
SELECTIVE INDUCTION OF MITOCHONDRIAL GENES IN THE PARAHIPPOCAMPAL BUT NOT IN THE ENTORHINAL CORTEX OF SUICIDE VICTIMS

by

TAMARA HAJDU

supervisors:

FANNI DÓRA

Research fellow

Semmelweis University

Human Brain Tissue Bank

Department of Anatomy, Histology and Embryology

DR. ÁRPÁD DOBOLYI, PhD, DSc

Professor

Eötvös Loránd University

Laboratory of Molecular and Systems Neurobiology

Department of Physiology and Neurobiology

EÖTVÖS LORÁND UNIVERSITY
FACULTY OF SCIENCE
INSTITUTE OF BIOLOGY



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ABSTRACT

Altered functional connectivity in human cortical networks has been reported in psychiatric disorders. One of these networks, the default mode network (DMN) is critical in mood disorders. Abnormal activity in the parahippocampal cortex (PHC), a moderating hub of the ventral DMN, has been reported in depressed patients and suicide attempters. Alterations in neuronal mitochondrial function may contribute to depression and suicidal behavior, however, little is known about the underlying molecular level changes in relevant structures. Specifically, expressional changes of mitochondrial genes have not been reported in the PHC related to suicide. Therefore, we addressed the protein level alterations of several mitochondrial genes including genes encoding tricarboxylic acid cycle enzymes in the PHC of suicide victims by reverse phase protein array (RPPA). Postmortem human brain samples were collected from 13 control and 11 suicide individuals. The entorhinal cortex (EC) was selected to serve as a control brain region because it is anatomically adjacent to the PHC but has distinct neuronal functions. RPPA analysis revealed that the protein levels of DLD, OGDH, SDHB, SUCLA2 and SUCLG2 were significantly induced in the PHC but not in the EC. With qRT-PCR, mRNA level changes of 3 out of the 5 genes were revealed suggesting transcriptional regulations. The observed changes support increased glutaminolysis in the PHC of suicide victims. It could increase energy supply of the neurons and support their increased activity during suicidal behavior.

LIST OF ABBREVIATIONS

ACC	anterior cingulate cortex
CN	caudate nucleus
DLD	dihydrolipoamide dehydrogenase
DMN	default mode network
EC	entorhinal cortex
ETC	electron transport chain
FWM	frontal white matter
HBTB	Human Brain Tissue Bank
KGDHC	alpha-ketoglutarate dehydrogenase complex
LTC	lateral temporal cortex
MDD	major depressive disorder
mPFC	medial prefrontal cortex
OGDH	oxoglutarate dehydrogenase
PCA	principal component analysis
PCC	posterior cingulate cortex
PDC	pyruvate dehydrogenase complex
PHC	parahippocampal cortex
PMI	postmortem interval
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
RPPA	reverse phase protein array
rs-fMRI	resting-state functional magnetic resonance imaging
SUCLA2	succinyl-CoA ligase ADP-forming subunit beta
SUCLG2	succinyl-CoA ligase GDP-forming subunit beta
TCA	tricarboxylic acid

1. INTRODUCTION AND AIMS

During the past decades, resting-state functional magnetic resonance imaging (rs-fMRI) provided information about the functioning of the resting brain in healthy and disordered brains. Among them, the default mode network (DMN) is considered a task-negative network of brain regions that deactivate during cognitive goal-directed tasks [1]. Seed-based resting-state functional connectivity divided the DMN into dorsal and ventral subsystems. The ventral subsystem consists of the lateral temporal cortex (LTC), hippocampal, parahippocampal, retrosplenial, and posterior parietal cortices while the dorsal subsystem consists of the medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), posterior cingulate cortex (PCC), and precuneus [2-6].

Structural and functional abnormalities have been identified in brain circuits involved in emotional processing in patients with major depression [7-9] and suicidal individuals [10-13]. These regions include the hippocampus, parahippocampal cortex (PHC), amygdala, ventral and dorsal mPFC, ACC, PCC and precuneus. The PHC is considered a key region of the limbic system and, together with the amygdala and hippocampus, is important in the operation of the reward system and plays essential roles in emotional regulation, motivation and cognitive control [14, 15]. Recall of the past suicide action was associated with increased activation in the ACC, mPFC and hippocampus, while recall of the mental pain when committing suicide was associated with the deactivation of frontal cortical regions and activation of the PHC [16].

A growing number of molecular investigations using high-throughput omics technologies have been carried out in recent years, however, relatively few examined the molecular alterations in individual regions of the human brain networks involved in psychiatric disorders. Previous studies have studied the proteomic and genomic differences in the parahippocampal and entorhinal cortices in neurodegenerative disorders [17-20], however, proteomic alterations related to depression and suicide have not been reported in these areas of the human brain. Microarray and RNA sequencing studies of psychiatric disorders reported on gene expression changes in glial, endothelial and mitochondrial functioning associated with major depression and suicide [21-25]. Mitochondrial dysfunction was highlighted as a crucial vulnerability factor for the development of depression [26-28] and it has also been proposed that mitochondrial dysfunction is associated with suicide completion [29, 30].

