



Plasma Ndel1 enzyme activity is reduced in patients with schizophrenia – A potential biomarker?

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ABSTRACT

Ndel1 oligopeptidase interacts with schizophrenia (SCZ) risk gene product DISC1 and mediates several functions related to neurite outgrowth and neuronal migration. Ndel1 also hydrolyzes neuropeptides previously implicated in SCZ, namely neurotensin and bradykinin. Herein, we compared the plasma Ndel1 enzyme activity of 92 SCZ patients and 96 healthy controls (HCs). Ndel1 enzyme activity was determined by fluorimetric measurements of the FRET peptide substrate Abz-GFSPFRQ-EDDnp hydrolysis rate. A 31% lower mean value for Ndel1 activity was observed in SCZ patients compared to HCs (Student's $t = 4.36$; $p < 0.001$; Cohen's $d = 0.64$). The area under the curve (AUC) for the Receiver Operating Characteristic (ROC) curve for Ndel1 enzyme activity and SCZ/HCs status as outcome was 0.70. Treatment-resistant (TR) SCZ patients were shown to present a significantly lower Ndel1 activity compared to non-TR (NTR) patients by t -test analysis ($t = 2.25$; $p = 0.027$). A lower enzymatic activity was significantly associated with both NTR ($p = 0.002$; $B = 1.19$; OR = 3.29; CI 95% 1.57–6.88) and TR patients ($p < 0.001$; $B = 2.27$; OR = 9.64; CI 95% 4.12–22.54). No correlation between Ndel1 enzyme activity and antipsychotic dose, nicotine dependence, and body mass index was observed. This study is the first to show differences in Ndel1 activity in SCZ patients compared to HCs, besides with a significant lower activity for TR patients compared to NTR patients. Our findings support the Ndel1 enzyme activity implications to clinical practice in terms of diagnosis and drug treatment of SCZ.

Objective of the study: To compare the Ndel1 enzyme activity levels of schizophrenia (SCZ) patients and healthy controls (HCs) and to correlate these values with the clinical profile and response to treatment by measuring the Ndel1 enzyme activity in human plasma.

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Abbreviations: SCZ, Schizophrenia; DISC1, disrupted in schizophrenia 1; Ndel1, nuclear-distribution element-like 1 or NudE-like 1; HCs, healthy controls; TR, treatment-resistant; NTR, non-treatment-resistant; CI, confidence interval; OR, odds ratio; endo A, endooligopeptidase A; endo B, endooligopeptidase B; POP, prolyloligopeptidase; ACE, angiotensin-converting enzyme; NT, neurotensin; BK, bradykinin; Dyn, dynorphin; FRET, Fluorescence Resonance Energy Transfer; PANSS, Positive and Negative Syndrome Scale; SCID, Structured Clinical Interview for DSM-IV; CGI, Clinical Global Impression; GAF, Global Assessment of Functioning; IPAP, International Psychopharmacological Algorithm Project; LSD, Fisher's Least Significant Difference; ROC, receiver operating characteristic; AUC, area under the curve; NO_{AB} inhibitor, anti-catalytic anti-Ndel1 antibody; ZIP, Z-pro-prolinal-insensitive peptidase; BD, bipolar disorder; FNDT, Fagerstrom Nicotine Dependence Test; BMI, body mass index.

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1. Introduction

The current body of evidence suggests that schizophrenia (SCZ) is a multifactorial disease influenced by genetic and environmental factors (Sawa and Snyder, 2002; Sullivan et al., 2003). The identification of a balanced translocation that segregates with SCZ and affective disorders in a large Scottish family has suggested DISC1 as a potential gene for susceptibility to this major psychiatric disorder (Millar et al., 2000, 2004). This gene product was proposed to be a multifunctional protein that interacts with several proteins of centrosome and cytoskeleton (Ozeki et al., 2003; Brandon and Sawa, 2011). In fact, the function of DISC1 protein is mainly regulated by its protein ligands (Camargo et al., 2007).

Thus the identification of Ndel1 as the major ligand of DISC1 led to further investigations on the interaction of DISC1 and Ndel1, and their involvement in neurite outgrowth and, brain development

and function (Ozeki et al., 2003; Duan et al., 2007; Hayashi et al., 2010). Disruption of the DISC1/Ndel1 interaction may determine perturbations of key events (e.g., neurite outgrowth, neuronal migration) that are essential to the formation of normal brain structures (Kamiya et al., 2005, 2006; Hayashi et al., 2005, 2010), potentially increasing the vulnerability to SCZ.

