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OXIDATIVE STRESS RESPONSES AND THEIR ALTERATIONS IN THE Nrf2-NMDA RECEPTOR PATHWAY IN THE BRAIN OF SUICIDE VICTIMS

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Suicide is a global public health concern. There is evidence of an association between suicidal behavior and depressive disorders (DDs). An increasing number of studies have suggested that nuclear factor erythroid-derived 2-like 2 (Nrf2), a major endogenous regulator of the oxidative stress response, can be a novel target for the neurobiology of suicide-related disorders (including depression). This study aimed to investigate the relationship between oxidative stress progression, Nrf2 regulation, and N-methyl-D-aspartate receptor (NMDA) subunit composition in the hippocampus (Hp) and frontal cortex (FCx) of suicide victims (n=14) and matched controls (n=8). Furthermore, zinc and magnesium concentrations and their potency to inhibit [^{3}H] MK-801 (radioactively labeled form of MK-801 - dizocilpine, a well-characterized NMDAR channel uncompetitive antagonist frequently used in receptor-binding assays) binding to NMDA receptor channels were measured. Our results revealed a statistically significant increase in protein carbonyl levels and thiobarbituric acid-reactive substances (TBARS) concentrations in Hp and FCx of suicide victims. Enhanced superoxide dismutase (SOD) activity (only in FCx) in suicides compared to controls was shown. These alterations were accompanied by an increase in Nrf2 protein levels in whole homogeneous tissue lysates and cytosolic fractions of Hp and FCx. Importantly, suicide victims presented a significant reduction in Nrf2 protein levels in the nuclear fraction of FCx. Finally, the observed decrease in N-methyl-D-aspartate receptor subunit 2B (GluN2B) and postsynaptic density proteins 95 (PSD-95) protein levels was associated with a statistically significant reduction in magnesium levels in the FCx of suicide victims. These results confirm for the first time that increased oxidative stress parameters are related to Nrf2 protein changes and alterations in the NMDA receptor complex in the pathophysiology of suicidal behavior.

Key words: *suicide, depression, oxidative stress, reactive oxygen species, nuclear factor erythroid-derived 2-like 2, N-methyl-D-aspartate receptor, postsynaptic density proteins 95, magnesium, zinc*

INTRODUCTION

Suicide is a serious public health problem worldwide. According to World Health Organization (WHO), over 700,000 people died of suicide each year, with suicide attempts occurring up to 20 times more often (1). Therefore, knowledge and determination of the most important risk factors for suicide are key to the development of appropriate preventive systems. A large body of evidence points to a close relationship between suicidal behavior and psychiatric disorders such as major depressive disorder (MDD), bipolar disorder (BD; types I, II), schizophrenia, and anxiety disorders (2-5). The suicide rate in patients with MDD is approximately 20-fold higher than that in the general population, and the increased rate of suicide attempts in patients with BD is associated with comorbid anxiety (2, 5).

The oxidative stress hypothesis of suicide-related disorders is critical and influential. Oxidative stress is an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defense, leading to DNA, protein, and lipid damage in the central and peripheral nervous systems. Prolonged oxidative stress can induce damage to brain regions that contribute to the pathophysiology of suicide and depressive disorders (DDs) (6). Suicidal behavior may be associated with pathological alterations in the cortical thickness of the frontal areas and hippocampus (7). Neuroimaging studies have shown that the hippocampal, frontal, and temporal lobe volumes of suicide victims are significantly reduced (8, 9). Moreover, increasing evidence supports the hypothesis that hippocampal volume could be a suicidal state marker for DDs (9, 10). The discovery of structural brain alterations and their relationship with molecular mechanisms is important for understanding the pathophysiology of suicide-related disorders.

In clinical studies, oxidative stress markers (such as decreased superoxide dismutase 1/2 (SOD1/2) and glutathione peroxidase 1

(GPX1) expression, higher frequency of severe DNA damage, increased concentration of malondialdehyde (MDA) and enhanced catalase (CAT) activity were observed in subjects with suicidal ideation and suicide victims with MDD (6, 11, 12). The strong association between the activation of oxidative stress pathways and suicidal ideation or attempts has been thoroughly discussed by Vasupanrajiy *et al.* (13). These data confirm that dysregulation of redox homeostasis is implicated in the pathophysiology of suicide-related disorders. In addition, some measurable biological markers of oxidative stress present different profiles of changes in suicidal and non-suicidal individuals. Regardless of this, it is still important to explore the underlying molecular mechanisms of oxidative stress and their potential applications in the diagnosis and treatment of mental disorders (including suicidal behavior). Recent studies indicate that nuclear factor erythroid-derived 2-like 2 (Nrf2) plays an essential role in the cellular antioxidant defense system, mainly through the regulation of antioxidant and cytoprotective gene expression (14-16). Importantly, the Nrf2 system is linked to various signaling and metabolic pathways (17). Alfaris *et al.* demonstrated that the hippocampal and memory function may be mediated by crosstalk between Nrf2 and nuclear factor-κB (NF-κB) response (18). Moreover, evidence suggests that the Nrf2/NADPH oxidase 4/endothelial NO synthase modulation pathway is involved in preventing vascular oxidative stress (19). This integration of Nrf2 activity with multiple cellular networks makes Nrf2 a critical target in the pathogenesis and pharmacotherapy of many diseases. In the context of depressive disorders, preclinical and clinical results support the hypothesis that downregulation of the Nrf2 pathway and modulation of Nrf2 activity may be new molecular targets in the development of psychiatric disorders (14). Indeed, the regulation of the GSK3-Nrf2 axis has been related to the pathophysiology of suicide (20), and Nrf2 activators such as sulforaphane induce antidepressant-like activity and suppress oxidative stress (21-23). Desipramine and duloxetine (tricyclic antidepressant (TCA) and selective serotonin and norepinephrine reuptake inhibitor (SNRI), respectively) have antioxidant properties through Nrf2 upregulation (24). Interestingly, Nrf2 activation is important for protection against glutamate toxicity (25). It has recently been reported that the kynurenine pathway (KP) is related to Nrf2 downregulation and may lead to the development of depressive symptoms (26). Increased concentrations of quinolinic acid (QA), a metabolite of KP, a potent agonist of the ionotropic glutamate N-methyl-D-aspartate receptor (NMDAR), and dysregulation of KP enzymes have been observed in suicidal patients (27). Similarly, the intracellular regulation of KP is associated with the modulation of NMDARs structure and function (28). In fact, all KP metabolites act on NMDARs (29). For example, QA binds to the glycine site in the NMDAR complex and consistently agonizes it. In turn, kynurenic acid (KA) binds to the glutamate recognition site of NMDARs and induces antagonistic effects (26). Zinc and magnesium (NMDA receptor antagonists) are also important for the synaptic modulation of NMDAR function (30). Numerous studies have linked zinc and magnesium interactions with the glutamatergic system (including NMDA receptors regulation) in the pathogenesis of depressive disorders (31-33). Moreover, our previous *post-mortem* study showed a statistically significant decrease in the potency of zinc and magnesium to inhibit [^{3}H] MK-801 (a radioactively labeled form of MK-801 (dizocilpine), a well-characterized NMDAR channel uncompetitive antagonist frequently used in receptor-binding assays) binding to NMDA receptors in the HP of suicide victims (30). Importantly, knowledge of the molecular mechanisms involved in NMDA receptor-mediated oxidative stress remains insufficient. In the molecular background, there is little evidence of a potential relationship between Nrf2 regulation and NMDA receptor function in antioxidant responses. Additionally, no published study has evaluated this mechanism in the pathophysiology of suicide-related disorders.