To better understand the connection between mitochondrial dysfunction and suicidal behavior, in the present study, we measured the protein expression of 11 tricarboxylic acid (TCA) cycle enzyme subunits in the parahippocampal cortex of suicide victims in comparison with controls using a microarray-based reverse phase protein array (RPPA) technology. We identified five differentially expressed subunits of TCA cycle enzymes between the control and suicide groups in the parahippocampal cortex but not in other brain areas.

2. MATERIALS AND METHODS

2.1. Human brain tissue samples

Human brain samples were collected in accordance with the Ethical Rules for Using Human Tissues for Medical Research in Hungary (HM 34/1999) and the Code of Ethics of the World Medical Association (Declaration of Helsinki). Tissue samples were taken during brain autopsy at the Department of Pathology, Forensic and Insurance Medicine of Semmelweis University and at the Clinical Centre of Pathology, University of Debrecen in the framework of the Human Brain Tissue Bank (HBTB), Budapest. The activity of the HBTB has been authorized by the Committee of Science and Research Ethic of the Ministry of Health Hungary (ETT TUKEB: 189/KO/02.6008/2002/ETT) and the Semmelweis University Regional Committee of Science and Research Ethic (No. 32/1992/TUKEB). The study was performed according to a protocol approved by the Committee of Science and Research Ethics, Semmelweis University (TUKEB 189/2015).

2.2. Tissue preparation

Postmortem human brain tissue samples from the parahippocampal and entorhinal cortices, frontal white matter and caudate nucleus were acquired from the HBTB (Semmelweis University, Budapest, Hungary). Samples were collected from 24 individuals with a postmortem delay of 1 to 10 h. The control group includes 13 subjects (5 females and 8 males, mean age of 50.6 ± 2.8) while the suicide group consists of 11 suicide victims (3 females and 8 males, mean age of 47.5 ± 4.5). The age of the subjects ranged from 35 to 67 years (Table 1). The selected control subjects were not diagnosed with any psychiatric disorder, while 9 out of 11 of the suicide victims were without evidence of acute or chronic depression. Samples were dissected from frozen sections between +10.0 and -10.0 mm from the origin of AC-PC coordinates (see [31]). Special microdissection needles with 8 and 15 mm inside diameters were used [32]. The parahippocampal and entorhinal cortical tissue pellets included both gray and white portions within the gyrus.

#Donor	Group	Sex	Age	Post mortem interval (PMI)	Cause of death	Clinical and pathological diagnosis
#1	Control	male	42	3.5 h	acute respiratory insufficiency	-
#2		male	47	1 h	cardiac insufficiency	chronic coronary sclerosis
#3		male	52	1.5 h	acute cardiac insufficiency	-
#4		female	67	5 h	respiratory insufficiency	brain tumor, decubitus ulcer, urinary tract infection
#5		female	26	6.5 h	acute cardiac insufficiency	acute myocardial infarction, stomach and small bowel ulcers, oedema cerebri
#6		female	56	6 h	cardiorespiratory insufficiency, oedema cerebri	oedema cerebri, coarctatio aortae, hepatitis alcoholica
#7		male	64	4 h	pneumonia	tumor radices linguae, coronary sclerosis, myocardial infarction, bilateral pneumonia, cerebral atherosclerosis, arteriosclerosis
#8		male	50	5.5 h	stroke, brain hemorrhage	large cortical and subcortical hemorrhage in the parietal lobe
#9		male	51	1 h	cardiorespiratory insufficiency	-
#10		male	53	2 h	acute cardiac insufficiency	acute myocardial infarction, oedema cerebri, ventricular hypertrophy
#11		female	56	5 h	myocardial infarction	-
#12		male	50	2 h	myocardial infarction	acute myocardial infarction
#13		female	44	5 h	acute cardiac insufficiency	hypertension, coronary atherosclerosis, myocardial infarction
#14	Suicide	male	71	1 h	suicide (jumping from a height)	without any clinical care during the past 6 months
#15		female	65	5 h	suicide (hanging - asphyxia)	pathological diagnosis: negative status (no pathological sign for any diseases)
#16		female	48	6 h	suicide (drug overdose)	-
#17		male	31	8 h	suicide (hanging - asphyxia)	without known drug treatment
#18		male	66	8 h	suicide (hanging - asphyxia)	without any clinical care
#19		male	43	4 h	suicide (hanging)	without any clinical care
#20		male	41	10 h	suicide (hanging)	without any clinical care
#21		male	48	6 h	suicide (hanging - asphyxia)	without known drug treatment
#22		male	35	6 h	suicide (hanging)	past suicidal behavior, under clinical care
#23		female	49	6 h	suicide (drug overdose)	without known drug treatment
#24		male	25	8 h	suicide (hanging)	no sign of any previous diseases, under psychiatric control, drug treatment: Risperdal (risperidone treatment), laboratory test: alcohol: positive

Table 1. Demographic and clinical data of the human samples.