Other research approaches to investigate Ndel1 was focused on its oligopeptidase activity. Initially isolated from rabbit brain cytosol due to its ability to degrade small peptides such as bradykinin (BK) and neurotensin (NT) (Camargo et al., 1983), the study of this peptidase, originally named as endo A, stimulated the development of the first internally quenched fluorescent peptide designed to quantitatively measure oligopeptidase activity (Juliano et al., 1990). This fluorescent peptide was the precursor of the Fluorescence Resonance Energy Transfer (FRET) substrates, largely used to quantify peptidase activity in biological fluids and homogenates of tissue and cells (Quinto et al., 2000; Hayashi et al., 2005, 2010).

Ndel1 (Hayashi et al., 2004, 2005, 2010), is preferentially expressed in the brain (Hayashi et al., 2000; Guerreiro et al., 2005; Myöhänen et al., 2008). Interestingly, the Ndel1 substrate NT has been implicated in the pathophysiology of SCZ, and NT receptor agonists were also suggested to be potential antipsychotics (Cáceda et al., 2003; Kinkead and Nemeroff, 2004, 2006; Boules et al., 2007). Increased levels of Ndel1 substrates, e.g., NT and pro-enkephalin products (Hayashi et al., 2004), were observed under antipsychotic treatment (Merchant et al., 1994; Bauer et al., 2000; Binder et al., 2001).

Lipska et al. (2006) reported a significant reduction of Ndel1 in the brain tissue of SCZ patients and also showed a significant epistatic interaction between Ndel1 and DISC1 influencing the risk for SCZ (Burdick et al., 2008; Nicodemus et al., 2010). Therefore we hypothesized that Ndel1 enzyme activity would be reduced in SCZ patients. Thus, our primary objective was to compare the Ndel1 enzyme activity levels of SCZ patients and healthy controls (HCs). Secondly, we tried to correlate these values with the clinical profile and response to treatment. This study is the first to measure Ndel1 enzyme activity in human plasma.

2. Materials and methods

2.1. Subject enrolment and psychiatric assessment

Patients with SCZ or schizoaffective disorder diagnoses and followed for at least 1 year were consecutively recruited from an outpatient clinic, The Schizophrenia Program (PROESQ) of the Federal University of São Paulo (Universidade Federal de São Paulo, UNIFESP). A total of 86 patients with SCZ and 6 with schizoaffective disorder diagnoses agreed to participate. 14 Patients included in this study were affected by a non-paranoid type of disease (9 disorganized, 1 catatonic, 1 residual, and 3 undifferentiated). The Structured Clinical Interview for DSM-IV (SCID) applied by trained psychiatrists confirmed the diagnoses. The clinical assessment also included the Positive and Negative Syndrome Scale (PANSS), Calgary Depression Scale (Bressan et al., 1998), Global Assessment of Functioning (GAF), and Clinical Global Impression (CGI) (Lima et al., 2007). For diagnosis, all available information, including medical records, were used. Any doubt in the diagnosis, including the diagnostic subtype, was solved by the review of the interview by two additional trained psychiatrists. The exclusion criteria employed in this work were either the inability to diagnose SCZ or schizoaffective disorder or inability to provide informed consent. None of the patients assessed in this study were excluded.

To assess remission, we used the criteria proposed by Andreassen et al. (2005), with mild severity (score of 3 or lower) in

eight items of the Positive and Negative Syndrome Scale (PANSS) (e.g., delusions, unusual thought content, hallucinatory behaviour, mannerisms/posturing, blunted affect, social withdrawal, and lack of spontaneity). There was also a minimum time threshold of 6 months, in which the severe symptoms must be maintained. The duration was directly assessed with the patient and, when in doubt, a companion or informant was questioned and/or the information was recovered from the available clinical records. Treatment-resistant (TR) status was defined following the International Psychopharmacological Criteria (IPAP) [www.ipap.org] as a failure to respond to 4- to 6-week trials of monotherapy with two different antipsychotics in adequate doses (equivalent to 5 mg of risperidone or 400 mg of chlorpromazine).