Therefore, our study examined whether the selected intracellular elements of the antioxidant response are potentially

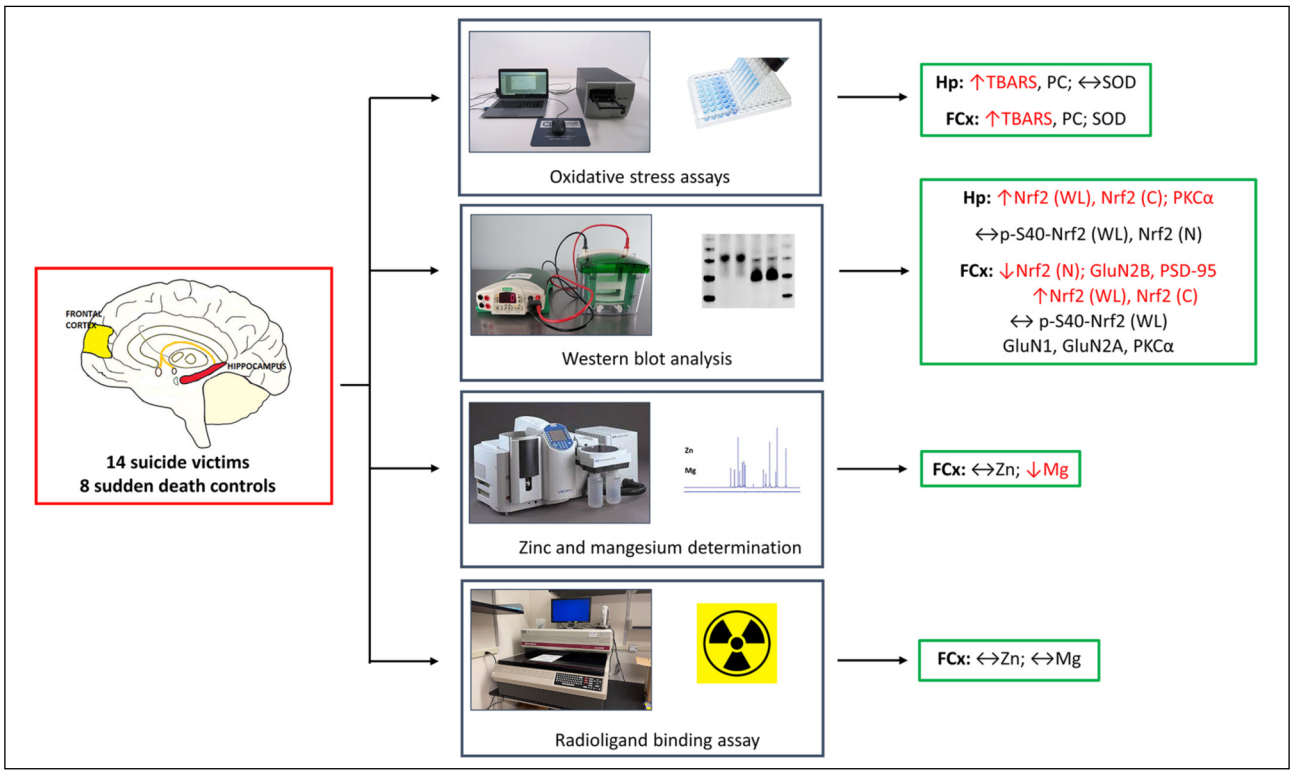

Fig. 1. Flow chart of analyses and major outcomes of this study.

related to changes in Nrf2 regulation and NMDA receptor function in the hippocampus and frontal cortex of suicide victims. In particular, this study assessed, for the first time the possible relationship between mediators of oxidative stress (protein carbonyl content (PC), TBARS, SOD, the Nrf2 pathway (Nrf2, p-S40-Nrf2), and modulation of NMDA receptor subunit composition (GluN1, GluN2A, GluN2B, PSD-95, PKCα, zinc, and magnesium)) in the pathophysiology of suicidality.

MATERIALS AND METHODS

Human subjects and tissue collection (Fig. 1)

Brain tissues (hippocampus (Hp) and frontal cortex (FCx) - Brodmann area 10) were obtained from two groups of subjects: psychiatrically non-diagnosed suicide victims (n=14) and unexpected sudden death controls (n=8) at the time of autopsy in the Department of Forensic Medicine, Jagiellonian University Medical College. All the collected samples were frozen and stored at –80ºC until the start of scheduled analyses.

The ethics committee approved a waiver of informed consent for this study knowing that participants will not and cannot be identified (we only have the data disclosed in our previous report, (34)) and consented to the collection of tissues and their use in this study (approval number 7/2000 of July 20, 2000).

Among the 22 study subjects were 8 females and 14 males. The mean age (±SEM) in the suicides (29.21±3.594) had no significant differences from the control group (31.0±4.89). According to available medical history, both suicides and controls included in the study were not treated for any chronic central nervous system disorders. It is known that 3 cases of suicide death were a consequence of drug overdose, but these people were not taking any medication permanently, including psychotropic. More detailed characteristics of each subject are presented in *Table 1*.

Oxidative stress assays

According to the manufacturer's protocols, oxidative parameters were determined in the whole tissue lysates using commercially available kits. The protein and lipid peroxidation levels were measured using a Protein Carbonyl Colorimetric Assay Kit or TBARS Assay kit (Cayman Chemical, Ann Arbor, MI, USA), respectively. Protein Carbonyl Colorimetric Assay Kit was based on a reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls in which the final product was analyzed spectrophotometrically at 385 nm. The concentration of carbonyls in each sample was calculated according to the manufacturer's recommendations (cat. 10005020, Cayman Chemical). The range of measured concentrations was 0.79-3.06 nmol/ml. In turn, to determine lipid peroxidation, the malondialdehyde (MDA)-thiobarbituric acid (TBA) adduct was measured fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Concentration values for each sample were calculated from the standard curve

Table 1. Demographic characteristics of controls and suicide subjects. Based on Ref. (34).

