Nicotine and saline treatments were given daily subcutaneously in the afternoon.

2.3. Preparation of lymphocytes from blood

Venous blood (10 mL) was collected in EDTA tubes (Sarstedt, Germany) and centrifuged at 1600 × *g* for 10 min. The buffy coat between the upper plasma layer and the lower layer of packed erythrocytes was harvested and diluted 1:5 with Phosphate-buffered saline (PBS). The leukocyte suspension was carefully laid on top of the Ficoll-Paque Plus solution (GE Healthcare, Germany). Centrifugation at 600 × *g* for 20 min resulted in separation of the mononuclear and polymorphonuclear cells into two distinct bands at the interphases. The lymphocyte ring in the upper layer was removed and washed twice with PBS and stored at −80 °C until further processing.

For the preparation of lymphocytes from blood of rats, blood was collected in EDTA tubes (Sarstedt, Germany) and isolation was performed according to manufacturer's instructions with the Ficoll-Paque Premium 1.084 solution (GE Healthcare, Germany). After

isolation the cell pellets were flash-frozen and stored at −80 °C until further processing.

2.4. Measurement of plasma cotinine

Cotinine is the main metabolite of nicotine [43]. Due to its longer half-life of 20 h, in contrast to nicotine which has a much shorter half-life of about 20 min, it is widely used as a biomarker of nicotine exposure. Based on the linear relationship between nicotine and cotinine levels in blood plasma, one can correlate smoking behavior to cotinine content in the blood [44].

Cotinine concentrations were determined using the commercial EIA based kit Inspec II (Mahasan Diagnostika, Germany). Plasma samples which had been stored at −80 °C were processed on 96-well microplates. All samples were diluted in water (1:100) and, in order to prevent blood clotting as the consequence of lowering the EDTA concentration in respect to the subsequent preparation steps, 25 μL of the diluted plasma aliquots were supplemented with 25 μL EDTA (20 mM, pH 7). After the addition of 100 μL enzyme conjugate, the samples were incubated for 30 min at room temperature. After six washing steps, substrate solution was added followed by a second 30 min incubation time. Color processing was stopped and the cotinine concentration which is inversely proportional to the color intensity was measured at 450 nm using the MRX microplate reader (Dynatech Laboratories, Germany). Test reliability was monitored using one negative and four positive calibrators in the concentration range of 0–50 ng/mL.

2.5. Western blot analysis of lymphocytes

Lymphocyte pellets were thawed on ice and immediately lyzed in VRL buffer: 50 mM HEPES (pH 7.5), 250 mM sucrose, 5 mM $MgCl_2$, 100 mM KAc, 2 mM PMSF (all Sigma–Aldrich, Germany), 2× Protease Inhibitor (Roche, Germany) supplemented with 1% Triton X-100, 1 mM PMSF (Sigma–Aldrich, Germany) and 40 U/mL DNaseI (Roche, Germany). After 30 min on ice, the lysate was incubated for 30 min at 37 °C for DNA digestion. Afterwards, the protein content of the lysate was determined using the *DC* Protein Assay Kit (Bio-Rad, Germany) and 30 μg of total protein per sample were loaded onto 10% SDS-PAGE gels. Afterwards the samples were blotted onto a 0.45 μm nitrocellulose membrane (GE Healthcare, Germany) for 16 h at 150 mA. After blotting, the membranes were directly treated with 100 mM KOH for 5 min at room temperature and subsequently blocked with 5% milk in PBS with 0.05% Tween-20 (PBST). DISC1 immunoreactivity was tested with the human DISC1 specific mAB 14F2. Analysis was done blind with regard to diagnosis.

Western blot procedure was identical for rat lymphocytes, but then the ratDISC1 specific polyclonal C-term antibody was used [45].

2.6. Isolation of lymphocytes by flow cytometry

Flow cytometry sorting of freshly isolated lymphocytes from peripheral blood with Ficoll-Paque Plus (GE Healthcare, Germany) was done by Cellsort, the Core Flow Cytometry Facility of the Medical School Düsseldorf, Germany. The resulting pure lymphocyte fraction was lyzed as described above and used for Western blot analysis.