Healthy control volunteers (HCs) were selected and paired by age, sex, and educational level from a governmental unemployment agency. First, the HCs were submitted to a checklist to screen for psychiatric diagnoses, and then they underwent the SCID and a family history of mental disease questionnaire, adapted from the SCID screening questions. Exclusion criteria were personal diagnosis of psychiatric disease and any degree of a family history of psychosis. Even after the checklist, nine HC candidates who fulfilled DSM-IV criteria for depression or substance dependence were excluded. Ultimately, 96 HCs were studied. The Fagerstrom Nicotine Dependence Test was used to evaluate smoking status in both groups.

The interviewers inferred ethnic background and four groups were considered: Caucasian, African, Native American (in this group we included Chinese and Japanese ancestry) and miscellaneous group (for the miscellany of Caucasian + African, Native American + African, native American + Caucasian).

This study was approved by the Research Ethics Committee of UNIFESP [CEP No. 1883/10], and a written informed consent was obtained from all recruited participants. Clinical and laboratory investigations were strictly conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Blood samples

Blood samples were collected from all subjects into heparin vacuum tubes (BD Vacutainer®, BD, NJ, USA). The samples were kept at 4 °C and they were centrifuged at 1500–2000×g for 10–15 min at room temperature to recover the plasma, which was then stored at –20 °C in sterile Eppendorf tubes (Axygen Inc., CA, USA) until use.

2.3. Activity measurements

Ndel1 enzyme activity of the SCZ patients and HCs plasma was measured using the FRET peptide substrate Abz-GFSPFRQ-EDDnp [10 μM], following the method previously described by the group (Hayashi et al., 2000, 2005). Hydrolysis of the substrate at 37 °C was monitored by measuring the fluorescence in a Shimadzu RF-5301 PC spectrofluorometer at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm. The 1-cm path length cuvette containing the substrate in 1 mL of buffer (50 mM Tris–HCl pH 7.4, and 100 mM NaCl buffer) was placed in a thermostatically controlled cell compartment for 5 min before the addition of the plasma samples. The time course increase in fluorescence (AFU, arbitrary fluorescence units) was continuously recorded for 5–10 min, either in the absence or in the presence of 50 μL of a heat-inactivated Ndel1 antibody (NO_{AB} inhibitor). Since this antibody shows specific inhibitory activity against Ndel1, the measured Ndel1 activity was defined as the rate of hydrolysis in the absence of minus the rate determined in the presence of this specific antibody.

Both the storage and activity measurement procedures for HCs and SCZ patient groups' samples were exactly the same; the collection of these samples was scheduled independently from the

clinical condition. Furthermore, the Ndel1 enzymatic activity measurements were performed by someone kept blind to the clinical condition.

No significant differences in the measurements were observed for triplicate measurements of few samples (total of 5 samples), showing that the deviation was smaller than 5% for these same samples, then the a duplicate measurement was adopted here and the shown data represent the average of these measurements.

2.4. Data analysis

The Student's *t*-test was used to compare the differences in the Ndel1 enzyme activity mean values for the SCZ patients and HCs. The possible associations of Ndel1 enzyme activity and variables that present non-parametric distributions were investigated using non-parametric correlations (Spearman's Rank Correlation test).

The clinical usefulness of Ndel1 enzyme activity measurements to discriminate SCZ patients and HCs was assessed by the generation of a Receiver Operating Characteristic (ROC) curve for Ndel1 enzyme activity and SCZ patient/Hc status as the outcome. Then, Ndel1 was dichotomized into groups of either high or low levels using the median value for the whole sample, and a logistic regression model with clinical condition as the dependent variable with correction for age and sex was generated.

Post-hoc power analysis was conducted to determine what was the statistical power of our findings. We first estimated Cohen's *d* effect size using our observed means and standard deviations for patients with schizophrenia and healthy controls. Then we used the effect size found at a 0.05 two-tailed significance threshold to estimate the post-hoc statistical power for a Student's *t*-test.

Data analyses were performed using the Statistical Package for Social Science (SPSS) Version 14.0.

3. Results

3.1. Subjects description

In this study, 92 schizophrenia (SCZ) patients and 96 healthy controls (HCs) from 16 to 68 years old were enrolled. There was no significant difference between groups in terms of sex, age, educational level, and ethnic background (Table 1).

