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Sodium nitroprusside as an adjunctive treatment for schizophrenia reduces the Ndel1 oligopeptidase activity

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Abstract

Objective: Schizophrenia (SCZ) is a disabling disorder that continues to defy clinicians and researchers. We investigated the effects of sodium nitroprusside (sNP) in an animal model of SCZ and as an add-on therapy in patients and the relationship between treatment with sNP and activity of the nDel1 enzyme, whose involvement in the pathophysiology of the disorder has been suggested earlier.

Methods: Ndel1 activity was measured following sNP infusions in spontaneously hypertensive rats (SHR; 2.5 or 5.0 mg/kg) and in a double-blind trial with SCZ patients (0.5 µg/kg/min).

Results: Ndel1 activity was significantly reduced after sNP infusion in blood of SHR compared to controls, and in patients receiving sNP ($t = 7.756$, $df = 97$, $p < 0.0001$, $d_{cohen} = 1.44$) compared to placebo. Reduced Ndel1 activity between baseline and the end of the infusion was only seen in patients after treatment with sNP.

Conclusion: Our findings suggest that SCZ patients may benefit from adjunctive therapy with sNP and that the Ndel1 enzyme is a candidate biomarker of psychopathology in the disorder. Future research should look into the role of Ndel1 in SCZ and the potential effects of sNP and drugs with similar profiles of action in both animals and patients.

Keywords: schizophrenia, sodium nitroprusside, Ndel1, animal model, biomarker.

1. Introduction

Schizophrenia (SCZ) is a chronic, devastating, multi-factorial brain disorder that is the 8th leading cause of disability-adjusted life years (DALYs) worldwide[1]. Despite its prevalence, the full understanding of SCZ etiology and pathophysiology remains elusive. While treatment with antipsychotic drugs is still the mainstay in SCZ treatment, a significant proportion of patients exhibit poor responses[2]. Alarmingly, approximately one in three patients have treatment resistant schizophrenia (TRS), a condition defined by the persistence of symptoms despite undergoing two or more trials with antipsychotic medications at appropriate dosage and duration[3].

Activation of the N-methyl-D-aspartate receptor (NMDAR) by glutamate results in calcium (Ca^{2+}) influx into the cell. This intracellular Ca^{2+} binds to calmodulin and stimulates neuronal nitric oxide synthase (nNOS) to produce nitric oxide (NO) within the central nervous system (CNS). This intricate process subsequently influences the release of neurotransmitters, including glutamate and dopamine, with direct effects on learning and memory, as well as neurodevelopment[4,5].

Notably, deficits in NO have been associated with SCZ, suggesting that enhancement of nitrergic activity might be beneficial to patients. Specifically, both SCZ and bipolar disorder patients have exhibited lower NO levels compared to control groups[6]. In addition, a subtle negative correlation of NO levels and disease duration has been reported in bipolar patients, as well as a subtle positive correlation of NO levels and disease severity in SCZ patients[7].

As a result, novel molecules with the ability to increase NO production (such as NO donors) emerge as promising candidates for the treatment of

SCZ[8]. In a translational clinical trial investigating the effects of the NO donor sodium nitroprusside (sNP) in acute SCZ, compelling evidence emerged showing that sNP infusion led to a rapid amelioration of symptoms in SCZ patients who were concurrently receiving antipsychotic treatment, with noticeable improvements occurring within hours[9]. This reduction in psychiatric symptom severity and cognitive deficits in SCZ by sNP was also demonstrated by others, both in clinical studies and using animal models[10,11,12,13].

Furthermore, it has also been proposed that sNP may have the ability to modulate the dopaminergic system[14]. This suggests that NO-based therapies may offer substantial value for patients who have a poor response to the treatment with antipsychotics or for clinical treatment failure, particularly when employed in combination with other therapeutic approaches[15,16].

Nuclear distribution element-like 1 (Ndel1) is an oligopeptidase and neurodevelopmental protein, whose oligopeptidase activity has been investigated in relation to chronic SCZ, revealing its enzyme activity to be reduced in SCZ generally compared to healthy controls, but to a greater extent in patients with treatment-resistant SCZ (TRS) compared to non-resistant SCZ patients[17]. This implies that Ndel1 activity holds promise as a potential biomarker for SCZ diagnosis and as a support for treatment decisions[18]. Furthermore, Ndel1 activity was positively associated with symptom amelioration in a one-year follow-up study of first episode psychosis patients, who were treated mainly with the atypical antipsychotic risperidone, suggesting that Ndel1 activity may also have potential as a biomarker of clinical improvement, at least in first episode psychosis patients[18].

We have also demonstrated that Ndel1 enzyme activity in peripheral blood reflects changes in the CNS in a rodent animal model for SCZ studies (the SHR strain)[19], as assessed before and after the acute administration of psychostimulants or following a long-term treatment mainly with atypical antipsychotics, under conditions in which the SCZ-like animal behaviors were reverted[20]. This supports the idea that Ndel1 activity, as measured in peripheral blood samples, could act as a surrogate to monitor changes in the CNS[20,21]. Interestingly, Ndel1 has also been implicated in serotonin signaling pathways, based on nematode worm studies, using strains that lack genes orthologous to Ndel1[22,23], which we believe to be in line with evidence of a significantly lower Ndel1 activity in TRS[17], as the only FDA-approved antipsychotic for TRS (*i.e.*, the atypical antipsychotic clozapine) acts through blockade of 5-HT2A/5-HT2C serotonin receptors and the D1-4 dopamine receptor[24].

sNP has demonstrated its potential in both preventing and reversing the development of SCZ-like behaviors in SHR rats[25]. Furthermore, genetic deletion of nNOS appears to increase the binding of Ndel1 to the Disrupted-in-Schizophrenia 1 (DISC1) protein[26]. DISC1 is a scaffold protein and known inhibitor of Ndel1[27], and this nNOS deletion was seen to regulate neurite outgrowth, implicating nNOS signaling in cortical development and prefrontal cortex functioning[26]. This latter effect may occur due to the Ndel1-DISC1 interactions, given that decreases in Ndel1 activity due to the binding with DISC1 have previously been seen to correlate with decreased neurite outgrowth and neuronal migration[28,29,30,31]. Curiously, S-nitrosylation at cysteine 203 of Ndel1 accelerates dendritic arborization, and enhances N-methyl-D-aspartate

receptor (NMDAR)-mediated neuronal activity, which is the main regulator of dendritic formation[32].

Given the potentially convergent findings regarding the role of Ndel1 and sNP in SCZ symptom amelioration, as well as its underlying biology, we assessed for the first time the Ndel1 enzyme activity following the acute administration of sNP in a rodent SCZ animal model and in a cohort of chronic SCZ patients in a double-blind randomized clinical trial.