2.7. Preclearing of 14F2 antibody

NHS-Activated Sepharose 4 Fast Flow (GE Healthcare, Germany) was activated with 1 mM HCl (VWR, Germany) according to manufacturer's instructions. Fifty microliter beads were coupled with 1 mg of either BSA (GE Healthcare, Germany) or recombinant DISC1 (amino acids 316-854) protein [34], both dialyzed to 10 mM sodium phosphate buffer (NaPi, pH 8), or buffer only for 16 h at 4 °C. Afterwards, beads were blocked with 100 mM Tris–HCl (pH 8.5) for 2 h at room temperature and washed with hybridoma cell medium (MEM media supplemented with FCS, Pen/Strep, HT supplement, L-Glutamine; all Gibco, Germany). For the preclearing, 2 mL fresh antibody supernatant of 14F2 was mixed with 2 mL of PBS supplemented with 0.05% Tween-20 (Sigma–Aldrich, Germany) and incubated with either NHS beads coupled to BSA, rDISC1 (316-854), or uncoupled and preincubated for 1 h at room temperature. The antibody solution was then centrifuged for 10 min at 2000 × *g* to pellet the beads and supernatant was used as primary antibody on the Western blots.

2.8. Quantitative expression analysis

Total RNA was prepared from human lymphocyte pellets with the RNeasy Mini Kit (Qiagen, Germany). From this preparation, 1 μg total RNA was used as template to synthesize cDNA using the random hexamer primers of the RevertAid First Strand Synthesis Kit (Thermo Scientific, USA) in a total volume of 20 μL. For quantitative Real-Time PCR, the cDNA template was diluted 1:10 and 5 μL were used as template for the qPCR reaction.

Primers targeting huDISC1 spanning exon 5 and 6: forward 5′-ACACCCCACTGAGAATGGAG-3′; reverse 5′-GTTGCTGCTCTTGCTCCTCT-3′ (300 nM each). Primers for the housekeeping gene ARF1: forward 5′-GACCACGATCCTCTACAAGC-3′; reverse 5′-TCCCACACAGTGAAGCTGATG-3′ (300 nM each). PCR conditions for both primer pairs: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 °C for 1 min. Real-Time PCR was conducted with the StepOnePlus Real-Time PCR System (Applied Biosystems, Germany) and the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Germany). The data was processed with the StepOne Software v2.3 and DISC1 expression was normalized to expression level of the housekeeping gene ARF1 and set in relation to a control cDNA sample that was used in all individual qPCR runs.

2.9. Nicotine treatment of rats

For testing the effect of nicotine on the aggregation of endogenous DISC1, male Wistar rats were treated for 15 days with either saline (1 mL/kg bw injection volume) or increasing amounts of nicotine. For the injections (−)-Nicotine hydrogen tartrate salt (Sigma–Aldrich, Germany) was dissolved in PBS calculated on the basis of the nicotine component. Animals were weighed daily before the injections. The nicotine group was treated for 5 days with 0.25 mg/kg, the next 5 days with 0.5 mg/kg and last 5 days with 1 mg/kg nicotine subcutaneously. Blood withdrawal and brain dissection was carried out 24 h after the last injection.

2.10. Aggregome assay of rat brain material

For the preparation of the insoluble aggregome of Wistar rats, brain tissues underwent a low-stringency aggregome assay due to the low expression of endogenous rat DISC1.

For the preparation of 10% homogenate, the mPFC was homogenized in ice-cold VRL buffer (see Section 2.5). The homogenate (200 μL) was mixed with 100 μL of buffer A3: 50 mM HEPES pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 100 mM KAc, 15 mM GSH, 2 mM PMSF, 1× PI, 3% NP-40, 0.6% sarcosyl and 120 U/mL DNaseI and rotated overnight at 4 °C to digest the DNA. Next day, 450 μL of a 2:1 VRL:A3 mix and 530 μL buffer B3 (50 mM HEPES pH 7.5, 2.3 M sucrose, 5 mM GSH, 1% NP-40, 0.2% sarcosyl; final sucrose concentration of 1.1 M) was added to the tube. Samples were mixed by vortexing and ultracentrifuged for 45 min at 4 °C and 100,000 × *g* (TLA-55 rotor in an Optima ultracentrifuge; Beckman Coulter).