3.2. Enzymatic activity of SCZ patients and HCs

A mean value of 6.7 ± 4.2 and 9.6 ± 4.9 nM/min for the Ndel1 enzyme activity of SCZ patients and HCs, respectively, was observed. The mean value for Ndel1 enzyme activity in SCZ patients

was significantly lower compared to HCs (Student's $t = 4.361$; $p < 0.001$; Cohen's $d = 0.64$) (Fig. 1A and Supplemental data S1A).

3.3. Statistical and comparative analysis

The statistical analysis was performed in two ways: for the whole sample and also for the separated groups to determine the influence of age, sex, educational level, and ethnic background on the measured enzymatic activity. However, no significant differences were found in either analysis (Supplemental data S2). A statistical power calculation considering our sample size was performed, and the observed power for the two-tailed *t*-test was of 0.994.

Our study sample presented a predominance of chronic patients with a mean duration of illness of 13 years ($SD = 8$). Attempts to correlate Ndel1 activity with the duration of illness and age of onset, looking for a possible confounding effect, did not show significant correlation with the duration of illness as indicated by the values for Spearman's $\rho = -0.27$ ($p = 0.098$). The Spearman's ρ for age of onset was 0.171 ($p = 0.100$). Differences in lifestyle that might have influenced our results were also addressed here. Smoking status was available for 182 out of 188 subjects enrolled in this study. A total of 93 subjects reported they have never smoked, 33 have smoked in the past but reported stopping, and 56 reported themselves as current smokers. There was no significant difference in smoking status frequencies between SCZ patients and HCs ($p = 0.650$). Also there was no significant difference between groups for Ndel1 enzymatic activity ($p = 0.301$). Considering the group of former and current smokers, there was a significant difference in Fagerstrom Nicotine Dependence Test (FNDDT) scores; SCZ patients had a higher total score compared to HCs, but there was no significant correlation for the FNDDT total score and Ndel1 enzyme activity ($p = 0.200$). There was no correlation between the mean Ndel1 enzyme activity and the reported number of cigarettes smoked per day ($p = 0.665$). For 43 SCZ patients, body mass index (BMI) values were also available since weight and height were recorded for clinical evaluation in the same day of blood collection. No correlation between BMI and Ndel1 enzyme activity could be observed (Spearman's $\rho = -0.147$; $p = 0.347$).

3.3.1. Ndel1 enzyme activity and symptom severity

We then investigated the association of symptom dimensions and severity with Ndel1 enzyme activity. Utilizing the five dimensions proposed by Levine and Rabinowitz (2007), we performed a non-parametric correlation analysis in reference to Ndel1 enzyme activity. A significant correlation was found only for the negative dimension (Spearman's $\rho = 0.218$; $p = 0.041$). We also

Table 1
Sociodemographic characteristics.

		SCZ patients (N = 92)		Healthy controls (N = 97)		Statistics	
		(%)	N	(%)	N	Test value	p
Gender	Male	59.2	60	63.5	61	0.058	0.810
	Female	32.8	32	36.5	35		
Educational level	>10 ^a	67.5	28	70.5	73.2	0.024	0.877
	<10 ^a	24.5	64	25.5	26.8		
Age mean	Years-old	35.5 ± 10.3	92	34.2 ± 10.1	96	0.856	0.393
Ethnic background	Caucasian	66.3	61	63.5	61	4.962	0.175
	African	4.3	4	11.5	11		
	Native American	10.9	10	5.2	5		
	Miscellaneous ^b	18.5	17	19.8	19		
Mean dose of antipsychotic	Chlorpromazine equivalents (mg)	556.3 ± 400	92	—	—	—	—
Mean no. of hospitalizations		2.33 ± 3.2	92	—	—	—	—

^a Education years.

^b Caucasian + African, Caucasian + Native American, or African + Native American.