2. Experimental procedures

2.1. Animals

Male drug-naïve normotensive Wistar rats (NWRs) and spontaneously hypertensive rats (SHRs), aged 3-5 months, obtained from our own colony (at Escola Paulista de Medicina) and weighing approximately 250-300 grams, were housed in groups of four animals, in Plexiglas cages (41 × 34 × 16.5 cm), under controlled temperature (22 - 23°C), and lighting conditions (12/12 h light/dark cycle, lights on at 07:00 AM), with free access to food and water. Animals were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources (National Research Council, USA). This study was approved by the Ethics Committee of the Universidade Federal de São Paulo (UNIFESP/EPM), certificate CEUA Nº 7290170315.

2.2. Treatment of animals and plasma preparation

Treatment of animals with sodium nitroprusside (sNP) was performed essentially as previously described[19]. Briefly, sNP (NITROPRUS - Cristália) diluted in vehicle (0.9% NaCl saline solution) was administered by

intraperitoneal (ip) injection (1.0 mL/kg) to adult (4-months old) NWR or SHR animals, n = 5 per group.

Blood was collected by caudal punction in tubes containing heparin from animals both before (baseline) and after (4, 24, and 48 h) the ip administration of vehicle or sNP (2.5 or 5.0 mg/kg). Plasma samples were then recovered after centrifugation at 1000 - 2000 × g for 10 min at 4 °C, and aliquots were stored at –20 °C until used, as described previously[18,33].

2.3. Patients

This study is part of a larger project that investigated symptom improvement in patients with schizophrenia (SCZ) following the intravenous administration of sNP. The protocol of the study was registered in REBEC:RBR-2zhgjw clinical trials database (UTN: U1111-1213-9511)[12].

Participants were recruited from community mental health facilities in Ribeirão Preto (Brazil) and surrounding towns. Patients who met the inclusion criteria (detailed in Figure 1) underwent a psychiatric assessment before being considered for inclusion in the present study.

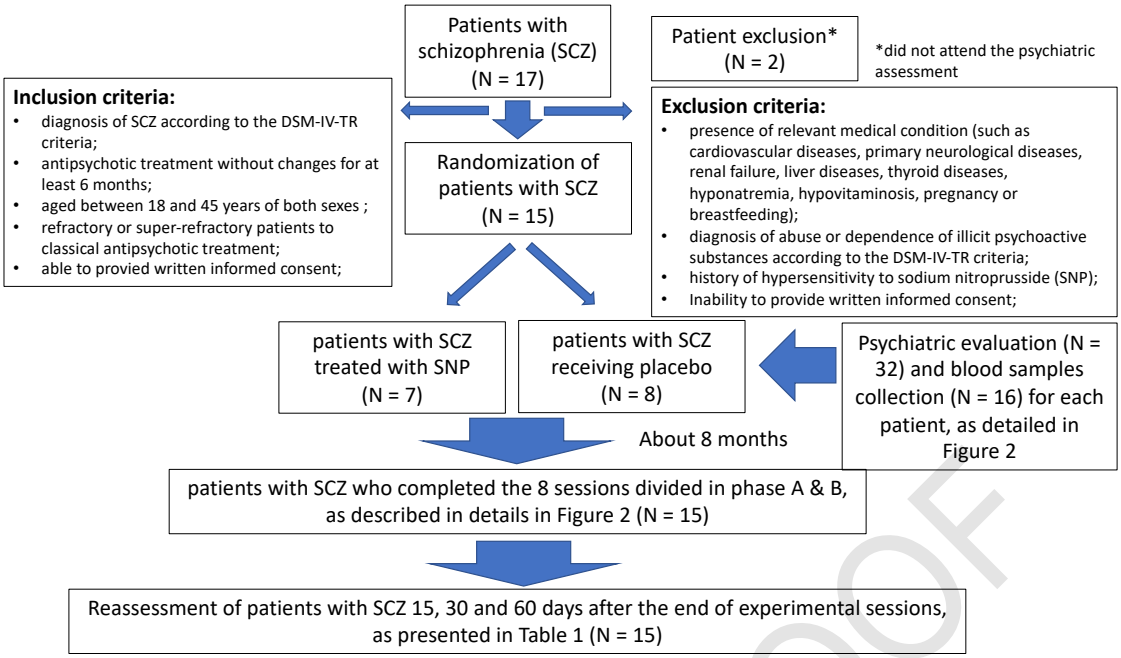

Figure 1. Flow chart of the sample recruitment process of the present study.

All patients were invited to participate in the presence of a family member. The patient and the patient's relatives received complete information on the study procedures and were informed about the characteristics and implications of this study. Participation was conditioned on the signature of a consent form, the design of which was approved by the Ethics Committee of the Ribeirão Preto Medical School University Hospital (HCFMRP-USP; Resolution 466/2012 of the National Health Council on Research Involving Human Beings of the Ministry of Health).

All participants had undergone previous assessments to identify relevant general medical conditions, including a detailed clinical anamnesis (clinical history and physical examination), collected from both the patient and their relative.

2.4. Clinical Study Procedure

According to the recruitment procedures detailed in Figure 1, 15 SCZ outpatients (15 men and 2 women; mean age, 32.94 ± 6.49 years) in treatment with typical or atypical antipsychotics at the time of the trial were randomly assigned to receive either sNP or placebo according to a pseudo-randomization process (allocation ratio 1:1). All patients and front-line study staff were blinded as to which patients received each treatment. A fully trained anesthetist was present during each infusion to ensure safety during the experiment.

sNP was administered as an infusion of 0.5 μg/kg/min, for 4 h, while placebo was a 5% glucose solution infused under the same conditions. Patients received two rounds of treatment (phases A and B) and the infusions were administered on a weekly basis for 4 weeks (Figure 2). Each participant was interviewed by the same psychiatrist, using the 18-item Brief Psychiatric Rating Scale (BPRS-18 - Bech's version) and the negative subscale of the Positive and Negative Syndrome Scale (PANSS - negative subscale) (Supplemental Table 1).

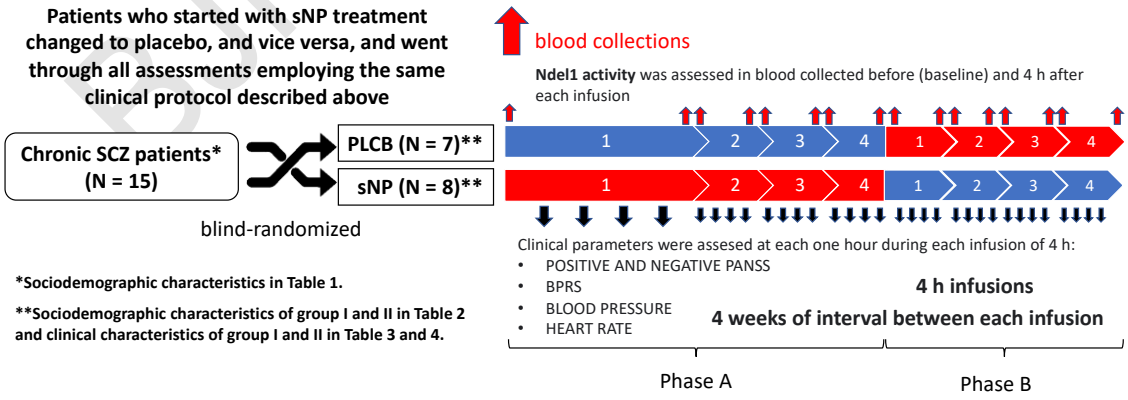

Figure 2. Flow chart of the present study. The patients with chronic schizophrenia (SCZ) were blind-randomized to form group I (N = 8) and group II (N = 7) which received four infusions of placebo (PLC, in blue) or sodium nitroprusside (sNP, in red), respectively, in phase A of treatment. In the second phase of treatment (phase B), those receiving four infusions of

PLC in phase A received SNP, while the other group receiving SNP in the phase A then received PLC in phase B of treatment. Blood was collected before (at baseline) and 4 h after each infusion of PLC or sNP, while the clinical assessment occurred at each hour during the 4 h of each infusion (in a total of 4 assessments for each patient for each infusion). The interval between each infusion was of one-month (*i.e.*, 4 weeks each).