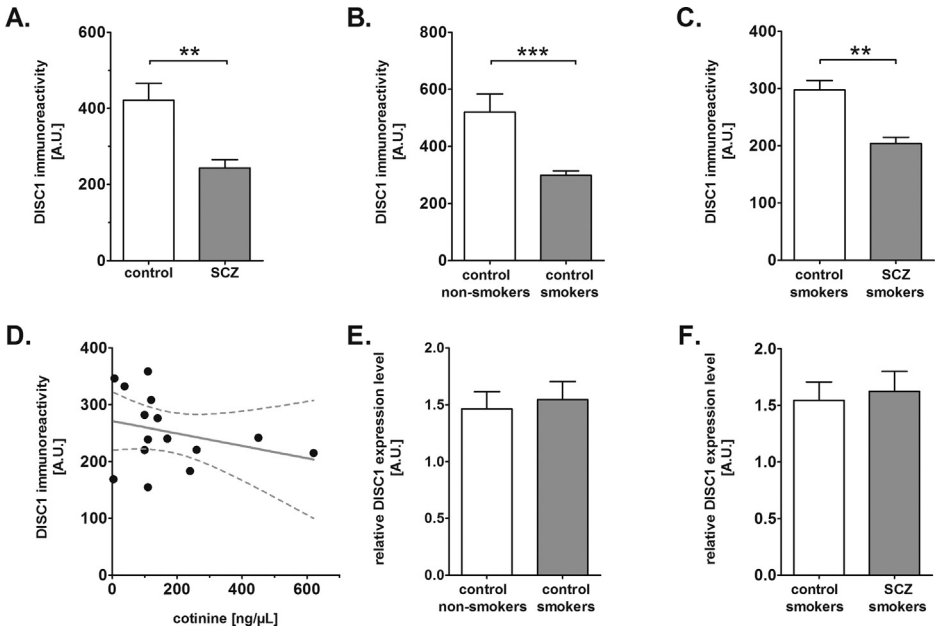

Fig. 1. Altered DISC1 immunoreactivity in lymphocytes of schizophrenic (SCZ) and control patients and the influence of smoking behavior. (A) Comparison of the 100 kDa DISC1 immunoreactive band in lysates of lymphocytes derived from blood of either schizophrenic ($n = 12$) or control patients ($n = 18$). SCZ cases display significantly reduced DISC1 band intensity. Unpaired two-tailed t-test $^{**}p = 0.004$ (means ± SEM). (B) Comparison of DISC1 band intensity in lysates of control group only. Control cases included smokers ($n = 8$) and non-smokers ($n = 10$). Lymphocytes from smoking controls had significantly less DISC1 immunoreactivity than non-smoking controls. Unpaired two-tailed t-test $^{**}p = 0.008$ (means ± SEM). (C) Comparison of the DISC1 protein levels between smoker groups. SCZ smokers ($n = 8$) express less DISC1 than control smokers ($n = 8$). Unpaired two-tailed t-test $^{*}p = 0.037$ (means ± SEM). (D) No significant correlation between DISC1 Western blot signal and cotinine content of plasma derived from smokers. DISC1 immunoreactive signal is plotted against cotinine content in the blood plasma of control and SCZ smokers. Regression line (full line) and 95% confidence interval (dashed line) are depicted. Pearson's correlation coefficient $r = -0.287$, $p = 0.300$. (E) No DISC1 expression changes in lymphocytes from control non-smokers ($n = 10$) and control smokers ($n = 8$). Quantitative Real-Time PCR did not show a difference of DISC1 expression between the two groups. Unpaired two-tailed t-test $p = 0.718$ (means ± SEM). (F) Quantitative Real-Time PCR of lymphocyte samples from control smokers and SCZ smokers. SCZ smokers had the same DISC1 expression as controls. Unpaired two-tailed t-test $p = 0.747$ (means ± SEM).

Buffer B3 was mixed 7:3 with buffer C3: 50 mM HEPES (pH 7.5), 5 mM GSH, 1% NP-40, 0.2% sarcosyl (total sucrose concentration 1.6 M). The pellet was resuspended in 700 μL of this buffer and after addition of another 700 μL the sample was spun again at 4 °C and 100,000 × *g*. The pellet was resuspended in 1 mL buffer D3: 50 mM HEPES (pH 7.5), 1.5 M NaCl, 5 mM GSH. After another ultracentrifugation, the pellet was washed in 1 mL E3 (50 mM HEPES pH 7.5, 0.2% sarcosyl), ultracentrifuged and the final pellet was dried in a speedvac centrifuge (Eppendorf, Germany). The insoluble aggregome pellet was taken up in 2× SDS-loading buffer, separated by SDS-PAGE and blotted onto 0.45 μm nitrocellulose membrane. Endogenous rat DISC1 was detected with the polyclonal C-term ratDISC1 antibody.