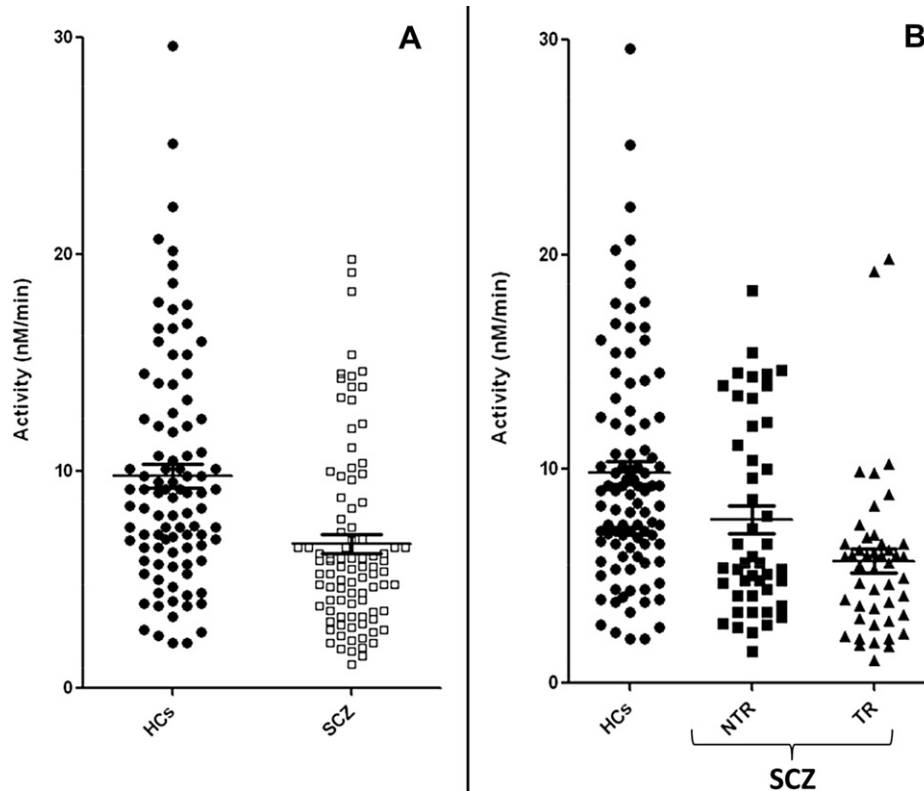


Fig. 1. Scatter plot for Ndel1 enzymatic activity measurements. Scatter plot for Ndel1 enzymatic activity measurements of healthy controls (HCs) vs. SCZ patients (A), and of HCs vs. treatment-resistant (TR) and non-TR (NTR) SCZ patients (B). There is a significant difference between groups, with TR patients showing the lowest Ndel1 activity mean value.

looked for a relationship between Ndel1 enzyme activity and symptom severity using Clinical Global Impression (CGI) and Global Assessment of Functioning (GAF) values, but no correlation with Ndel1 enzyme activity or its mean differences were found when categorizing patients by the median values for each scale.

The enzyme activity was also categorized as high or low by assessing the whole sample median values. Then, a logistic regression was performed with clinical condition (HC or SCZ) as the dependent variable, and the dichotomized enzymatic activity, sex, and age as covariates. We observed that low activity could predict the patient condition using this model ($p < 0.001$; $B = 1.680$; $OR = 5.35$, $CI\ 95\% = 2.27\text{--}12.57$; Nagelkerke $R^2 = 0.23$). We generated a Receiver Operating Characteristic (ROC) curve to better estimate the usefulness of this measurement to differentiate HCs from SCZ patients, and an area under the curve (AUC) of 0.703 was determined (Fig. 2).

3.3.2. Ndel1 enzyme activity for patient subgroups based on treatment-resistant criteria

As a specialized clinic, severe cases are referred to us and 50% of patients in this sample are treatment-resistant (TR) according to The International Psychopharmacology Algorithm Project (IPAP) criteria [<http://www.ipap.com>], as described in the [Material and methods](#) section. Based on these criteria, the patients were divided into two subgroups of TR and non-TR (NTR), and a t -test analysis to identify differences between these groups was performed. The mean value for the TR group was significantly lower compared to the NTR patients ($t = 2.25$; $p = 0.027$). Overall, the highest mean value was observed for the HC group (9.6 nM/min), and the lowest value was observed for the TR group (5.7 nM/min), with an intermediate value for the NTR group (7.6 nM/min) of SCZ patients (Fig. 1B and [Supplemental data S1B](#)).