	Age (years)	**Sex**	**Cause of death**
Controls			
1	17	M	Cranial/brain injure
2	44	F	Road accident
3	20	M	Carbon monoxide poisoning
4	54	M	Myocardial infarction
5	29	M	Homicide
6	42	M	Myocardial infarction
7	21	F	Homicide
8	21	F	Road accident
Mediana	*25.0*		
Mean	*31.0*		
SEM	*4.89*		
Suicides			
1	33	M	Hanging
2	29	M	Hanging
3	21	F	Hanging
4	17	M	Hanging
5	47	M	Hanging
6	19	M	Jump under train
7	21	F	Self-poisoning/drug overdose (doxepine + clomipramine)
8	29	F	Jumping
9	20	M	Self-poisoning/drug overdose (hydroxyzine + perazine)
10	55	M	Hanging
11	20	F	Self-drowning
12	55	F	Self-poisoning/drug overdose (diazepam + ethanol)
13	24	M	Jumping
14	19	M	Hanging
Mediana	*22.5*		
Mean	*29.2*		
SEM	*3.59*		

F, female; M, male.

(y=0.87050x + 144.1; r^2=0.999) according to the manufacturer's protocol (cat. 10009055; Cayman Chemical, Ann Arbor, MI, USA). The range of measured concentrations was 0.068-0.184 μM. The activities of SOD, as an endogenous antioxidant, were measured with the use a of Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The SOD Assay Kit uses a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzymes needed to exhibit 50% of the dismutation of the superoxide anions (U/ml). The absorbance was measured at 450 nm. The SOD activity in each sample was calculated according to the manufacturer's instructions (cat. 706002; Cayman Chemical, Ann Arbor, MI, USA) using the equation

(y=70.787x + 0.9916; r^2=0.999)

obtained from the linear regression of the standard curve. The range of measured activities was 0.027–0.043 units/ml.

Finally, the concentrations of carbonyls and TBARS as well as SOD activity in whole tissue lysates were converted into protein concentrations (nmol/mg; uM/mg and U/mg of protein; respectively).

Western blot analysis

Most of the Western blot analyzes were performed in the whole tissue lysates (homogenates), while the remaining -in selected tissue fractions, i.e. cytosolic and nuclear. The expression level of Nrf2, p-S40-Nrf2, GluN2A, GluN2B, PSD-95, and PKCα in whole tissue homogenates and Nrf2 in cytosolic and nuclear fractions from post-mortem brain samples were investigated.

Whole tissue lysates. The brain samples [in a 2% aqueous sodium dodecyl sulfate (SDS) solution; ratio: 50 mg tissue:1000 μL SDS] were homogenized, denatured (10 min, 95ºC), and finally centrifuged (5 min at 10000 × g at 4ºC). The clear supernatant was then transferred to new tubes, to obtain a complete tissue homogenate.

Tissue fractions. Cytosolic and nuclear fractions were obtained according to the procedure described by (35). Briefly, the brain samples were homogenized in the isotonic homogenization buffer (0.32 M sucrose, 10mM HEPES (pH=7.4), 2 mM EDTA, protease (BioShop, Burlington, Canada) and phosphatase (Sigma-Aldrich, Steinheim, Germany) inhibitors) at a ratio of 50 mg tissue to 1000 μl buffer and next centrifuged (760 × g, 10 min, 4ºC). The resulting supernatant (S1) was transferred to fresh tubes, and the pellet was resuspended in a homogenization buffer and centrifuged again (760 × g, 10 min, 4ºC). The obtained pellet (nuclear fraction) was suspended in the fraction concentration buffer (0.32 M sucrose, 10 mM HEPES, 2 mM EDTA, 0.5% Triton X-100, 1% SDS, protease and phosphatase inhibitors), while the supernatant (S2) was pooled with S1 and centrifuged at 10000 × g (15 min, 4ºC). The resulting supernatant (cytosolic fraction) was transferred to new tubes. In order to verify the correctness of the fractionation procedure and the purity of the obtained fractions, marker verification (with the use of specific antibodies; Hsp90 as a marker of cytoplasmic proteins and HDAC2 for nuclear fraction) was performed.

Immunoblotting. Immunoblotting was performed according to our standard lab protocol (30, 34, 36). First total protein concentration in the prepared samples (whole tissue lysates and tissue fractions) was determined using the bicinchoninic acid method (Pierce Biotechnology, Waltham, MA, USA). Next, the samples containing 50 μg (whole tissue lysates) and 20 μg (tissue fractions) of total protein and loading buffer were fractioned by 8–10% (depending on the molecular weight of the tested protein) SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Feldkirchen, Germany). After incubation in the 1% blocking solution (BM Chemiluminescence Western Blotting Kit (mouse/rabbit); Roche, Basel, Switzerland), and next with the respective primary antibodies (*Table 2*), the membranes were washed with TBS-T. In order to visualize specific proteins, the membranes were incubated with the secondary antibody (goat anti-rabbit/anti-mouse IgG peroxidase-conjugated antibodies; dilution: 1:20.000; Bio-Rad, Feldkirchen, Germany) and after washing in TBS-T (Tris-buffered saline with 0.1% Tween20) developed using enhanced chemiluminescence reaction. The Nrf2, p-S40-Nrf2, GluN1, GluN2A, GluN2B, PSD-95, and PKCα signals were visualized with Syngene G-Box Chemi XT4 and quantified with the GeneSys software. The density of each protein band was normalized to the density of the β-actin band (control for transfer and loading).

Zinc and magnesium determination

Determination of zinc and magnesium was carried out by flame atomic absorption spectrometry (FAAS) method (Pye Unicam SP-9 800 AA Spectrophotometer with deuterium (D2) background correction (Cambridge, UK). The Hp and FCx were

Table 2. The list of primary antibodies (with regard to the applied dilutions) used in the study.

Antibody	Host species	Clonality	Cat. number	Dilution factor	Company
anti-GluN1	Mouse	Monoclonal	32-0500	1:2000	Invitrogen, Carlsbad, CA, USA
anti-GluN2A	Rabbit	Polyclonal	Ab14596	1:1000	Abcam, Cambridge, UK
anti-GluN2B	Rabbit	Polyclonal	Ab65783	1:1000	Abcam, Cambridge, UK
anti-PSD-95	Rabbit	Monoclonal	Ab76115	1:1000	Abcam, Cambridge, UK
anti-PKCα	Mouse	Monoclonal	Sc-8393	1:200	Santa Cruz Biotechnology, Dallas, TX, USA
anti-Nrf2	Rabbit	Monoclonal	12721S	1:1000	Cell Signaling Technology, Danvers, MA, USA
anti-p-S40-Nrf2	Rabbit	Polyclonal	12811	1:1000	SAB-Signalway Antibody, Greenbelt, MD, USA
anti-β-actin	Mouse	Monoclonal	A5441	1:10000	Sigma-Aldrich, St. Louis, MO, USA

weighted (0.018–0.198 g) and digested in 1.5 ml of concentrated nitric acid (SupraPur, Merck, Darmstad, Germany). Brain tissue samples were mineralized using water bath for 5 h at 80ºC. After cooling the samples, demineralized water was added to the total volume of 5 ml. FAAS measurement conditions were the following: compressed air flow 4.5 l/min; acetylene flow 1.1 l/min; analytical wavelength 213.9 nm for zinc and 285.2 nm for magnesium; slit width 0.5 nm; time of measurement 5 s for every sample. The method's relative standard deviation (RSD) (the whole analytical procedure: digestion + zinc/magnesium determination) did not exceed 2.4%. For preparing the standard solutions a standard Tritisol solution (Merck, Darmstad, Germany) was used. The calibration curve was in the range of 0–50 µg (for zinc) and 0–200 µg (for magnesium). The accuracy of