2.11. Densitometric analysis and statistics

Densitometric analysis was performed using the ImageJ 10.2 software (National Institute of Health, USA). For statistical analyses the IBM SPSS Statistic 20 program was used.

3. Results

3.1. Decreased DISC1 immunoreactivity in lymphocytes of patients diagnosed with schizophrenia compared to healthy controls

To investigate DISC1 levels in mononuclear cells from schizophrenic patients and healthy controls, cells were purified on a Ficoll gradient, lyzed and DISC1 was detected by immunoblotting with the monoclonal antibody 14F2 highly specific for human DISC1 [35]. Densitometric analysis blind to clinical diagnosis of samples of the predominant approximately 100 kDa DISC1 immunoreactive band revealed a significant reduction of DISC1 protein in schizophrenia (SCZ) cases compared to mentally healthy controls (Fig. 1A).

We also performed a separate flow cytometry analysis with the goal to identify the cell population within mononuclear cells that was the origin of the DISC1 signal. We identified the main DISC1 signal in a mainly lymphocytic cell population with only minor content of monocytes and granulocytes (data not shown). Western blotting of sorted lymphocytes revealed a strong DISC1 signal (Fig. 2A, left panel) comparable to that of the whole lysates of the Ficoll purified lymphocytes. We, therefore, conclude that the majority of the DISC1 immunoreactivity stems from lymphocytes.

To validate the identity of the approximately 100 kDa immunoreactive band as DISC1, we precleared mAB 14F2 with recombinant human DISC1 (316-854) protein coupled to NHS beads. Subsequent incubation of blots with the precleared antibody supernatant revealed a signal reduction on Western blots both of cell lysates transfected with full length human DISC1, as well as the lymphocytic 100 kDa immunoreactivity (Fig. 2B), indicating that the approximately 100 kDa immunoreactive band on Western blots is a lymphocyte-specific form of DISC1.

3.2. DISC1 protein levels in lymphocytes of mentally healthy smokers

When we investigated DISC1 protein levels in lymphocytes of smokers and non-smokers in healthy controls in a blinded analysis, we observed less DISC1 immunoreactivity in control subjects that were smokers than in non-smokers (Fig. 1B). This could indicate that, to some degree, DISC1 protein levels are modulated by smoking, but not to the extent of influencing the overall effect of schizophrenia itself since the smoking cohort of patients diagnosed

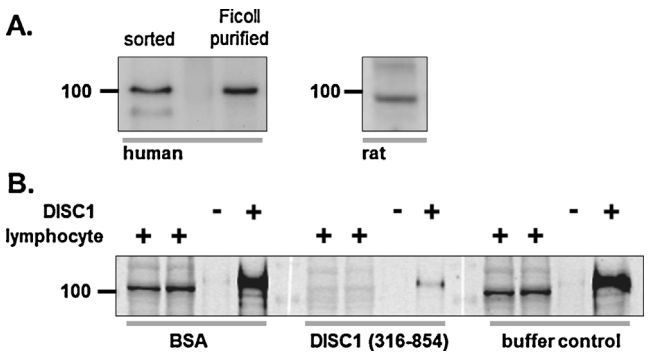

Fig. 2. Validation of the 100 kDa DISC1 immunoreactive band in lymphocytes. (A) Derivation of the 100 kDa DISC1 band from lymphocytes. Flow cytometry sorting of the Ficoll purified lymphocyte fraction from peripheral blood and subsequent Western blotting showed that the DISC1 signal of the mAB 14F2 results exclusively from lymphocytic DISC1 species (left panel). Also in lymphocytes from the rat a 100 kDa endogenous rat DISC1 species can be visualized by the polyclonal C-term antibody (right panel). (B) Reduction of the 100 kDa DISC1 band intensity by preclearing of the mAB 14F2 with recombinant DISC1 protein. Depicted are two different lymphocyte samples, lysates from SH-SY5Y cells without (−) or with (+) expression of human full-length DISC1. Preincubation of the mAB 14F2 with NHS-beads coupled to recombinant DISC1 (316-854) led to a decrease of the 100 kDa band as well as the DISC1 overexpression control, whereas preincubation with NHS coupled to BSA or buffer only did not lead to a diminished signal intensity.