As this result could reflect the effects of antipsychotic treatment, especially under use of clozapine, a possible correlation between the dose of clozapine and the enzymatic activity values was also tested. From the 46 TR patients, 34 were under treatment with clozapine. No significant correlation was observed for the dose of clozapine and the levels of measured enzyme activity (Spearman's

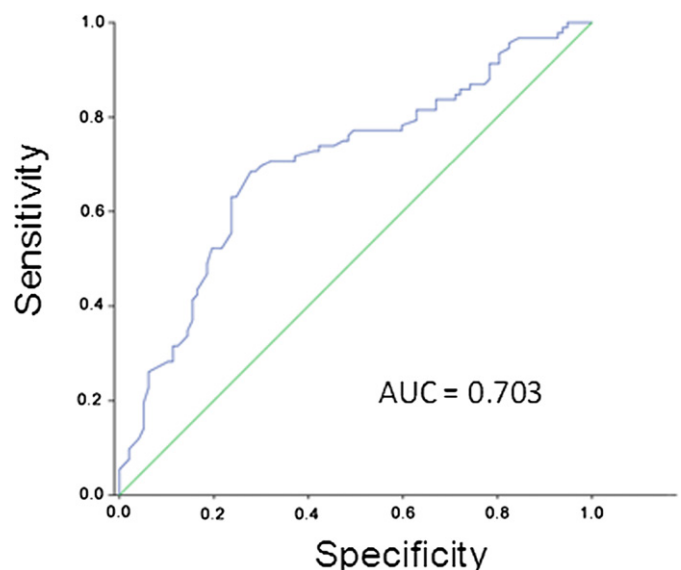


Fig. 2. Receiver operating characteristic (ROC) curve analyses. The area under the ROC curve (AUC) for Ndel1 enzyme activity and SCZ vs. HCs status as outcome was determined as 0.703. This curve combines the information of the true positive rate and the true negative rate, and the AUC is a measure of the overall discriminative power.

$\rho = 0.021$; $p = 0.908$). The mean values of enzymatic activity for TR patients under treatment with clozapine or not were of 5.5 and 6.2 nM/min, respectively, and this difference was not significant ($z = -0.27$; $p = 0.790$).

To evaluate the possible confounding effects of other antipsychotic drugs on plasma Ndel1 enzyme activity of NTR patients, the correlation with other antipsychotics was also examined. Among these patients, 30 patients were using olanzapine, which is the second most commonly used antipsychotic drug in our sample, after clozapine. These patients had a mean value of 7.2 nM/min for Ndel1 enzyme activity, with no significant correlation observed with dose (Spearman's $\rho = 0.281$; $p = 0.130$). Small numbers of patients were treated with other antipsychotic drugs, including the following: risperidone (8 patients), haloperidol (4), quetiapine (6), aripiprazole (6), or with paliperidone, conda, clopixol, sulpiride, or trifluoperazine (1 patient for each drug). The mean value of Ndel1 activity for this group was 7.5 nM/min. For this heterogeneous group, a dose conversion to chlorpromazine equivalents was performed, and then a dose correlation with Ndel1 enzyme activity was also determined to be non-significant (Spearman's $\rho = -0.2$, $p = 0.300$).

To further address the clinical potential of Ndel1 enzyme activity analysis, we performed a multinomial logistic regression with clinical condition (HCs, TR, and NTR) as the dependent variable. The dichotomized enzymatic activity and sex were fixed factors, and age was the covariate. The lower enzymatic activity was significantly associated with both NTR ($p = 0.002$; $B = 1.19$; OR = 3.29; CI 95% 1.57–6.88) and TR patients ($p < 0.001$; $B = 2.27$; OR = 9.64; CI 95% 4.12–22.54). The Nagelkerke Pseudo R square for this model was 0.22.