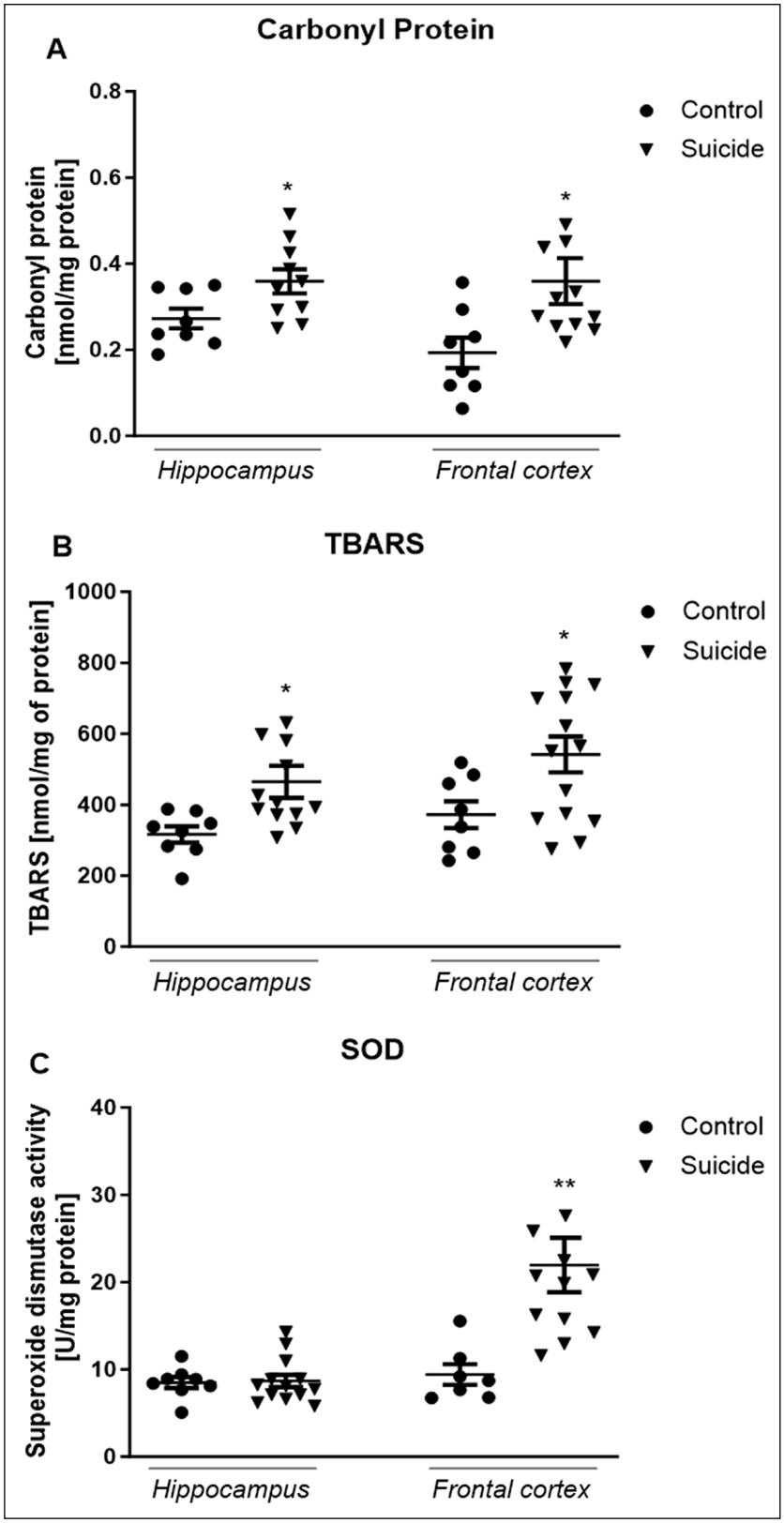

Fig. 2. Alterations in the oxidative stress parameters: protein carbonyl (A) and thiobarbituric acid reactive substances - TBARS (B) levels, and superoxide dismutase (SOD) activity (C) in the hippocampus and frontal cortex of suicides (n=14) in relation to the sudden death controls (n=8). The values are expressed as mean ±SEM. *p<0.05; **p<0.001 vs. Control.

the method was verified (in each series of assessments) against an international standard, Seronorm (Nycomed, Oslo, Norway). The results are presented in µg/g wet weight of brain tissue.

Radioligand binding assay

Radioligand binding assay was performed as described previously by (30). In short, frozen cortical tissues were homogenized in ice-cold 5 mM HEPES/4.5 mM Tris buffer (HTS, pH 7.4) and centrifuged at 20000 × g for 20 min (4ºC). The resulting pellets were resuspended in HTS containing 1 mM EDTA and then centrifuged at 20,000 × g for 20 min. The obtained tissue pellet was resuspended once more in HTS (plus 1mM EDTA) and then centrifuged at 20000 × g for 20 min.Obtained pellets were resuspended in 5 volumes of fresh HTS and stored at –70ºC for at least 3 days before assay. Just before the assay, the frozen aliquots were thawed in 20 volumes of HTS and centrifuged at 20000 × g for 20 min. Radioligand binding assays were performed in microtiter plates (MultiScreen, Millipore, Bedford, MA, USA). The final volume (300 µl) of incubation mixture consisted of: 240 µl membrane suspension (~50 µg of protein), 30 µl of a 5 nM [^{3}H]MK-801 (28.8 Ci/mmol; PerkinElmer, Waltham, MA, USA) and 30 µl buffer containing six concentrations (ranged 10^{-2}–10^{-7} M) of zinc or magnesium. Nonspecific binding was assessed using 100 µM phencyclidine. The assay mixture was incubated for 2 h at 25ºC and terminated by rapid vacuum filtration over glass fiber filters. The filters were then washed 2 times with 0.1 ml of ice-cold HTS, placed in scintillation vials with 4 ml of liquid scintillation cocktail and bound radioactivity was measured in Beckman LS-6500 scintillation counter.

Data analysis

The data were evaluated using GraphPad PRISM software (ver. 8, San Diego, CA, USA). Oxidative stress parameters were normalized to total protein concentration. Western blot results are presented as Nrf2, p-S40-Nrf2, GluN1, GluN2A, GluN2B, PSD-95, PKCα/β-actin ratio. Radioligand binding data were analyzed using iterative curve fitting routines. All results are presented as means ±SEM (standard error of the mean). The Shapiro-Wilk test was performed in order to evaluate the normal distribution of quantitative data. Because all data showed the normal distribution, group differences were assessed using an unpaired Student's t-test. $p<0.05$ was considered as statistically significant.