2.3. Sample handling and reverse phase protein array (RPPA) measurement

We performed RPPA technology on cortical and subcortical samples to determine protein expression changes in the parahippocampal and entorhinal cortices, frontal white matter and caudate nucleus related to suicidal behavior. The key steps of this procedure are sample

preparation, antibody validation, printing samples, fluorescent signal detection, and data analysis [33]. Protein isolation from brain tissues and RPPA analysis were accomplished at the RPPA Facility hosted in the Department of Biochemistry of Semmelweis University with cooperation of project leader, Dr. Christos Chinopoulos. The procedure was carried out as described previously [34]. Briefly, the purification of proteins was carried out by Benzonase buffer combined with protease inhibitors. The protein concentration was adjusted to 1.0 mg/ml for each sample, then lysates were serially diluted in lysis buffer to produce four serial two-fold dilutions. Two technical replicates were then printed onto ONCYTE nitrocellulose slides (Sigma-Aldrich, cat. no. 705170) using an Aushon BioSystems 2470 microarrayer (Quanterix, Billerica, MA, USA). After washing and blocking procedures, slides were incubated with avidin-biotin and peroxidase blocking reagents (both VECTOR), then incubated with primary antibodies. Bound antibodies were detected by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Europe, Ely, UK). To amplify the signals, slides were incubated with amplification reagent (Bio-Rad, Watford, UK, cat. no. 1708230), then washed and probed with IRDye800CW-coupled streptavidin fluorophore. For normalization against total protein, an additional slide was prepared, and total protein was stained with Fast Green FCF (Sigma-Aldrich, cat. no. F7258). Slides were read using InnoScan 710-IR slide scanner (Innopsys) at an excitation wavelength of 785 nm for IRDye800CW and an excitation wavelength of 670 nm for Fast Green FCF. The relative fluorescence intensity of each sample spot was quantified using Mapix software (Innopsys). Nonspecific signals were determined by omitting the primary antibody incubation step. Processed signal intensities were normalized using the corresponding total protein stain signal intensity. Means of technical replicate sample spots were calculated, and data were written to a comma-separated values (CSV) file.

2.4. RPPA data analysis

Normalized RPPA data defined a log₂-transformed single expression value for each biological sample, based on the entire serial dilution curve and all technical replicates. The transformed normalized data was stored as matrices arranged in rows of different samples and columns of different antibodies. The median of all samples was calculated and this was subtracted from each element in that column. Matrices of antibody-wise median-centered data were horizontally integrated into a single matrix. Normalized and log₂-transformed data were written to a CSV file.

2.5. Gene expression analysis with qRT-PCR

The procedure was carried out as described previously [35]. Briefly, total RNA was isolated from approximately 20 mg of frozen postmortem brain tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as lysis buffer combined with RNeasy Mini kit (Qiagen, Germany) following the manufacturer's instructions. The quality and quantity of extracted RNA were determined using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of RNA was adjusted to 1000 ng/ μ L, and it was treated with Amplification Grade DNase I (Invitrogen, Carlsbad, CA, USA). The isolated RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). After 10-fold dilution, 2.5 μ L of the resulting cDNA was used as a template in PCR performed in duplicates using SYBR Green dye (Sigma, St Lou-is, MO, USA). The PCR reactions were performed on CFX-96 C1000 Touch Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) with iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA) in total volumes of 12.5 μ L under the following conditions: 95°C for 3 min, followed by 35 cycles of 95°C for 0.5 min, 60°C for 0.5 min and 72°C for 1 min using specific primers (Table 2). The primers used for qRT-PCR were synthesized by Integrated DNA Technologies, Inc., (IDT, Coralville, IA USA) and used at 300 nM final concentration. Housekeeping genes ACTB, GAPDH and LDHA were used as internal controls. The applied primers are listed in Table 2. The relative gene expression values were calculated from the ratio of genes and the averages of housekeeping genes using the $2^{-\Delta\Delta C_t}$ method.