Patient recruitment and all clinical procedures occurred between 2016 and 2018. For this trial, 15 subjects with chronic SCZ were randomized into two groups (Group I: n = 7; Group II: n = 8), with one group receiving placebo and the other sNP in phase A. Clinical interviews were made every hour during the 4 h infusion, and this procedure was repeated 4 times at intervals of one week in both phases A and B. Physiological cardiovascular and pulmonary measures were also recorded every hour to assess the safety of sNP throughout the infusion. After infusion, the patient was asked how he/she felt during the infusion, and if he/she experienced any unexpected bodily or psychological sensation or discomfort throughout the session. Participants were also allowed to rest for at least 1 h after this debriefing with the researcher.

The second round of treatment (phase B) was then carried out in exactly the same manner as phase A, except that the group who previously received placebo in phase A were treated with sNP in phase B and vice-versa (flow chart in Figure 2).

2.5. Outcome Measures

The Structured Clinical Interview for DSM-IV (SCID) was used to confirm the diagnoses of SCZ. The BPRS-18 (Bech's version) and the positive and negative PANSS subscales were used to measure changes in symptoms. Although the BPRS-18 and the PANSS were not originally intended to track

short-termed changes in psychopathology, we opted to follow the same method used in the trial by Hallak and colleagues[9] that first described the effects of sNP in SCZ patients.

Safety and tolerability parameters were recorded throughout the infusions. Cardiac function was monitored using an automated monitor (model 2020, Dixtal Medical) with electrocardiography, blood pressure, and blood oxygen saturation levels recorded hourly throughout the experimental sessions. The structured UKU rating scale was used to prospectively assess the treatment adverse effects during this study.

2.6. Human blood collection for plasma preparation

Blood was collected into heparin tubes both before and 4 h after the infusion of sNP (or placebo). Plasma samples were then recovered following centrifugation at 1000 - 2000 × g, for 10 min, at 4 °C. Aliquots of plasma were stored in microcentrifuge tubes at –20 °C until use, as previously described[18,33].

2.7. Ndel1 enzyme activity measurements

Ndel1 activity was measured in blood plasma collected from all patients both before and 4 h after each infusion of placebo or sNP, in a total of eight samples per patient per round of treatment (phase A or B). Over both phases, a total of 240 measurements were therefore obtained for the 15 randomized SCZ patients (120 in placebo infusions and 120 in sNP infusions.

Ndel1 enzyme activity was measured as described elsewhere[17]. Briefly, hydrolysis of a Ndel1 peptide substrate (Abz-GFSPFRQ-EDDnp) was monitored at 37°C by measuring fluorescence ($\lambda_{Ex} = 320$ nm and $\lambda_{Em} = 420$ nm) in a F-

7000 spectrofluorimeter (Hitachi Ltd., Ibaraki, Japan). Samples for these measurements consisted of 10 µL of plasma and 10 µM of substrate in 200 µL of buffer (NaCl 100 mM, 50 mM Tris-HCl pH 7.4). Ndel1-specific activity was determined through use of 10 µL of a heat-inactivated Ndel1 polyclonal antibody (NO_{AB} inhibitor), which has specific inhibitory activity against Ndel1[27]. Ndel1-specific activity was therefore defined as the rate of hydrolysis of the substrate peptide in the absence of NO_{AB} inhibitor minus the rate when NO_{AB} was present[34,35].

2.8. Statistical Analysis

Variable distribution was verified by the Gaussian distribution using the Kolmogorov-Smirnoff tests for the total sample and in each comparison group. Data was also tested for homogeneity and sphericity, with Welch's or Greenhouse–Geisser respectively, and correction was applied when necessary. Chi-square was used to analyze categorical variables such as sex and ethnic background, represented here in sociodemographic tables. Standard parametric (Paired and unpaired Student's *t* test, two-way ANOVA for repeated measures and Pearson correlation) tests were applied accordingly to variable type and distribution, and Bonferroni's multiple comparison test was adopted for post-hoc analysis. Ndel1 activity values were log transformed to suit normality. Additionally, we calculated the difference in Ndel1 activity (□Ndel1) in nM/min, between post sNP or placebo measurements and their respective baseline, aiming to enhance the clarity of the observed effects. Effect size analysis was conducted using the Cohen's D method. All results are expressed as the value of mean ± standard deviation (SD). Outlier analysis was performed by the

ROUT method (Q = 1%), identifying outliers from nonlinear regression based on the False Discovery Rate (FDR), with Q being the maximum desired FDR. Both graph and table were used to represent clinical outcomes, while Ndel1 activity was represented as graphs. Data analyses were performed using SPSS Statistics software version 22.0 (IBM Corporation, Endicott, NY) and graphs were generated using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). In all experiments, *p* values $\leq$ 0.05 were considered to be statistically significant.

3. Results

3.1. Ndel1 activity in plasma from a rat model of schizophrenia

The SHR strain of rats, a non-pharmacological animal model of SCZ, has been used to demonstrate decreases in social interaction and attenuated contextual fear conditioning deficits following acute administration of sNP at various doses[19]. Here, we used similar conditions and doses to measure Ndel1 activity in control normotensive Wistar rats (NWR) and in SHR, both before and after (4, 24 and 48 h) infusion with vehicle (Figure 3A) or sNP (2.5 or 5.0 mg/kg) (figures 3B and 3C, respectively). When compared to baseline (9.35 ± 1.89 nM/min), a significant decrease in Ndel1 oligopeptidase activity was seen in the plasma of SHR 4 h after the infusion of sNP (at both concentrations of 2.5 and 5.0 mg/kg) (6.51 ± 0.53 nM/min and 6.66 ± 1.59 nM/min respectively). No significant changes in Ndel1 activity was observed in the control NWR after the infusion of sNP in both concentrations (Figure 3).