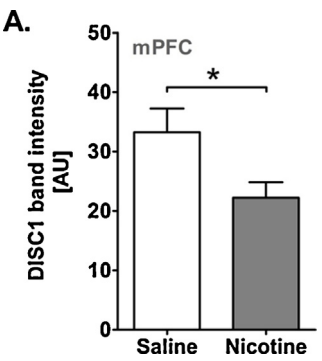

Fig. 3. Nicotine-dependent reduction of DISC1 aggregation in rat brains. Densitometric analysis of the 200 kDa endogenous ratDISC1 species in the mPFC of saline ($n = 6$) and nicotine-treated animals ($n = 7$) detected with the polyclonal C-term ratDISC1 antibody. Treatment with nicotine leads to significantly reduced aggregation of endogenous DISC1. Unpaired two-tailed t-test $^{*}p = 0.037$ (means ± SEM).

with schizophrenia still had significantly less DISC1 immunoreactivity than control smokers (Fig. 1C).

To investigate the relation between nicotine and lymphocytic DISC1 levels in a more quantitative way, we determined the levels of cotinine in the plasma of the patients at the same time the lymphocytes were taken.

We did not identify a significant correlation between plasma cotinine levels and lymphocytic DISC1 protein levels in either control smokers (Pearson's correlation: $p = 0.072$, $r = -0.664$) or schizophrenic smokers analyzed separately (Pearson's correlation: $p = 0.146$, $r = 0.610$) or by combining the smoking groups (Pearson's correlation: $p = 0.300$, $r = -0.287$), indicating that the difference in DISC1 levels of patients with schizophrenia and healthy controls is not due to a different quantity of nicotine intake (Fig. 1D).

For these same patients, we also determined expression of DISC1 mRNA in lymphocytes by quantitative Real-Time PCR, but did not find significant differences (Fig. 1E and F), neither by comparing control smokers and non-smokers, nor comparing control smokers and SCZ smokers.

3.3. Nicotine-dependent reduction of aggregated DISC1 in rat brains

To investigate the possible modulatory effect of nicotine on DISC1 protein assembly in the brain, adult wild type Wistar rats were treated for 15 subsequent days with either saline or nicotine solution (increasing doses every 5 days up to 1 mg/kg). Preparation of the insoluble aggregome displayed a significant reduction of an endogenous 200 kDa DISC1 species in the frontal cortex in nicotine treated animals compared to vehicle-treated controls (Fig. 3). Although endogenous DISC1 expression and thus, aggregation, is relatively low in rats, systemic nicotine administration had an effect on its aggregation propensity in the brain. When, in the same rats, DISC1 protein levels in lymphocytes were investigated as described above, a prominent 100 kDa band was detectable with the rat DISC1 specific polyclonal C-term antibody (Fig. 2A, right panel), but no reduction in DISC1 protein levels was detected between the two treatment groups (data not shown).

4. Discussion

In this study, we investigated whether peripheral DISC1 protein levels in lymphocytes have the potential to serve as a candidate biomarker of schizophrenia. We found a significant reduction of DISC1 levels in lymphocytes derived from patients, apparently not through a change in expression but rather due to a change in its biological half-life time. In addition, it was found that lymphocytic DISC1 levels differed between mentally healthy smokers and non-smokers independent of the clinical diagnosis schizophrenia.

Our results show that, at least for DISC1 protein levels, lymphocytes can be seen as windows to the brain, mirroring brain disease. A subset of mental illness patients has already been characterized by the presence of insoluble DISC1 protein in *post mortem* tissue [34]. Here we showed that DISC1 levels are also dysregulated in one peripherally accessible tissue, namely blood, of patients diagnosed with schizophrenia.