Target name	Primer	Sequence (5' - 3')	Amplicon length (bp)	GeneBank accession number
DLD	DLD-forward	ACGCATCCTTCAAAAACAGG	352	NM_000108.5
	DLD-reverse	ACCAGCCATTCTTCAACAC		
OGDH isoform 1,2	OGDH1-forward	CTGATCCCTGCCCTCAAGAC	349	NM_002541.4
	OGDH1-reverse	CTGATCCCTGCCCTCAAGAC		
SDHB	SDHB-forward	AGAAACTGGACGGGCTCTAC	215	NM_003000.3
	SDHB-reverse	GCAGTTCATGATGGTGTGGC		
SDHC	SDHC-forward	TGTTGCTGAGACACGTTGGT	238	NM_001035512.2
	SDHC-reverse	GAGACCCCTGCACTCAAAGC		
SUCLA2	SUCLA2-forward	TGCAAGAAGCTGGTGTCTCC	259	NM_003850.3
	SUCLA2-reverse	CTGCCCTTTTCTCCCGTTTG		
SUCLG2	SUCLG2-forward	GAAGCTCTCGAGGCTGCTAA	236	NM_003848.4
	SUCLG2-reverse	GCTTCAGCAACCATCACCTT		
EGFR	EGFR-forward	TCACACATACTCCTCCTCT	239	NM_005228.5
	EGFR-reverse	ATCTCCATCACTTATCTCCT		
NOTCH1	NOTCH1-forward	TGCTGGACGAGTACAACCTG	214	NM_017617.5
	NOTCH1-reverse	GACTTCTCTCCGTGCCTT		
GNAQ	GNAQ-forward	GGCTATTGAAGATACACAAG	227	NM_002072.5
	GNAQ-reverse	AAATACACTGAGTCACAAGG		
Actin-beta	Actin-beta-forward	GTGCTATCCCTGTACGCCTC	271	NM_001101.5
	Actin-beta-reverse	TGGCCATCTCTTCTCGAAG		
GAPDH	GAPDH-forward	CCAGAACATCATCCCTGC	275	NM_002046.7
	GAPDH-reverse	GTGGGTGTCGCTGTTGAA		
LDHA	LDHA-forward	GGAGTTGTGTCATGTTGTCC	290	NM_005566.4
	LDHA-reverse	CAGTGAAGGAGCCAGGAAGT		

Table 2. Information on primer sequences used in the study.

2.6. Statistical analysis

Demographics were compared using the Chi-Square test or Welch's unequal variances t-test ($p < 0.05$). Statistical analysis of RPPA data was performed using a Python Statistical Analysis Tool (Python 3.8.5) to identify differentially expressed proteins between control and suicide groups. The signal intensities of $< 1\%$ of undiluted samples were filtered out. A principal component analysis (PCA) plot was generated for PHC samples across all the measured proteins using *ggfortify* package in R Studio [36]. Two-way ANOVA was used for the statistical analysis of altered protein expression for each brain region followed by Sidak's multiple comparison test, whereas a Welch's unequal variances t-test was carried out to compare mRNA expression between groups (GraphPad Prism 8.0.1). Differences were considered statistically significant when $p < 0.05$ and plotted as mean $\pm$ SEM (standard error of the mean).

3. RESULTS

3.1. Differentiate suicide and control samples

Reverse phase protein array (RPPA) was used to examine mitochondrial protein expression changes in suicidal individuals. A total of 11 TCA cycle enzyme subunits were profiled in the PHC, EC, FWM and CN regions prepared from 13 control individuals and 11 suicide victims. The demographic information for the control and suicide groups is listed in Table 1. We compared the age, sex and PMI covariates between the control and the suicide individuals using Welch's unequal variances t-test or Chi-Square test. We found no significant differences between the two groups for the age and sex covariates, however, there was a significant difference between controls and suicide samples considering the PMI covariate (Table 3). To check whether difference in PMI can have an effect on protein expression, we performed a PCA analysis. With this method, we can identify clusters including those samples that have similar expressional features. Based on the expression of the examined proteins PCA confirmed that most of the variance in the data (58%, PC1) was associated with expression changes between the suicide and control samples (Fig. 1). PCA plot indicated that samples were mostly clustered by two individual groups indicating differences between the proteomes of control and suicide groups, however, one control and one suicide sample showed some similarity to the opposite cluster (Fig. 1).

Brain region		Control	Suicide	p-value
Σ area	Age (mean $\pm$ SEM)	50.6 $\pm$ 2.8 (n=13)	47.5 $\pm$ 4.5 (n=11)	0.56
	Sex ratio (m/f)	8/5	8/3	0.56
	PMI (hours; mean $\pm$ SEM)	3.7 $\pm$ 0.5	6.2 $\pm$ 0.7	0.013
PHC	Age (mean $\pm$ SEM)	50.8 $\pm$ 3.6 (n=10)	49.7 $\pm$ 4.3 (n=10)	0.84
	Sex ratio (m/f)	7/3	7/3	>0.99
	PMI (hours; mean $\pm$ SEM)	3.9 $\pm$ 0.6	6.0 $\pm$ 1.05	0.04
EC	Age (mean $\pm$ SEM)	77.7 $\pm$ 3.4 (n=7)	39.8 $\pm$ 3.1 (n=5)	0.32
	Sex ratio (m/f)	5/2	4/1	0.74
	PMI (hours; mean $\pm$ SEM)	3.0 $\pm$ 0.8	6.8 $\pm$ 1.0	0.02
FWM	Age (mean $\pm$ SEM)	58.6 $\pm$ 3.1 (n=5)	49.2 $\pm$ 5.7 (n=6)	0.18
	Sex ratio (m/f)	2/3	4/2	0.37
	PMI (hours; mean $\pm$ SEM)	5.1 $\pm$ 0.3	6.8 $\pm$ 0.9	0.12
CN	Age (mean $\pm$ SEM)	50.6 $\pm$ 3.7 (n=10)	53.4 $\pm$ 5.2 (n=7)	0.67
	Sex ratio (m/f)	5/5	5/2	0.37
	PMI (hours; mean $\pm$ SEM)	3.9 $\pm$ 0.6	6.0 $\pm$ 1.05	0.11