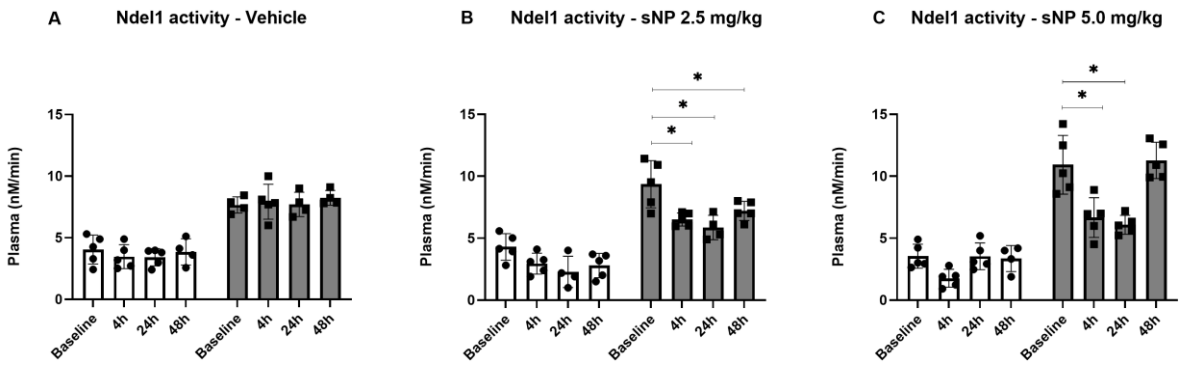

Figure 3. Ndel1 activity in rat plasma 4 h after the infusion of sNP (2.5 or 5.0 mg/kg). Normotensive Wistar rat (NWR, white bars) and spontaneously hypertensive rats (SHR, black bars) received the infusion of sodium nitroprusside (sNP) and their blood were collected in heparinized tubes 4, 24 and 24 h after the infusion of vehicle or sNP (2.5 or 5.0 mg/kg). (A) vehicle (0.9% NaCl saline solution); (B) sNP 2.5 mg/kg; (C) sNP 5.0 mg/kg. The Ndel1 oligopeptidase activity was measured using 10 µL of plasma of each animal, and the data are presented here as µM/min. Statistical analysis by two-way ANOVA, for N = 5, *$p \leq 0.05$.

Specifically, Ndel1 activity was not affected by administration of the vehicle in either strain (Figure 3A). In line with previous reports of SCZ-like behavior in SHR animals[13,19], SHR showed significant reductions in Ndel1 activity 4, 24 and 48 h (6.51 ± 0.53 nM/min, 5.86 ± 0.98 nM/min and 7.20 ± 0.77 nM/min respectively) after the infusion of the lowest sNP dose studied here (2.5 mg/kg) when compared to the baseline before infusion (9.35 ± 1.89 nM/min) (F = (1.422, 10.43) = 13.62, $p = 0.002$ and F (1, 8) = 84.15, $p < 0.001$ for time and strain factors respectively and (F (1.42, 10.43) = 13.62, p = 0.002) for the interaction) (Figure 3B). With the higher dose of sNP (5.0 mg/kg), Ndel1 activity also decreased 4 and 24 h (6.66 ± 1.59 nM/min and 6.08 ± 0.75 nM/min respectively) after treatment, but not 48h (11.27 ± 1.46 nM/min) after (F = (2.117, 21.87) = 13.67, $p < 0.001$ and F (1, 31) = 169.3, $p < 0.001$ for time and strain factors, respectively and (F (2.11, 21.87) = 13.67, p < 0.001) for the interaction) (Figure 3C). These findings were used to select time points for the

clinical phase of this work, with Ndel1 activity being measured in SCZ patients from blood collected 4 h after infusion with sNP or placebo.

3.2. Ndel1 activity in plasma of SCZ patients

Ndel1 activity was examined in a blind-randomized group of SCZ patients (groups I: n = 7 and group II: n = 8). Sociodemographic data for the whole cohort is in Table 1, and data divided by group in Table 1. There were no significant differences in sex, educational level, ethnic background and age between the blind-randomized groups I and II. Clinical characteristics of these groups are described in Supplemental table 2 and 3. The sample consisted almost exclusively of clozapine users (92.86%), with some using enhancers of conventional treatment such as lamotrigine and/or AFG or AGF.

Groups had their Ndel1 activity analyzed before and 4 h after infusion with sNP or placebo, and the change in Ndel1 activity (□Ndel1) between these two time points was determined. This trial was conducted twice (phases A and B), with patients receiving sNP in phase A and placebo in phase B, and vice versa. There was a significant decrease in Ndel1 activity following sNP infusion compared to placebo, across the whole dataset (Figure 4A), and this was also seen when data from each phase (A and B) was analyzed separately, as shown in Figure 4B.