4. Discussion

Considering the lack of definite results in genetic association studies, the emergence of CNVs and the increasing number of studies investigating the estimated role of rare variants in SCZ investigation (Allen et al., 2008; Stefansson et al., 2008; Moens et al., 2011), new approaches are sought. This rationale led our team to direct our efforts towards protein function. More specifically, we focused on Ndel1 enzyme activity. Ndel1 was chosen due to its roles in the neuronal differentiation and migration processes (Ozeki et al., 2003; Hayashi et al., 2004, 2010; Sasaki et al., 2005; Kamiya et al., 2006), and due to the evidence suggesting a direct correlation of Ndel1 enzyme activity and SCZ (Brandon et al., 2004; Hayashi et al., 2005). Ndel1 endooligopeptidase activity and some of its potential substrates, namely bradykinin (BK), dynorphin (DYN), and neurotensin (NT), have also been implicated in SCZ (Binder et al., 2001; Cáceda et al., 2003; Kinkead and Nemeroff, 2006; Schwarzer, 2009). Alterations in plasma endopeptidase activity have been described for psychiatric disorders such as bipolar disorder and SCZ (Maes et al., 1995; Breen et al., 2004), and more recently, some of these peptidases were also suggested as potential targets for the treatment of several mental diseases, including SCZ (Männistö et al., 2007; Lawandi et al., 2010).

The thiol-activated Ndel1 endopeptidase is the responsible for more than 90% of the BK inactivation observed in rabbit brain cytosol (Hayashi et al., 2000). The biochemical and enzymatic features of Ndel1 have been widely studied and characterized, and although there is currently no specific chemical inhibitor for this enzyme, its activity has been determined by using highly specific antibodies with anti-catalytic activity (NO_{AB} inhibitor) combined with the use of selective peptide substrates that allow the specific measurement of Ndel1 enzyme activity (Hayashi et al., 2000, 2005, 2010). The internally quenched fluorescent substrate, based on Fluorescence Resonance Energy Transfer (FRET) used herein

allowed a highly sensitive and quantitative monitoring of Ndel1 enzyme activity in human plasma.

However, considering the previously demonstrated hydrolysis rates of the Ndel1 oligopeptidase activity against this same FRET substrate (Hayashi et al., 2005, 2010), considering the activity levels measured here, we could foresee that Ndel1 protein amount present in the human plasma is significantly lower compared to that detected in homogenates of PC12 cells or brain tissues. In fact, attempts to detect Ndel1 in human plasma by Western blot, even after depletion of albumin, was unsuccessful (data not shown), suggesting that only highly sensitive fluorimetric measurements (Juliano et al., 1990), as the one employed here, would allow a rapid, adequate, quantitative, and reliable comparative evaluation of the Ndel1 levels in this type of biological material.

To our knowledge, this study is the first to investigate Ndel1 enzyme activity in plasma. Our study reports a lower Ndel1 activity in chronic schizophrenia (SCZ) patients compared to healthy controls (HCs). This finding is also in line with the demonstration of a lower Ndel1 RNA expression in SCZ patients (Lipska et al., 2006). We also found that treatment-resistant (TR) patients have significantly lower enzyme activity levels compared to non-TR (NTR) patients, although this difference was not of the same magnitude as that observed between SCZ patients and HCs (Fig. 1 and Supplementary material S1). Indeed, the median values for Healthy Controls and SCZ patients highlight the observed mean difference (respectively 8.9 and 5.6 nM/min), whereas suggest some caution interpreting the finding on the difference observed TR and NTR means, as the median values were, respectively, 5.5 and 5.6 nM/min.

Diagnostic methods for SCZ have been described by others (Schwarz et al., 2010; Moens et al., 2011). Although these methods demonstrated higher AUC values, they employed a panel of genetic or biochemical biomarkers that could introduce confounding factors and increase the complexity of the interpretation of results. The observed AUC of 0.7 is promising considering that is derived from one single measurement, but at this points it is still of low to moderate clinical utility (Greiner et al., 2000). Unfortunately, it is not possible to make a direct comparison of our methods with those of other studies solely based on published data, although our method described here is clearly easier to perform and interpret. A side-by-side study using the same cohort would be necessary to evaluate the comparative advantages and disadvantages of each method and to evaluate the potential convenience of using them as complementary methods for diagnosis or treatment follow-up.