Table 3. Analysis of sample data. Mean age, sex ratio and PMI proportions of samples in control (n=13) and suicide (n=11) groups. There were no significant differences between the groups for age and sex covariates, but there was a significant difference between controls and suicide individuals considering PMI covariate (PMI: p=0.04; indicated with a yellow box). For mean age and PMI, we used Welch's unequal variances t-test and for sex ratio, the Chi-Square test was used. The mean age, sex ratio and PMI for each brain region are also listed along with the statistical analysis for this study. We found that the PMI of the PHC and EC differed significantly between the two groups. CN, caudate nucleus; FWM, frontal white matter; EC, entorhinal cortex; PHC, parahippocampal cortex.

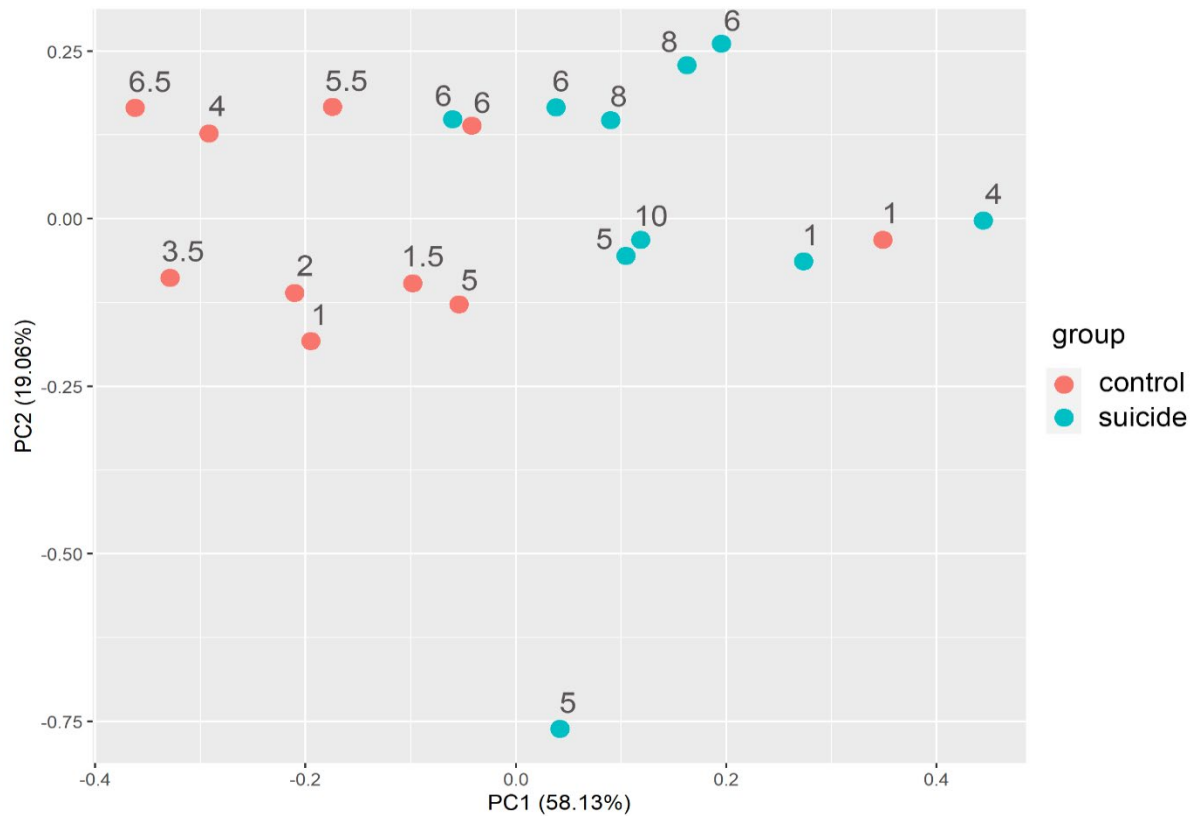


Fig. 1. Analysis of protein expression of the PHC between suicide and control groups. A Principal Component Analysis (PCA) was used to cluster the log-transformed normalized data of the samples of the PHC from both groups for visualizing the overall effect of experimental covariates. Red dots represent the control while blue dots refer to suicide subjects. The PMI values (in hours) for each sample are indicated on the plot.