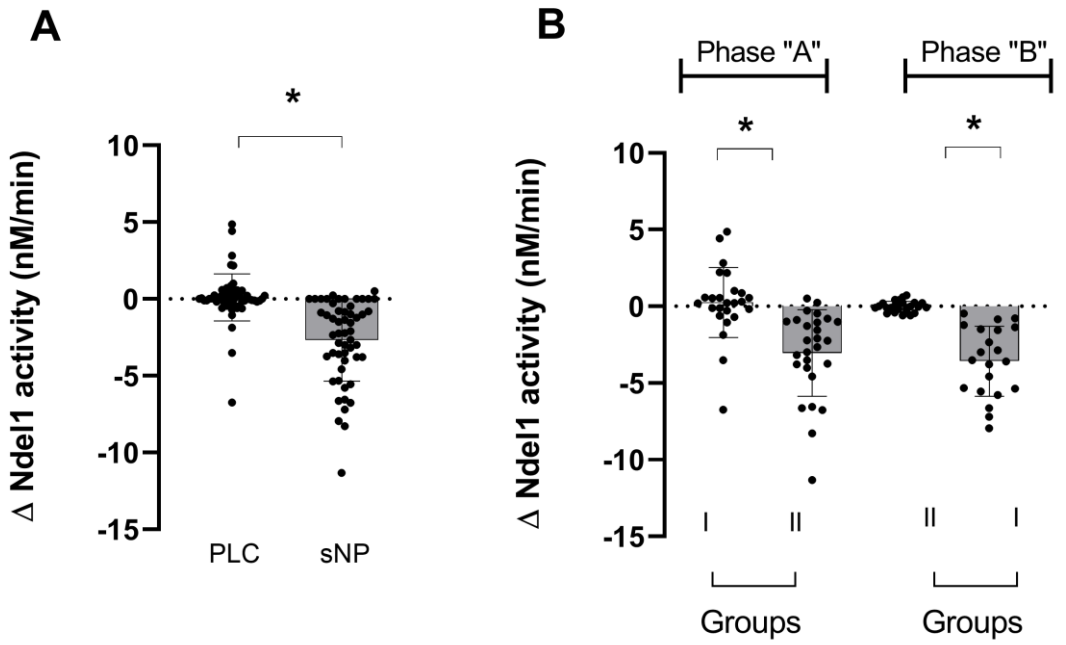

Figure 4. Delta Ndel1 activity in plasma of SCZ patients, treated with placebo (PLC) or sodium nitroprusside (sNP). (A) The Ndel1 activity was measured in the serum of all patients with chronic SCZ (N = 15) just before and 4 h after they have received PLC or sNP infusion for 4 h. The Ndel1 activity (in μM/min) correspond to the Ndel1 activity after each infusion (of PLC or sNP) minus the value at baseline for each SCZ patient. As the blood was collected before and 4 h after each infusion (N = 4 infusion in each session) of PLC or sNP, in a total of 8 samples collected for each SCZ patient (a total of 120 values for Ndel1 activity which correspond to the 8 samples for each of 15 SCZ patients, presented for each PLC and sNP infusions. (B) The Ndel1 activity of blind-randomized group I (N = 7) and II (N = 8) of SCZ patients separately. Group I received PLC and group II received sNP, in first round of treatment (phase A), while in the second round of treatment (phase B), conversely, the group II received PLC and group I received sNP. Each round of treatment (phase A and B) involved 4 infusions of 4 h each of PLC or sNP, and the blood was collected just before and 4 h after each infusion. The statistical analysis employed unpaired t-test, $*p \leq 0.05$ for the comparison of the values differences observed after the infusion of PLC or sNP minus their respective baseline values.

There were no significant differences in Ndel1 activity before and after placebo infusion in either phase, with Ndel1 activity close to zero (-0.27 ± 0.45 nM/min, t = 1.113, df = 98, p = 0.268) (Figure 4A). However, mean values of Ndel1 activity decreased after administration of sNP, both when all SCZ patients from both phases were considered together (-3.39 ± 0.43 nM/min, t = 7.756, df = 97, p < 0.0001) (Figure 4A) and when each phase was studied

individually (phase A: -3.29 ± 0.71 nM/min, t = 4.604, df = 50, $p < 0.0001$; phase B: -3.56 ± 0,45 nM/min, t = 7.889, df = 48, $p < 0.0001$, Figure 4B).

Ndel1 activity was significantly reduced in all subgroups of SCZ patients following the infusion of sNP ($F (1, 107) = 25.86$, $p < 0.001$) regardless of whether the patients were in the group that received treatment with placebo or sNP first (mean difference of -2.77 nM/min, 95% CI [-1.95 to 3.59]) (Figure 5A). Furthermore, in both phases of the trial, patients receiving the sNP infusion showed improvement in symptoms, as assayed by positive and negative PANSS values and the BPRS score (Figure 5B-D, statistical values in supplemental table 2 and 3). The only exception was the PANSS positive score after sNP infusions in phase B, corresponding to the patients who received sNP in phase B, after receiving placebo in phase A (Figure 5B, statistical values in supplemental table 2 and 3). We additionally conducted a comparative analysis of the Ndel1 activity and psychiatric symptoms scores after each session of sNP, reaffirming the observed effect (Supplemental Figure S2).

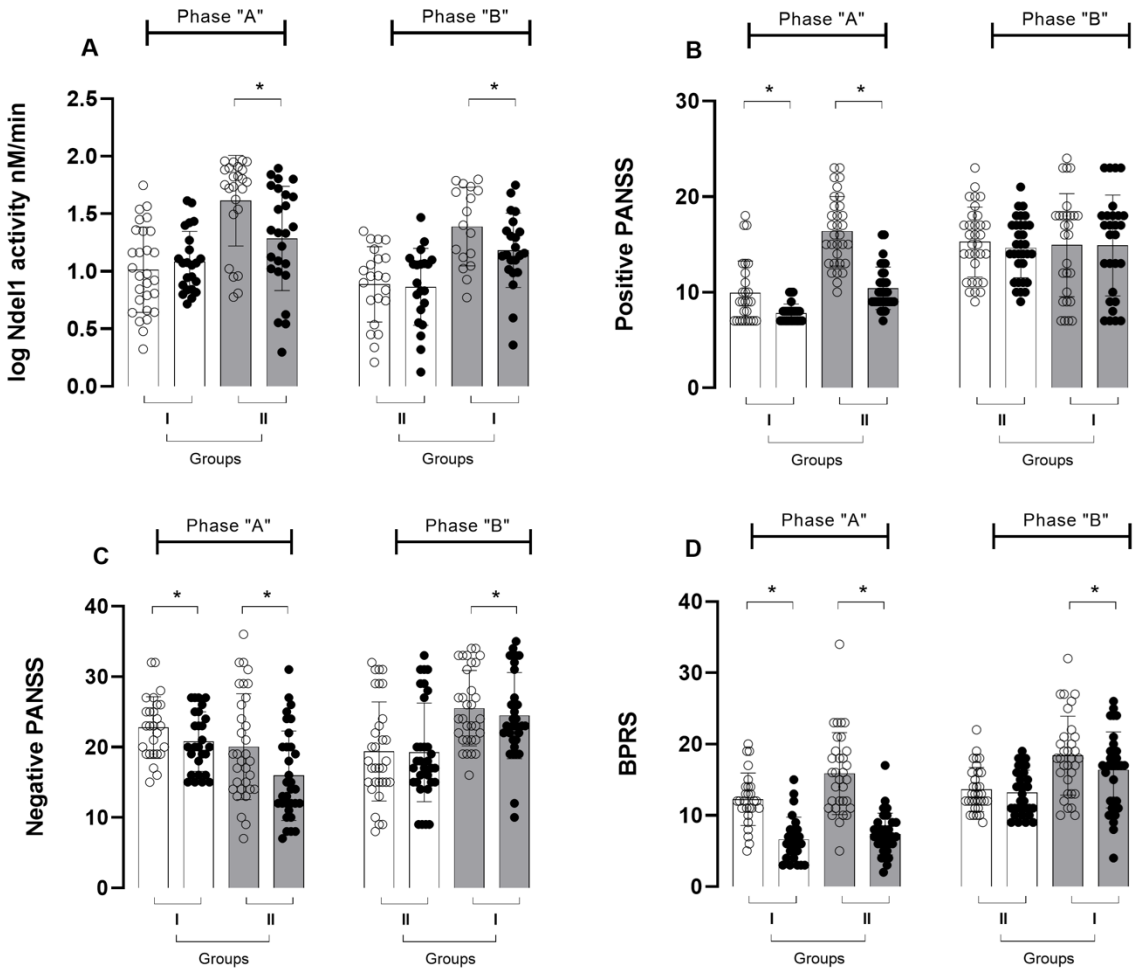

Figure 5. Values of log Ndel1 activity (A), positive (B) and negative PANSS (C) and BPRS (D) of patients of Group I and group II after the first round (phase A) and second round of treatment (phase B). These values represent the four infusion sessions of PLC or sNP for each SCZ patient, assessed before and 4 h after infusion for Ndel1 activity and in each hour along the 4 h of each infusion of PLC or sNP for the clinical assessments. Paired t-test, *$p \leq 0.05$.