RESULTS

Concentration or/activity of the oxidative stress parameters

The levels of oxidative stress markers in the hippocampus and frontal cortex of the suicides and control group are shown in *Fig. 2*.

The carbonyl proteins level was significantly higher in the suicide group than in the appropriate control both in the hippocampus and frontal cortex (0.359±0.028 vs. 0.273±0.023; t(20)=2.317, p=0.0341 and 0.360±0.053 vs. 0.193±0.035; t(20)=2.394, p=0.0285, respectively) (*Fig. 2A*). The TBARS concentration was also statistically increased in the suicide victims (by ~47% in the hippocampus and ~49% in the frontal cortex) compared to sudden death subjects (t(20)=2.512, p=0.022 and t(20)=2.316, p=0.031, respectively) (*Fig. 2B*). While, sodium dismutase (SOD) activity was only significantly elevated in the frontal cortex of suicides compared to the those obtained in the controls (21.96±3.122 vs. 9.415±1.183; t(20)=2.965, p=0.009). In the hippocampus, there are no alterations in the SOD activity (1.239±0.023 vs. 1.217±0.029; t(20)=0.600, p=0.555) (*Fig. 2C*).

Levels of the Nrf2 and phospho-S40-Nrf2 in hippocampus and frontal cortex of suicide victims and control subjects

Quantitative results and representative immunoreactive bands corresponding to Nrf2 (100 kDa), p-S40-Nrf2 (90 kDa)

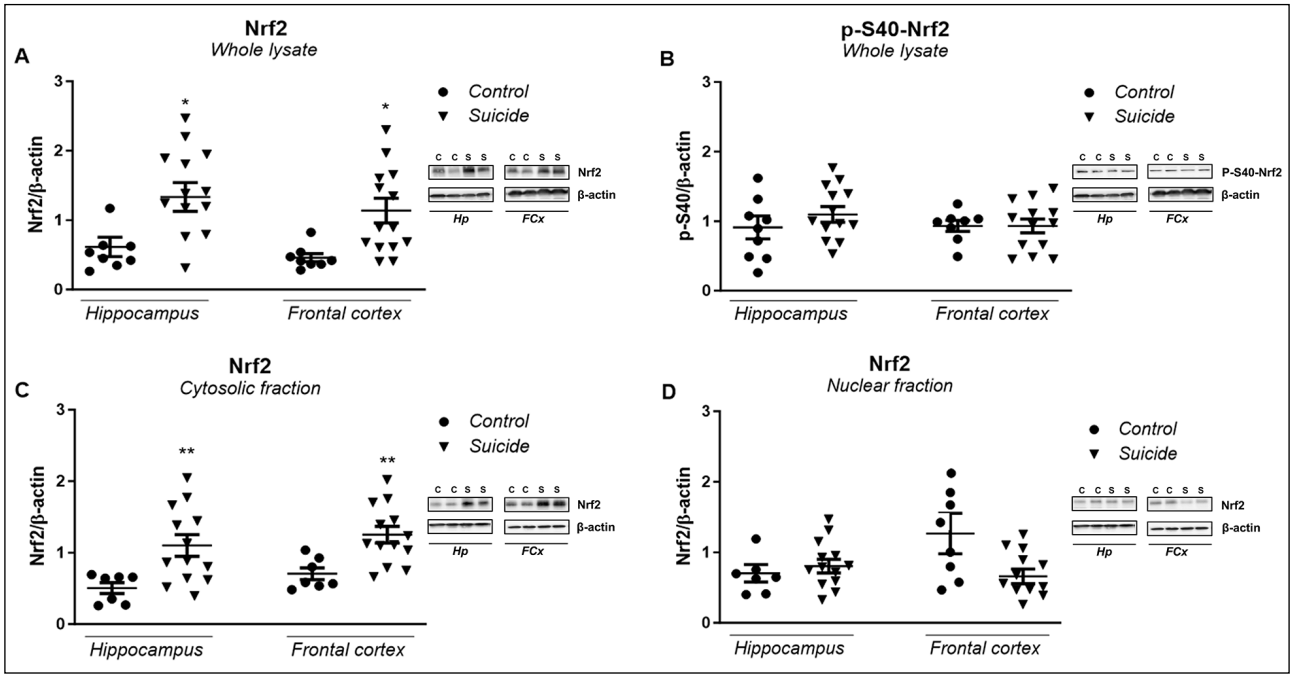

Fig. 3. Alterations in the Nrf2 and p-S40-Nrf2 protein levels in the whole lysates (A and B, respectively) and Nrf2 protein level in the cytosolic (C) and nuclear (D) fractions of the hippocampus and frontal cortex of suicides (n=14) compared to the controls (n=8). The values (mean ±SEM) represent normalized (to β-actin) optical density. *p<0.05; **p<0.01 vs. Control.

and adequate β-actin (42 kDa) protein in the whole lysates, cytosolic and nuclear fractions are presented in *Fig. 3*.

In WL, a significant increase in Nrf2 level in the suicide group than in the appropriate control both in Hp and FCx was noted (1.138±0.178 vs. 0.459±0.059; t(20)=2.809, p=0.011 and 1.333±0.207 vs. 0.613±0.140; t(20)=2.649, p=0.017] (*Fig. 3A*). Importantly, various changes in the level of total Nrf2 protein were found in the cellular fractions. In the cytosolic fraction, significantly elevated levels, both in Hp (by 118%; t(20)=3.972; p=0.009) and FCx (by 79%; t(20)=3.629, p=0.003) were noted (*Fig. 3C*). On the contrary, in the nuclear fraction, a statistically significant decrease in Nrf2 protein level was revealed, but only in FCx (0.659±0.104 vs. 1.266±0.286; t(20)=2.272, p=0.038), not in Hp (0.803±0.096 vs. 0.702±0.124; t(20)=0.640, p=0.531) (*Fig. 3D*). Simultaneously no alterations in the phosphorylated form (on serine 40) of Nrf2 protein was observed (*Fig. 3B*).

NMDA receptor composition: levels of the GluN1, GluN2A, GluN2B and PSD-95 in FCx, and PKCα in frontal cortex as well as hippocampus of suicide victims and control subjects

Quantitative results and representative immunoreactive bands corresponding to GluN1 (103 kDa), GluN2A (177 kDa), GluN2B (178 kDa), PKCα (80 kDa) and adequate β-actin (42 kDa) protein in the whole lysates are presented in *Fig. 4* and *Fig. 5*.

The levels of GluN1 (2.423±0.333; *Fig. 4A*) and GluN2A (2.499±0.319; *Fig. 4B*) in FCx of suicide victims were not statistically different from the control group (2.600±0.281; t(20)=0.384, p=0.706 and 1.983±0.382; t(20)=1.007, p=0.326, respectively). Simultaneously, a significant reduction (by approx. 48%) in GluN2B (*Fig. 4C*) and PSD-95 (by approx. 49%; *Fig. 4D*) in suicides compared to the matched controls was observed (t(20)=2.916, p=0.009 and t(20)=3.231, p=0.006, respectively).