3.2. Characterizing the expression levels of TCA cycle enzymes in suicide victims

The expression values for the 11 TCA cycle enzyme subunits were compared across 20 samples from the parahippocampal cortex (PHC) and 13 samples from the entorhinal cortex (EC) to determine differentially expressed proteins between the control and suicide groups. Two of the validated antibodies can recognize transcript variants. The anti-OGDH antibody (Proteintech, cat. no. 15212-1-AP) recognizes two transcripts, the OGDH isoform 1 and isoform 2. Another anti-OGDH antibody (Sigma-Aldrich, cat. no. HPA020347) recognizes the OGDH isoform 3. Significant changes were observed between groups, the expression of DLD, OGDH isoforms 1 and 2, SDHB, SUCLA2 and SUCLG2 enzymes were upregulated in the PHC of suicide victims (Fig. 2A). However, we did not find a difference for any of the examined proteins in the EC between groups (Fig. 2B).

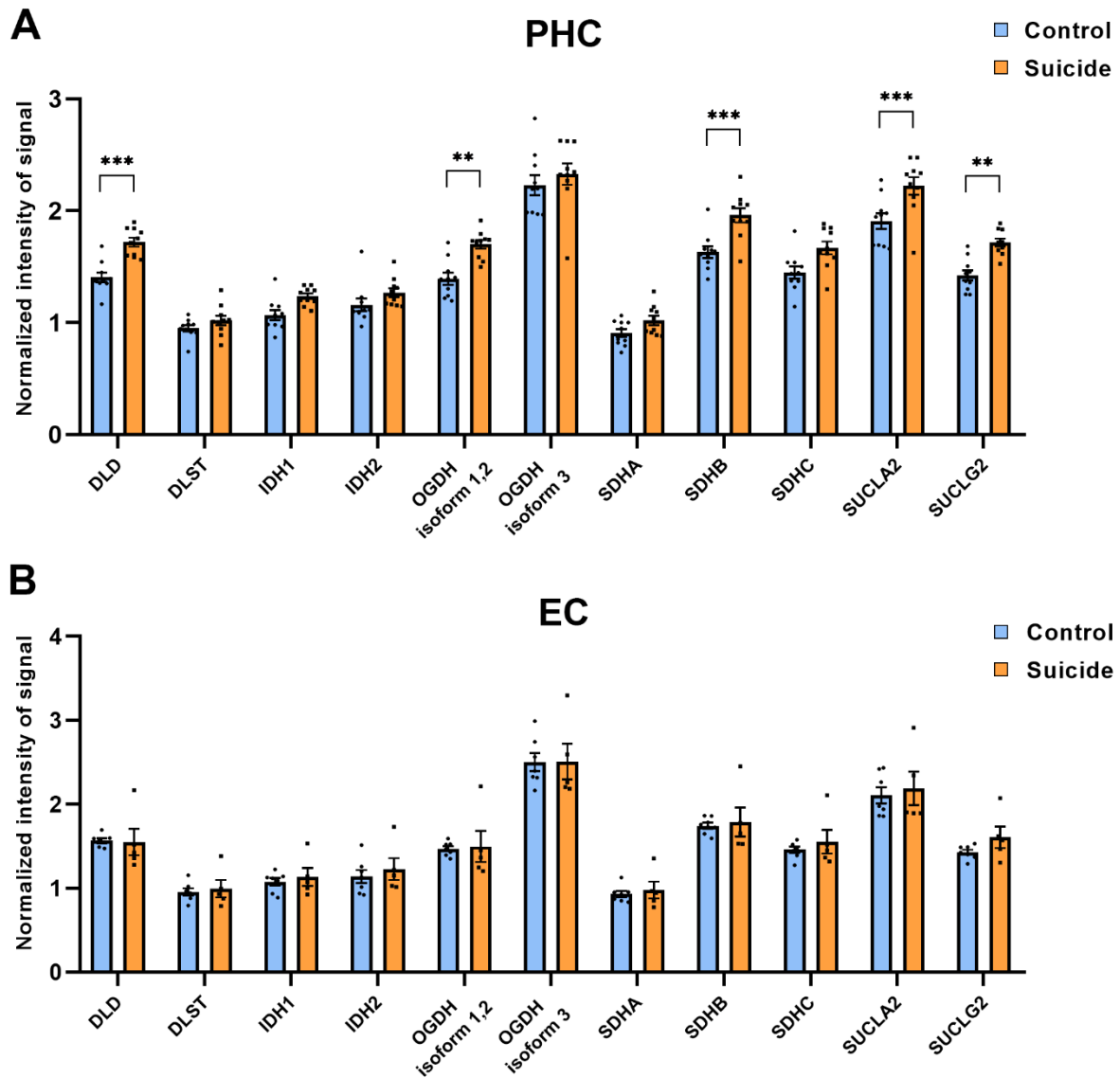


Fig. 2. Reverse phase protein array (RPPA) of DLD, DLST, IDH1, IDH2, OGDH isoforms 1 and 2, OGDH isoform 3, SDHA, SDHC, SDHB, SUCLA2 and SUCLG2 in human PHC and EC. (A) Bar graphs show representative examples of 5 differentially expressed subunits of the TCA cycle in the PHC. (B) At the same time, there were no significant changes in the expression of these subunits in the EC. The data are based on 10 control and 10 suicide individuals for PHC, and 7 control and 5 suicide