Our results seem to point more towards the use of Ndel1 activity as a tracer rather than as a state biomarker because the observed differences were more strongly associated with diagnosis and treatment response, e.g., TR and NTR, than to the severity of symptoms or the duration of illness. Another result that supports this hypothesis is the fact that patients with disorganized SCZ, also called hebephrenia, present a significantly lower Ndel1 enzyme activity level ($t = 3.13$; $p = 0.005$) compared to paranoid patients. Especially interesting are the clear differences in the mean values (7.1 nM/min for paranoid vs. 4.5 nM/min for disorganized SCZ) and the standard deviations (4.5 nM/min for paranoid vs. 2.0 nM/min for disorganized SCZ), suggesting that disorganized SCZ patients represent a distinct group in terms of Ndel1 enzyme activity. However, this result should be cautiously considered due to varying number of subjects in each group (73 in the paranoid group vs. 9 in the disorganized group). There was also a clear difference in treatment response status, with 8 of 9 disorganized SCZ patients being diagnosed as TR and under use of clozapine. Other point that must be addressed in future studies is the relation of Ndel1 enzymatic activity and both duration of illness and age of onset, since their correlation was only slightly above significance threshold (p -value of approximately 0.1 for each correlation). Ndel1 is related to

several processes linked to neurodevelopment, and thus, we could not exclude the possibility of some kind of correlation based only on these preliminary results. Additionally, in opposition to evidences towards a trait marker, at this moment it is not possible to rule out an influence of factors associated to chronicity and severity in Ndel1 enzymatic measure, i.e. the cumulative effect of antipsychotics. These points must be more deeply evaluated in a prospective study for better clarification.

Despite these considerations, the treatment response analysis is quite promising if we take into account that Ndel1 is a potential convergence point of the DISC1 pathway and neuropeptidases. In addition, the putative physiological Ndel1 substrate NT has been proposed to mediate some actions of clozapine and other atypical drugs (Prus et al., 2007; Gruber et al., 2011). The putative regulation of NT concentration by the action of Ndel1 may help understanding the differences observed for the mean Ndel1 activity values of TR and NTR patients (Fig. 1B). This result is especially interesting if we consider that subjects from both groups are under antipsychotic treatment. Therefore, the observed differences would reflect either native differences between the groups or they may also be the result of a specific clozapine effect. In either case, this investigation can shed further light on the underpinnings of TR studies.

Of course, this study should be considered more as a proof-of-concept rather than a validation study, and several study limitations must be taken in account, for instance: (1) we do not know how stable these values are over time because these data are transversal, and consequently, we cannot fully understand the role of the progression of illness by this activity. The marginal non-statistical result for the age of onset reinforces the necessity of investigating this measurement in larger samples in prospective studies, keeping in mind that Lipska et al. (2006) found a correlation between Ndel1 expression and age. Although we tried to rule out the possible confounding effects of antipsychotic use and/or its dosage (all results were non-significant), this issue would be better clarified in a prospective study. (2) Since this measurement is conducted using plasma, the true correspondence to brain activity values and function is uncertain. Although several studies have shown that Ndel1 is a key protein in different processes related to neurodevelopment, the extension and ultimate mechanisms that determine its presence in blood still remain to be clarified and are currently under assessment by our research group. (3) To confirm the value of using the Ndel1 enzyme activity measurements as a diagnostic method, we should also evaluate first episode SCZ patients who have not received long-term treatment with several antipsychotic drugs. We are also currently studying these patients. (4) Although we adopted standard operation procedures to reduce the chances of storage and measurements differences influencing our result, we cannot completely rule out this possibility. Otherwise, it remains to be clarified the effect of diurnal variation, though storage delays did not affect the Ndel1 enzymatic activity measured in these plasma samples up to one year later of storage at -20°C (data not shown).

In conclusion, Ndel1 enzymatic activity is a potential convergence point of the DISC1 pathway and neuropeptides, both of which have been linked to SCZ in several studies. Further comprehension of the underlying factors associated with Ndel1 plasma levels could be a useful tool to unravel Ndel1 and DISC1 complex relationship. Our results also suggest that Ndel1 is a biomarker with potential clinical use in supporting diagnosis and drug-choice, although the latter potential application still needs to be further investigated. Filling in these gaps in knowledge will result in a more robust and detailed understanding of the neuropathology of SCZ, and further efforts to this highly desirable end are ongoing in many laboratories, including ours.

Conflict of interest

The authors declare no conflicts of interest.

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João R. Sato – statistic analysis of the data.

Maria A. Juliano – peptide substrate synthesis and biophysical analysis.

Vitor Oliveira – biochemist and kinetic analysis and enzymatic protocols design.

Rodrigo A. Bressan – clinical psychiatrist also responsible for the patients trials, exams and biological material collection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpsychires.2013.01.009>.

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