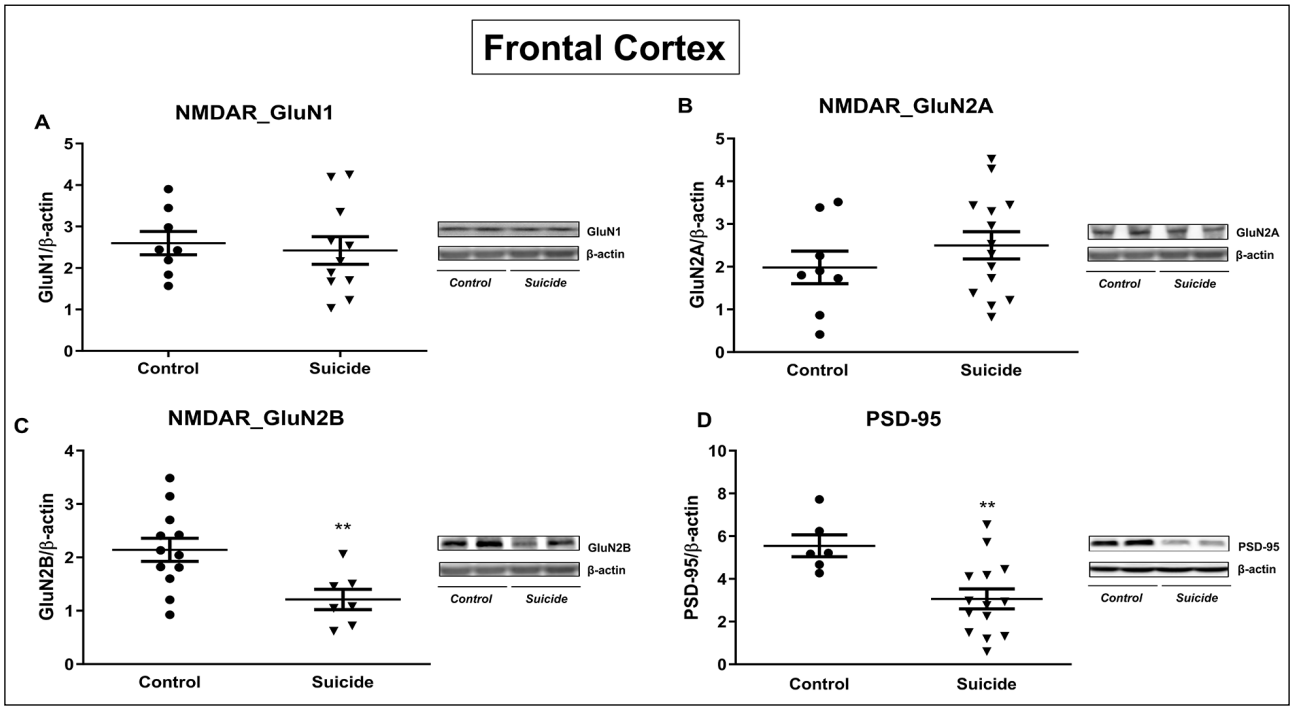

Fig. 4. Alterations in the protein levels of GluN1 (A), GluN2A (B) and GluN2B (C) subunit of the NMDA receptor as well as PSD-95 (D) in the whole lysates of the frontal cortex of suicides (n=14) compared to the controls (n=8). The values (mean ±SEM) represent normalized (to β-actin) optical density. **p<0.01 vs. Control.

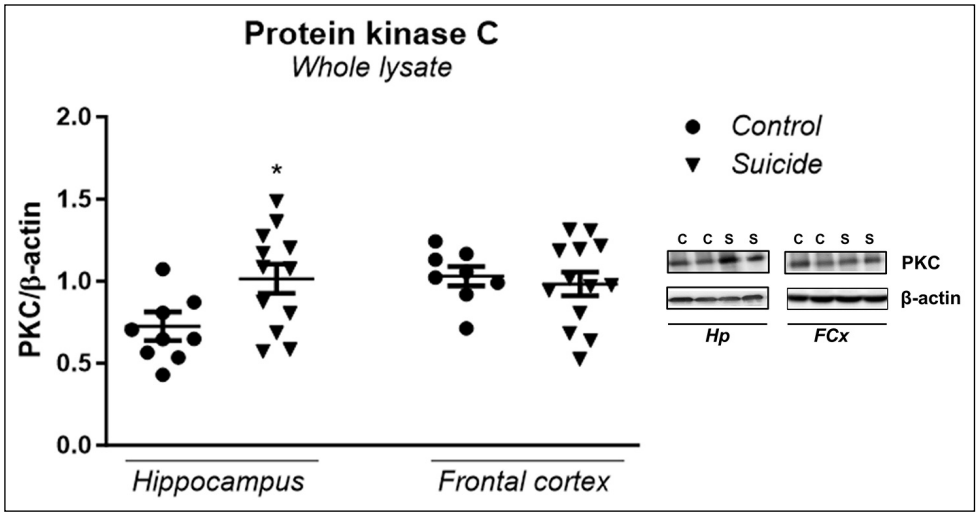

Fig. 5. Alterations in the PKCα protein level in the whole lysates of the hippocampus and frontal cortex of suicides (n=14) compared to the controls (n=8). The values (mean ± SEM) represent normalized (to β-actin) optical density. *p<0.05 vs. Control.

Table 3. Tissue concentration of zinc and magnesium ions in the frontal cortex of suicide victims and matched controls.

Group	***n***	**ZINC**	**%**	**MAGNESIUM**	**%**
Control	*8*	10.27 ± 0.35	100	129.6 ± 3.5	100
Suicide	*14*	10.84 ± 0.36	105	**116.4 ± 2.5****	**89**

Data are expressed in micrograms Zn^{2+} or Mg^{2+}/g tissue and represent mean ± S.E.M. **p=0.01 vs. Control.

Table 4. The potency of zinc and magnesium to inhibit [^{3}H] MK-801 binding to the NMDA channel in the frontal cortex of suicide victims and matched controls.

Groups	**IC_{50}** [mM]	**%**	**Specific binding** [pmol/g tissue]
Zinc			
Control	0.271 ± 0.021	100	3.456 ± 0.250
Suicide	0.303 ± 0.014	112	3.538 ± 0.205
Magnesium			
Control	0.810 ± 0.128	100	3.541 ± 0.326
Suicide	0.960 ± 0.066	118	3.524 ± 0.202

Data are expressed as IC_{50} (mM) values and represent mean ±S.E.M (n=8 controls and 14 suicides).

Moreover, approx. 40% increase in PKCα level in suicides in relation to the control group was revealed but only in Hp (1.014±0.088 vs. 0.728±0.087; t(20)=2.278, p=0.034), not in FCx (0.982±0.072 vs. 1.030±0.058; t(20)=0.465, p=0.647) (*Fig. 5*).

Zinc and magnesium measurement

The concentration of magnesium (Mg^{2+} µg/g tissue) in the frontal cortex of suicide victims (116.4±2.5) was significantly decreased (by 11%) compared to matched controls (129.6±3.5, t(21)=2.857, p=0.009). Instead, there are no alterations in zinc level (Zn^{2+}µg/g tissue; 10.27±0.35 vs. 10.84±0.36, t(21)=0.882, p=0.388) (*Table 3*).