individuals for EC as indicated in Table 3. Bar graphs represent mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The mRNA levels for each of the 5 significantly altered proteins were analyzed in the PHC and EC of both groups. As shown in Fig. 3, the quantitative PCR analyses identified three significantly altered genes, thus, we verified an increased expression of the OGDH isoforms 1 and 2, SDHB, and SUCLA2 whose expression level alterations confirmed their protein

expression (Fig. 3A). The mRNA expression of SDHC was also significantly increased in the PHC despite its protein level did not change significantly, however, the p-value of its expression level was less than 0.1 ($p=0.056$) (Fig. 3A). Although the mRNA expression of DLD and SUCLA2 was higher in suicide victims than in control subjects, differences are not statistically significant (not shown; SUCLA2: $p=0.109$, DLD: $p=0.233$).

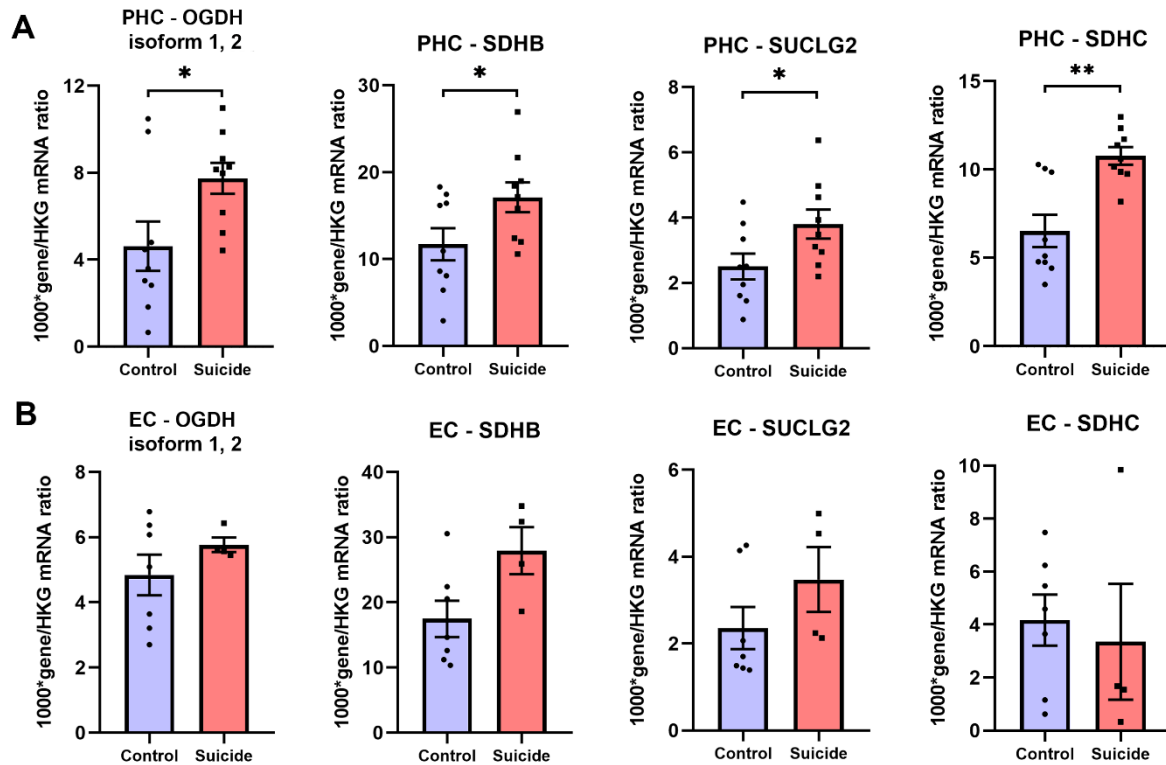


Fig. 3. Bar graphs showing mRNA levels assessed by real-time PCR in the PHC and EC of suicide victims. The mRNA expression analysis of the altered TCA enzyme subunit DLD, OGDH isoforms 1 and 2, SDHB, SUCLA2 and SUCLG2 showed that gene expression of OGDH isoforms 1 and 2, SDHB and SUCLG2 were significantly elevated in the PHC as expected (A), while in the EC the gene expression did not change significantly (B). Bar graphs represent mean $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$).