Ndel1 activity was also analyzed within each group of patients (I and II) to compare Ndel1 in each phase. That is, to see whether sNP and placebo (administered at different time points: phases A and B) had differing effects on the same individual patients. All groups showed a significant decrease in Nde1l activity following sNP compared to placebo infusion, in contrast to the larger values observed for sNP infusions (Supplemental Figure S1). This was seen both for the cohort as a whole (effect size Cohen's d = 1.44), and in the

individual groups that received either placebo first (group I, effect size Cohen's d = 1.68) or sNP first (group II, effect size Cohen's d = 1.13).

It is also important to note that the clozapine doses used by patients in each group had no significant differences (group I: 550.0 ± 342.3 mg, group II: 471.4 ± 138.0 mg, t = 0.566, df = 13, p = 0.581). In addition, during the course of these experiments, there were no significant changes in cardiovascular conditions and/or blood pressure of the patients, as shown by systolic blood pressure, diastolic blood pressure and heart rate measures. The only exception was for the increased heart rate seen in group II following placebo infusions, but not sNP infusions (Supplemental Table 4). Crucially, the UKU rating scale was used to prospectively assess the secondary outcomes of this study (including safety and tolerability), and showed no observable adverse effects arising from the treatments used in this study (*data not shown)*.

4. Discussion

Treatment of SHR animals with sNP under conditions previously shown to decrease social interaction and attenuate contextual fear conditioning deficits was associated with a significant decrease in plasma Ndel1 activity in SHR, whereas this effect was not observed in control NWR animals. Intriguingly, after infusion of sNP (at doses of 2.5 or 5.0 mg/kg), Ndel1 activity in the plasma of SHR animals was similar to those observed in NWR. This suggests that 4 or 24 h after sNP infusion, a condition more closely aligned with a physiological balance was evident in SHR animals, which is in close agreement with improvements in animal behavior previously described by others[19]. Moreover, it was determined that the 4 h after the infusion of sNP period was sufficient to

observe a significant decrease in Ndel1 activity in blood of animal models. Consequently, all clinical samples collected to monitor changes in Ndel1 enzyme activity in patients were obtained 4 h after placebo or sNP infusions.

A significant decrease in Ndel1 activity was consistently observed in all patients with chronic SCZ following the sNP infusion. This was evident when comparing the post-sNP infusion values to the baseline levels in the same SCZ patients, which were measured at different times, as well as when comparing them to patients who received a placebo infusion at the same time. Interestingly, this decrease in Ndel1 activity was even more pronounced in the first round of treatment with sNP (phase A) compared with the second round of treatment (phase B). These effects seemingly did not arise due to differences in the patients' main pharmacological treatments, since there was no significant difference in the mean amount of clozapine that each group was receiving.

The use of adjunct sNP treatment for patients with SCZ was suggested as a strategy to allow lower doses of antipsychotics while reducing the risk of side effects, based on behavioral and biochemical analysis of an animal model receiving low doses of sNP (1 and 1.5 mg/kg) aiming to improve the efficacy of the atypical antipsychotic risperidone[36].

The data presented here show that sNP not only improves the symptoms of patients with TRS, but also leads to a significant decrease in Ndel1 activity in their plasma. Previous studies has suggested a correlation between Ndel1 activity in the plasma and the brain[20]. In light of the various previous studies that implicate Ndel1 activity in SCZ and symptom severity[17,18], we hypothesize that these two effects of sNP are linked, with Ndel1 being part of the molecular

mechanism(s) underlying the effects of sNP infusions on both SCZ-like animal behavior improvement[19] and SCZ symptoms amelioration[9].

Previous studies by our group have demonstrated a significant decrease in Ndel1 oligopeptidase activity in SHR animal model , but not in NWR, animals after a long-term treatment with the typical antipsychotic haloperidol or the atypical antipsychotic clozapine[20], suggesting that the specific and selective effects of sNP seen in pathological conditions may not observed in physiological healthy conditions (represented by control drug-naïve NWR animals)[20]. Decreases in Ndel1 activity was also previously demonstrated to be associated with the amelioration of symptoms in first episode psychosis (FEP) patients[18]. Additionally, significant lower Ndel1 activity was reported in medicated patients with chronic SCZ relative to healthy controls, with even lower Ndel1 activity in treatment-resistant compared with treatment non-resistant SCZ patients[17].

Although we could also confirm the significant decrease in Ndel1 activity following the sNP infusion in both SCZ animal model and patients with chronic SCZ, it was not possible to demonstrate a clear association of these changes with the improvements in the animal behavior deficits (including the reported decreases in social interaction and attenuated contextual fear conditioning deficits) or with clinical symptoms amelioration of the patients (increases in several clinical scores, including PANSS).

The interaction between Ndel1 and other cytoskeletal proteins, particularly DISC1, has been proposed as a significant component of the neurodevelopmental hypothesis of SCZ. DISC1 binding to Ndel1 and inhibits its oligopeptidase activity[28,30,33]. One potential mechanism by which Ndel1 activity could be decreased following sNP treatment, would be because it affects Ndel1

nitrosylation, a post-translational event previously reported by others[32], affecting its interaction with DISC1. Further studies are needed to demonstrate this as a potential mechanism.

In statistics, an effect size is a number measuring the strength of the relationship between two variables in a population, or a sample-based estimate of that quantity. If the difference between two groups' means is less than 0.2 standard deviations, the difference is considered negligible, even if statistically significant, while Cohen suggested that d = 0.8 or higher could be considered as a 'large' effect size[37]. Remarkably, in our study, a significant difference in Ndel1 plasma activity was observed between the placebo controls and sNP treated groups, showing a strong effect of the sNP infusion. This effect was evident both for SCZ patients who had previously received the placebo in the initial phase of treatment (phase A), namely group I (effect size Cohen's d = 1.68), and for those who did not receive a placebo in the first phase, namely group II (effect size Cohen's d = 1.13). Therefore, a 'large' effect size for the Ndel1 activity reduction following the administration of sNP was demonstrated here.

We have also noticed a decrease in psychiatric symptoms scores following the PLC infusion, but only in the first round of treatment (phase A), which also led to significant alterations in the cardiovascular parameters of all patients receiving placebo or sNP (belonging to the groups I or II, respectively), which were not observed in the second round of treatment (namely phase B) (Supplementary Table 4).

This may explain the decreases in the psychiatric symptoms scores even after the administration of placebo observed only in phase A of treatment

(Figure 3). However, our study clearly establishes that this placebo-induced effect does not impact Ndel1 activity. Instead, Ndel1 activity was exclusively modulated by sNP infusions and remained unaffected by placebo infusions in both treatment phases. Moreover, we believe that the lack of correlation between the reductions in Ndel1 activity and symptom improvement in this study may be attributed to both the sample size limitations and the distinct effects observed between placebo and sNP treatments.