The impact of zinc and magnesium on [^{3}H]MK-801 binding to the NMDA receptor

To study the influence of Zn^{2+} and Mg^{2+} on [^{3}H]MK-801 binding to the NMDA receptor channel we used the radioligand receptor binding assay. MK-801 (dizocilpine) is a well-characterized NMDAR channel uncompetitive antagonist and its radioactively labeled form is frequently used in receptor-binding assays. This investigation was carried out on extensively washed neuronal membrane preparations from the human frontal cortex samples. The obtained data are expressed as IC_{50} (mM) values and represented as mean ±SEM.

As shown in *Table 4*, there were no alterations in the IC_{50} value of zinc (0.303±0.014 vs. 0.271±0.21; t(20)=1.197, p=0.245) and magnesium (0.810±0.128 vs. 0.960±0.066; t(20)=1.118, p=0.276) inhibition of [^{3}H]MK-801 binding to NMDA receptors between the control and suicide tissue, respectively. There were also no specific [^{3}H]MK-801 binding changes between the study groups.

DISCUSSION

A significant finding of the present study is that suicidal behavior is related to increased oxidative stress in both the Hp and FCx of the post-mortem brain. We report that the carbonyl protein level was significantly higher in the Hp and FCx of suicide victims than in sudden death controls. We also found an increase in TBARS concentration in the Hp and FCx of the suicidal group. These alterations were associated with the increased SOD activity observed in the FCx (but not in the Hp) of suicides. It is well known that oxidative stress and dysfunction of the antioxidant defense system play an essential role in the pathophysiology of depressive disorders (37-41). This molecular mechanism may also be critical for suicidal behavior (considering the observed relationship between depression and suicide). The link between oxidative stress and suicide attempts in patients with depression is poorly understood. Moreover, the number of post-mortem brain studies that have evaluated oxidative stress parameters in suicide or depressive disorders is limited (11, 12). Most of them, have analyzed the peripheral levels of selected oxidative stress biomarkers. Importantly, our findings are consistent with those of previous studies, which revealed that oxidative stress and lower total antioxidant levels might be involved in the pathophysiology of underlying suicidality (11, 13, 42). Furthermore, Vargas *et al.* reported a significantly higher level of lipid peroxidation in blood samples from individuals who attempted suicide (42). Increased TBARS concentrations have also been associated with suicidal ideation (39). Thus, the present results confirm the increase in lipid peroxidation in Hp and FCx of suicide victims. These data suggest the importance of oxidative stress in suicidal behavior and suicide-related disorders (such as depression). Nevertheless, a fundamental question arises regarding the potential molecular targets that link oxidative stress changes with the development of depressive disorders. Oxidative stress responses are regulated by different factors/proteins, among which Nrf2 is critical for mental disorders' cellular/molecular basis (15). Therefore, we investigated the Nrf2 protein level and phosphorylation of a specific serine residue (phospho-S40-Nrf2) in the Hp and FCx of suicide victims. Our study is the first to demonstrate that Nrf2 protein levels were significantly higher in whole homogenates of Hp and FCx in a group of suicide victims than in matched controls. In specific cellular subfractions, increased Nrf2 protein levels were observed in the cytosolic fractions of both brain structures. Importantly, we found decreased Nrf2 levels in the

nuclear fraction of FCx (but not Hp) in the suicidal group. In addition, no alteration in the phosphorylated form (serine 40) of Nrf2 was observed. Nrf2 signaling modulation has been analyzed in various animal models of depression (rodents with a depression-like phenotype). Martin-Hernandez *et al.* observed a statistically significant decrease in Nrf2 mRNA and protein expression levels in the prefrontal cortex (but not in Hp) of rats subjected to chronic mild stress procedures (CMS, widely used animal model of depression) (24). In turn, the chronic social defeat stress (CSDS) model has also indicated a lower Nrf2 protein level in the CA3, dentate gyrus of the hippocampus, and prefrontal cortex in mice with a depression-like phenotype (43). Zhang *et al.* observed a similar effect in a rat's learned helplessness (LH) model. Susceptible rats have decreased Nrf2 protein levels in the dentate gyrus of the hippocampus and prefrontal cortex (44). Studies focusing on the modulation of the Nrf2 pathway in the context of mental disorders in post-mortem human brain samples are limited. Zhang *et al.* show a significant decrease in Nrf2 expression in the parietal cortex collected from BD and MDD subjects (44). In addition, reduced Nrf2 expression in the dorsolateral prefrontal cortex of MDD patients was also noticed (45). These data suggest that the downregulation of Nrf2 (Nrf2 reduced gene expression or protein level) may be linked to stress resilience (especially evidence from animal models) and the pathophysiology of mental disorders (14, 46). Our study supports this hypothesis, as Nrf2 changes were found in the nuclear fraction of FCx. This alteration was associated with increased oxidative stress (PC and TBARS) parameters in suicidal brain tissue. Lower Nrf2 protein levels in suicides (FCx, nuclear fraction) probably led to a decrease in the capability of the cellular antioxidant defense system. Moreover, these results are consistent with the Nrf2 protein alterations observed in this brain region in MDD and BD patients in post-mortem studies (44, 45). In contrast, the observed increase in Nrf2 protein levels in whole homogenates of Hp and FCx of suicide victims may have resulted from compensatory mechanisms induced by oxidative stress. Furthermore, it cannot be excluded that under oxidative stress conditions, Nrf2 proteasomal degradation is enhanced. However, this aspect must be verified in future molecular studies. The profile of alterations suggests that Nrf2 function may be modulated and may involve the same molecular pathways in suicidal behavior as those in mental disorders. Interestingly, in the nuclear fraction (Hp), there were no changes in Nrf2 protein levels in suicides. One possible explanation is that the molecular mechanisms that respond to oxidative stress in the hippocampus of suicide victims may be related to the neurotrophin hypothesis. Reduced BDNF protein levels in the Hp of suicides have been reported in our previous post-mortem study (34). From a methodological perspective, our study showed that the Nrf2 protein should be analyzed in selected subfractions (especially in the nucleus). Researchers have left this issue in the methodology section multiple times. Initial data suggest that phosphorylation of Nrf2 at serine (Ser-40) may be crucial for its activation and signal transduction of antioxidant response element-mediated transcription (47). Accumulating evidence shows that other Nrf2 phosphorylations (like 568 tyrosine, 408 serine, 215 serine) are also involved in the molecular activation of this factor (48). In the present study, no changes in phospho-S40-Nrf2 protein levels were observed both in Hp or FCx of suicides. It is possible that in suicidal behavior, phospho-S40-Nrf2 is not required for Nrf2 stabilization/accumulation and that another type of Nrf2 phosphorylation is more important.