An interesting observation is that in this investigation, lymphocytes of patients diagnosed with schizophrenia revealed differential DISC1 protein levels whereas in a previous investigation, EBV-immortalized lymphoblasts did not show differences in DISC1 protein levels [46]. This could indicate that yet unidentified factors in the cellular and molecular machinery involved in maintaining cell proliferation are critical for schizophrenia-dependent DISC1 protein levels.

In our study, lymphocytic DISC1 levels are also a state marker, as the amount of DISC1 protein was decreased in control smokers, perhaps due to nicotine administration, although other covariates from smoking cannot be excluded. But this range of modulation did not exceed its value as trait marker for schizophrenia. These findings indicate that, to some extent, administration of the psychoactive substance nicotine is reflected in the peripherally accessible disease marker, DISC1 and, therefore, has a potential use as a marker in monitoring the effects of externally administered psychoactive substances in individuals.

The correlation between cotinine content, the main metabolite of nicotine, and DISC1 protein levels in the blood plasma of schizophrenics and controls was not significant (Fig. 1D), indicating that the intensity of smoking is related to DISC1 levels only to a minor degree. Other smoking- or schizophrenia-related factors, which have yet to be determined, may account for the reduced amount of DISC1. It is also conceivable that decreased DISC1 protein levels are related to a common biological cause for both schizophrenia and nicotine abuse or addiction.

It is also of importance that the difference in DISC1 protein levels was seen solely on the protein level, not on the mRNA level. This result points towards a cellular mechanism affecting

posttranslational modifications of DISC1, its protein clearance or half-life in the cell rather than changes in expression regulation by differential transcription the DISC1 protein. This is consistent with previous reports of differential posttranslational processing that potentially leads to misfolding and/or aggregation of DISC1 [33]. One caveat of our data is that although they were gathered in a blinded fashion, the case numbers are relatively low, so the results of this study need to be validated in a larger cohort.

In the second part of our study corroborating biological effects of smoking or nicotine on DISC1 protein expression and/or post-translational modifications, we showed that sub-chronic nicotine treatment of rats changed DISC1 insolubility in the rat brain. A subset of mental illness patients were previously characterized by insoluble DISC1 species in *post mortem* brain material [34,35]. The decrease of DISC1 aggregation in the mPFC of wild type rats treated with nicotine demonstrates that insoluble DISC1 assembly can be modulated by administration of small molecules. Although it is not clear whether nicotine actually decreases the existing aggregates or if it prevents its *de novo* assembly, our data show that nicotine decreases the aggregome load in the mPFC.

Other studies have shown that chronic nicotine treatment of rats favors an upregulation of ubiquitin and heat-shock proteins as well as members of the proteasome and chaperone pathways in the mPFC [47,48]. As protein degradation is a dynamic process, aggregated DISC1 protein could be cleared by the cell in a faster rate due to an upregulated proteasomal system to restore the homeostasis of the cell.

Reduction of aggregated DISC1 could also occur by preventing further accumulation of aggregated protein. Nicotine was identified to inhibit the aggregation of proteins prone to self-assembly like Aβ *in vitro* [49,50]. Therefore chronic nicotine might prevent the formation of further DISC1 aggregation rather than increasing the clearance of previously formed aggregated species.

Nicotine treatment of rats did not lead to changes in lymphocytic DISC1 levels as seen in the comparison of smoking versus non-smoking human controls. This suggests that 15 days of exposure to nicotine may not be sufficient and a chronic exposure to nicotine could be crucial to change lymphocytic gene expression or protein half-life. Also, the start of nicotine exposure might influence DISC1 levels, as the human participants normally started smoking during adolescence and were tested as adults, whereas nicotine treatment of the adult rats started after adolescence [51]. Alternatively, these data may support our previous reasoning that a common yet unidentified factor may account for both decreased DISC1 levels and schizophrenia, as well as nicotine abuse that is restricted to human pathophysiology.

Taken together, our results suggest that DISC1 expression in lymphocytes is a potential trait marker for schizophrenia. In addition, it represents a state marker for monitoring the effects of exogenously administered psychoactive substances. The change in lymphocytic DISC1 protein levels emphasizes that, at least for some candidate proteins, lymphocytes can represent a window to the brain and brain disorders. The influence of nicotine on DISC1 protein aggregation in the brain highlights its potential pharmacological reversibility and makes the nicotinic receptors in the brain a potential pharmacological target for DISC1-dependent disease [36].

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