It should be noted that not all the evidence available on the effects of sNP in SCZ points out in the same direction and that some controlled trials failed to find beneficial effects of the drug on either symptoms or cognitive measures[38,39]. The divergent findings of these trials might reflect their methodological differences, which include important aspects as the patient populations (early/late phase, treatment resistant), symptom severity (higher PANSS scores), and treatment contexts (monotherapy or polytherapy), leading to varying outcomes and highlighting the complexity of addressing SCZ. Our study aims to shed light on specific facets, including the significance of monotherapy, as the prevalent use of clozapine in our cohort, in the investigation of sNP adjunctive pharmacotherapy. An additional limitation of our study is the relatively small size of the clinical sample enrolled, as well as the impossibility to demonstrate the direct correlation of possible post-translational modifications or complex formation of Ndel1 with the decreased activity and/or with the improvement of symptoms. These points will be the focus of future studies aiming to clarify the real molecular role(s) of Ndel1 in the adjunctive effects of NO donors, such as sNP, on the add-on therapy with antipsychotics as evaluated herein. Furthermore, we intend to expand our investigations by

analyzing larger patient cohorts, as we hypothesize that these findings will bolster the implications of Ndel1 enzyme activity in clinical practice. This will aid in monitoring drug treatment for patients with SCZ and advancing our understanding of the mechanisms underpinning the therapeutic efficacy of sNP.

5. Conclusion

Our results support the hypothesis that Ndel1 activity is linked to the severity of SCZ symptoms, with changes in Ndel1 activity correlating with symptom/behavioral amelioration following treatment with antipsychotics and/or an NO donor in rats and patients.

The findings presented suggest that SCZ patients in treatment with clozapine may benefit from adjunctive therapy with sNP and that the Ndel1 enzyme is a candidate biomarker of psychopathology levels in the disorder. Future research should look into the role of Ndel1 in the pathophysiology of SCZ and the potential effects of sNP and drugs with similar profiles of action in both animals and patients.

Disclosure

The authors declare no conflict of interest.

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Supplemental material

Supplemental Table 1. Description of the experimental protocol

Time	Procedure
Admission	- Routine assessments of the psychiatric ward of the HCFMRP-USP - Screening and diagnosis (SCID-IV)
Biweekly trial session	
T0 (baseline)	- Start of monitoring HR, BP and O_2 saturation - Venous puncture - Clinical evaluation: BPRS, PANSS and UKU
Start of infusion (sNP or PLC)	
T1 (60 min)	- Clinical evaluation: BPRS, PANSS
T2 (120 min)	- Clinical evaluation: BPRS, PANSS
T3 (180 min)	- Clinical evaluation BPRS, PANSS
T4 (240 min)	- Clinical evaluation: BPRS, PANSS
End of infusion	
Closing of 8 experimental sessions	
Reassessment after 15 days	- Clinical evaluation: BPRS, PANSS, UKU
Reassessment after 30 days	- Clinical evaluation: BPRS, PANSS, UKU
Reassessment after 60 days	- Clinical evaluation: BPRS, PANSS, UKU

Supplemental Table 2. Clinical characteristics of the samples from the groups I and II, before (at baseline, t = 0) and 4 h after (t = 4) each infusion of placebo (PLC) or sodium nitroprusside (sNP) in the first treatment round, namely phase A, comprising four infusions of 4 h each followed by one-month intervals each.

		SCZ (N = 15)								Comparative analysis between groups at baseline	
		Group I (N = 7)				Group II (N = 8)					
		t = 0 (before)	t = 4 (after)	Test value	*p*-value	t = 0 (before)	t = 4 (after)	Test value	*p*-value	Test value	*p*-value
PANSS	Positive	9.9 (3.3)	7.7 (0.9)	4.273	<0.001*	16.3 (4.0)	10.1 (2.3)	10.53	<0.001*	7.056	<0.001*
	Negative	22.7 (4.3)	20.7 (4.2)	4.356	<0.001*	19.9 (7.1)	15.9 (6.0)	8.261	<0.001*	1.654	0.103
BPRS		12.2 (3.6)	6.6 (3.1)	9.353	<0.001*	15.8 (5.7)	7.3 (2.9)	10.631	<0.001*	2.811	0.006

Abbreviations: df, degrees of freedom; PLC, placebo; sNP; sodium nitroprusside; PANSS: Positive and Negative Syndrome Scale; BPRS: Brief Psychiatric Rating Scale.
Statistical significance was defined as $*p \leq 0.05$.

Supplemental Table 3. Clinical characteristics of the samples from the groups I and II, before (at baseline, t = 0) and 4 h after (t = 4) each infusion of placebo (PLC) or sodium nitroprusside (sNP) in the second round of treatment, namely phase B, comprising four infusions of 4 h each followed by one-month intervals each.

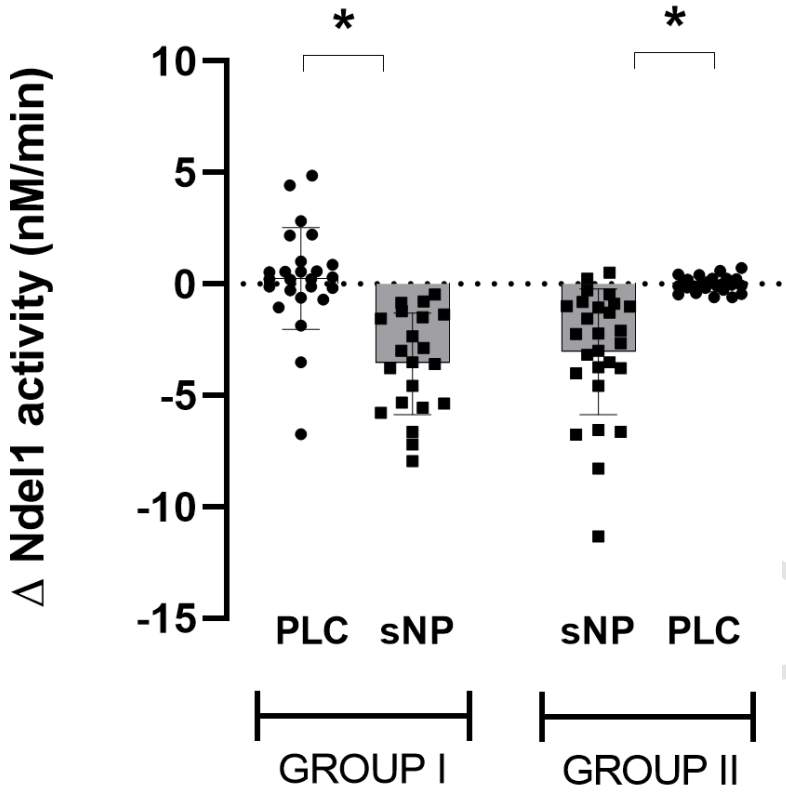
		SCZ (N = 15)								Comparative analysis between groups at baseline	
		Group II (N = 8)				Group I (N = 7)					
		t = 0 (before)	t = 4 (after)	Test value	*p*-value	t = 0 (before)	t = 4 (after)	Test value	*p*-value	Test value	*p*-value
PANSS	Positive	15.2 (3.6)	14.6 (3.1)	1.958	0.059	14.9 (5.3)	14.8 (5.2)	0.372	0.712	0.195	0.846
	Negative	19.3 (7.0)	19.2 (6.9)	0.387	0.701	25.5 (5.3)	24.4 (6.1)	2.143	0.040	3.940	<0.001*
BPRS		13.6 (3.1)	13.1 (3.1)	1.438	0.160	17.3 (5.0)	16.1 (5.3)	4.518	<0.001*	4.237	<0.001*

Abbreviations: df, degrees of freedom; PLC, placebo; sNP; sodium nitroprusside; PANSS: Positive and Negative Syndrome Scale; BPRS: Brief Psychiatric Rating Scale.
Statistical significance was defined as $*p \leq 0.05$.