The relationship between NMDA function and oxidative stress has been proposed to be a promising target in the molecular neurobiology of psychiatric disorders. Redox NMDARs modification may be important for cognitive deficits or memory impairment observed during aging or Alzheimer's disease progression (49, 50). In fact, patients with depressive disorders and suicidal ideation often present cognitive dysfunction in the clinical picture (51, 52). Currently, it is still unclear whether NMDAR complex modulation (decrease in NMDAR function) in suicide-related disorders (including depression) represents a direct consequence of oxidative stress or whether these alterations are linked to the Nrf2 pathway. Based on the Nrf2 changes observed in the FCx of suicide victims, we assessed the NMDAR receptor composition: GluN1, GluN2A, GluN2B, PSD-95 and PKCα protein levels in this brain region. Our results demonstrated a significant decrease in the GluN2B and PSD-95 protein levels in the FCx of suicide victims relative to controls, with no change in the PKCα, GluN1 and GluN2A. These findings showed a similar direction of biochemical changes as documented in the prefrontal cortex (PFC) of subjects with MDD (53). The PSD-95 protein is a synaptic scaffolding protein (a component of the postsynaptic density at excitatory synapses) critical for the trafficking and internalization of the NMDA receptor complex (54, 55). The potential role of postsynaptic proteins at excitatory synapses (including PSD-95) in the pathogenesis and pharmacotherapy of depressive disorders has been disused in our review paper (54). In the present study, the reduction of PSD-95 protein levels in cortical samples of suicide victims may have led to decreased expression of GluN2B NMDA subunits. These changes can also be associated with the development of NMDAR dysfunction. Therefore, in the next step, we investigated zinc and magnesium (inorganic NMDAR antagonists) concentrations and their potency to inhibit [^{3}H] MK-801 binding to NMDA receptor channels. We found, significantly lower concentrations of magnesium (but not zinc) in FCx of suicide, with no alterations in the potency of zinc and magnesium in inhibiting [^{3}H] MK-801. This may also explain the dysfunction of NMDAR (particularly GluN2B subunits). Clinical results have proven that hypomagnesemia is linked to depression (56). According to the radiological assay, the observed effects can be attributed to the analyzed brain regions. In contrast, our previous *post-mortem* study indicated a decrease in the potency of zinc and magnesium in inhibiting [^{3}H] MK-801 in the Hp of suicides ((30), *Table 5*). The present and previous data confirm that alternations in the NMDA receptor complex are involved in the psychopathology of suicide (30, 57). It cannot be ruled out that NMDAR-mediated oxidative stress responds *via* the Nrf2 signaling pathway. Zhang

Table 5. Summary of previous findings (changes in suicides compared to controls) obtained in the hippocampus of the same cohort. Based on Ref. (30).

	Observations
Protein levels	↑GluN2A; ↓GluN2B; ↓PSD-95
Ions concentrations	↔Zn; ↓Mg
The potency (expressed as IC_{50}) of zinc and magnesium to inhibit [^{3}H] MK-801 binding to the NMDAR	↑Zn; ↑Mg

et al. show that curcumin (naturally polyphenol which activates Nrf2) prevents ketamine (NMDAR antagonist) induced oxidative stress damage (58). Quinolinic acid induces pro-oxidant effects through Nrf2 modulation (26). In fact, the NMDAR-Cdk5-Nrf2 pathway has been suggested as an important target for astrocyte NMDA receptor activity (59).

In summary, this study is the first to describe alterations in Nrf2 levels in suicide victims under conditions of oxidative stress and NMDA receptor arrangement/dysfunction. These results contribute to a better understanding of the role of Nrf2 in the molecular mechanisms underlying suicidality. It can also initiate new directions of neurobiological research to search for novel targets for the development and treatment of suicide-related disorders. However, we are fully aware of the limitations of this study, mostly related to the subject's description. According to the demographic characteristics, we had no knowledge of psychiatric disorders among the suicide group. We only have information that suicide victims included in the study were not treated with antidepressants. Three of the fourteen suicide victims died from a drug overdose (in the following combinations: doxepin + clomipramine; hydroxyzine + perazine; diazepam + ethanol). The results from these three subjects did not indicate statistically significant differences when compared with the other 11 tissues. Therefore, our study proposes a hypothesis of Nrf2 regulation in suicidal behavior or suicide-related disorders. It is well-established that depressive disorders increase the risk of suicide. An increasing number of studies have shown a similar direction of biochemical changes in the FCX of patients with MDD and suicide victims (6, 60). Lack of information on potential comorbidities, post-mortem interval (PMI), or tissue pH is also a limitation of this type of study performed in subjects who die by sudden (suicidal) causes. Finally, the small size of the groups (particularly the control group) and gender disparities in the groups made it impossible to analyze the changes, taking into account gender differences.

Abbreviations: FCx, frontal cortex; GluN1, N-methyl-D-aspartate receptor subunit 1; GluN2A, N-methyl-D-aspartate receptor subunit 2A; GluN2B, N-methyl-D-aspartate receptor subunit 2B; Hp, hippocampus; NMDAR, N-methyl-D-aspartate receptor; Nrf2, nuclear factor erythroid 2-related factor 2; PC, protein carbonyl; PKCα, protein kinase C alpha; p-S40-Nrf2, Nrf2 phosphorylated at serine 40; PSD-95, postsynaptic density protein-95 kDa; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

Informed consent statement: All the tissues used in the present study were collected (in the period 2001–2004) during an autopsy (in Poland referred to as the forensic medical autopsy), which takes place at the request of the prosecutor (without the consent of the patient or closest relatives), to determine the cause of death (legal basis: Article 209 Code of Criminal Procedure of 6 June 1997). During an autopsy, it is permissible to collect tissues for research, however, at that time (in Poland), there was no legal regulation regarding the use of such tissues for research. Therefore, people interested in collecting these tissues and using them in scientific research applied (before storing and using tissues) to the Ethics Committee (legal basis: regulation of the Minister of Health and Social Welfare of 11 May 1999 on detailed rules for the appointment and financing as well as the mode of operation of bioethics committees) at the Institute of Pharmacology of the Polish Academy of Sciences in Cracow for approval for such activities. The Ethics Committee agreed (on July 20, 2000) to collect tissues and use them in research. The use of these tissues for research is also compliant with current legal regulations (Article 4 and 5, ACT of July 1, 2005 on the collection, storage and transplantation of cells, tissues and organs). In summary, the use of human material in this study (despite the lack of express consent of individual patients or their closest relatives) was consistent with Polish law and the code of ethics (both in 2001–2004 period and currently in force).

Authors' contribution: P. Panczyszyn-Trzewik: conducted Western blot analyzes and oxidative stress assays, participated in data analysis, prepared some of the figures and participated in the preparation of the first version of the manuscript; P. Misztak: conducted Western blot analyzes and oxidative stress assays; W. Opoka: conducted Zn concentration measurement; G. Nowak: contributed to the revising of the last version of the manuscript; M. Sowa-Kucma: initiated and supervised this study, designed experiments, participated in Western blot studies, conducted radioligand binding assay, analyzed the data, prepared some figures as well as created the final version of the manuscript.

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