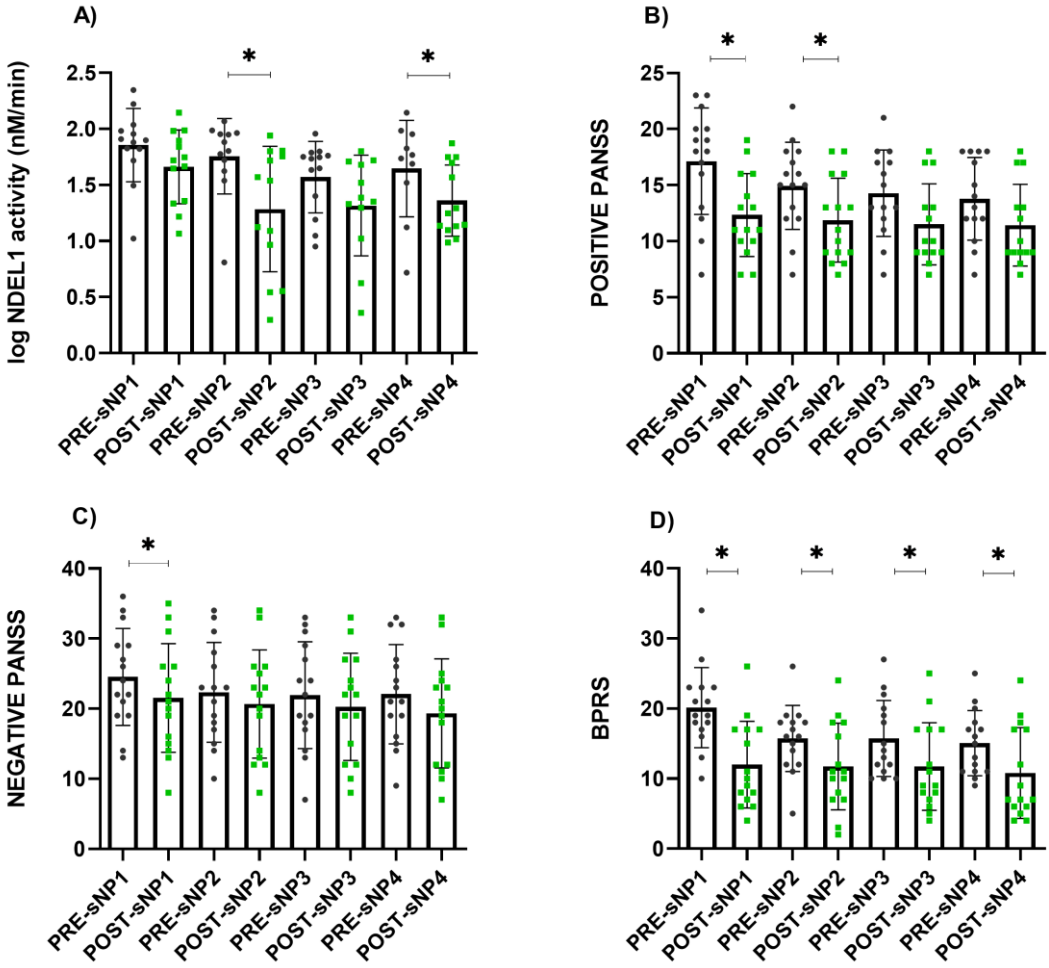
Supplemental Table 4. Cardiovascular characteristics of the samples from the groups I and II, before (at baseline, t = 0) and 4 h after (t = 4) each infusion of placebo (PLC) or sodium nitroprusside (sNP) in the first and second treatment round, namely phase A and B, respectively.

Treatment	Cardiovascular parameters	SCZ (N = 15)								Comparative analysis between groups at baseline	
		Group I (N = 7)				Group II (N =8)					
		t = 0	t = 4	Test value	*p*-value	t = 0	t = 4	Test value	*p*-value	Test value	*p*-value
Phase A	sBP mean (± SD)	125.2 (16.2)	114.5 (16.5)	4.996	<0.001*	124.5 (14.8)	112.5 (11.3)	4.895	<0.001*	0.171	0.864
	dBP mean (± SD)	68.6 (10.5)	61.45 (13.9)	3.529	0.001*	65.3 (8.3)	57.8 (8.9)	3.046	0.004*	1.126	0.212
	HR mean (± SD)	96.6 (15.2)	104.1 (14.7)	3.113	0.004*	92.5 (12.4)	96.6 (13.0)	1.853	0.073	1.109	0.272
		Group II (N = 8)				Group I (N = 7)					
Phase B	sBP mean (± SD)	125.5 (16.2)	127.9 (16.5)	0.939	0.355	124.5 (13.4)	123.8 (12.6)	0.259	0.797	0.287	0.774
	dBP mean (± SD)	69.1 (8.6)	70.4 (10.3)	0.659	0.514	69.2 (11.1)	69.1 (8.9)	0.013	0.989	0.035	0.972
	HR mean (± SD)	89.7 (10.2)	88.4 (10.7)	0.930	0.359	91.9 (14.3)	98.1 (13.4)	2.174	0.038*	0.684	0.496

Abbreviations: sBP: systolic blood pressure; dBP: diastolic blood pressure; HR: heart rate. Statistical significance was defined as $*p \leq 0.05$.



Supplemental Figure 1. Delta Ndel1 activity in serum of SCZ patients, treated with placebo (PLC) or sodium nitroprusside (sNP). The Ndel1 activity was measured in the serum of all patients with chronic SCZ (N = 15) just before (baseline) and 4 h after they have received PLC or sNP infusion. The present data correspond to the △Ndel1 activity (μM/min) for each double blind-randomized group, namely group I (N = 7) and group II (N = 8) of patients with chronic SCZ. The statistical analysis employed paired t-test, $*p \leq 0.05$ for the comparison of the values differences observed after the infusion of PLC or sNP minus their respective baseline value.



Supplemental Figure 2. Values of log Ndel1 activity (A), positive (B) and negative PANSS (C) and BPRS (D) of patients of both group I and group II before and after the every round of treatment with sNP. These values represent the four infusion sessions of sNP for each SCZ patient, assessed before and 4 h after infusion for Ndel1 activity and clinical assessments. Paired t-test, *$p \leq 0.05$.