3.3. Protein expression changes of non-mitochondrial proteins in suicide victims

Based on the previous results, changes in both RNA and protein levels of the presented TCA cycle enzyme subunits are prominent in the PHC. Therefore, we addressed whether changes in the levels of some non-mitochondrial proteins, which do not play a role in mitochondrial processes but are also involved in cell development and homeostasis occur in this region. For

this purpose, we selected three proteins, the EGFR, NOTCH1 and GNAQ, which are known to play a role in cell growth and differentiation [37-39]. Interestingly, we found that the protein levels of these three genes did not differ between the control and suicide groups in the PHC or the EC (Fig. 4A, B).

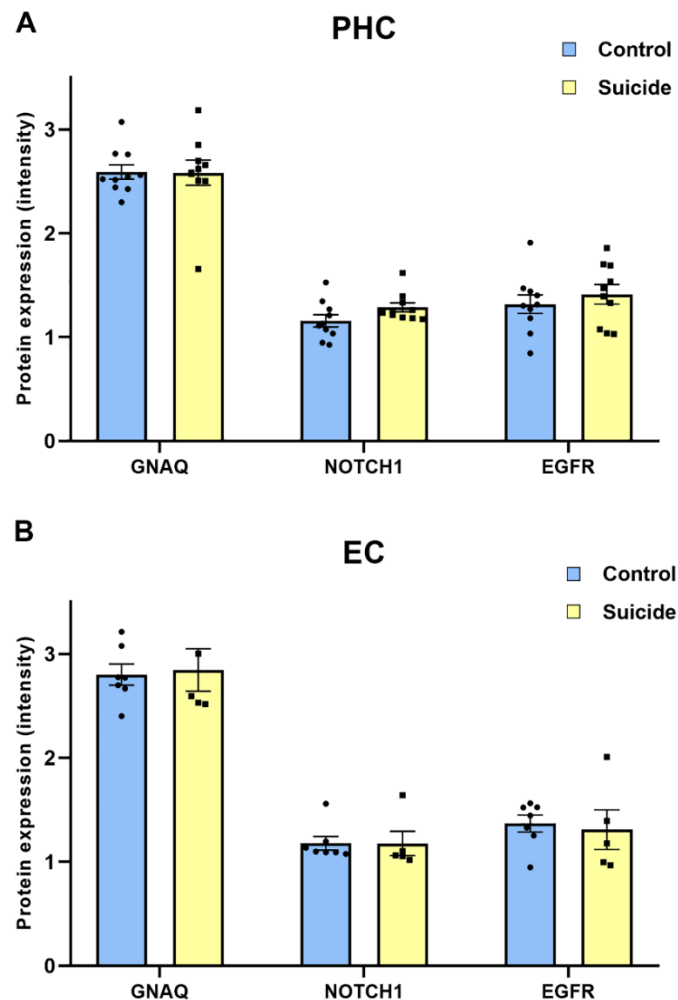


Fig. 4. Bar graph showing the representative protein expression of 3 not mitochondrial proteins, the EGFR, NOTCH1 and GANQ. For these proteins, statistical analyses showed no differences between the control and suicide groups in the PHC (A) and EC (B) ($p < 0.05$). Error bars represent the SEM.

3.4. Analysis of the post-mortem time (PMI)-dependent expression of samples

To test the effect of PMI on our results, we performed linear regression analysis for PMI-dependent gene and protein expression for the differentially expressed subunits of TCA cycle enzymes and protein expression for the three non-mitochondrial proteins of the PHC and EC samples. The experimental results showed that the p-values of F-test and t-test for all the

examined genes and proteins were more than 0.05 suggesting that PMI did not affect the expression levels of the examined proteins and genes.

Expression	Gene	Parahippocampal cortex		Entorhinal cortex	
		F-test	t-test	F-test	t-test
protein	DLD	3.062	0.097	1.786	0.211
	OGDH isoform 1,2	1.352	0.26	2.565	0.14
	SDHB	0.495	0.49	1.553	0.241
	SUCLA2	2.456	0.134	2.371	0.154
	SUCLG2	4.007	0.061	4.664	0.056
protein	EGFR	3.669	0.071	0.056	0.817
	NOTCH1	0.787	0.386	2.115	0.176
	GNAQ	0.019	0.891	2.296	0.16
mRNA	OGDH isoform 1,2	0.132	0.721	0.101	0.757
	SDHB	0.897	0.357	2.667	0.137
	SUCLG2	0.393	0.539	0.064	0.805
	SDHC	0.142	0.711	1.152	0.31

Table 4. Linear regression analysis of samples of the parahippocampal and entorhinal cortices for PMI-dependent protein and mRNA expression. The table shows that the p-values of F-test and t-test were > 0.05 .

3.5. Characterization of the protein expression of TCA cycle enzymes in cortical white matter and subcortical structures

To determine whether the dysregulated expression of TCA cycle enzymes occur in both the grey and white matter, we compared the expression level of all the 11 examined subunits in the frontal white matter (FWM) between the control and suicide groups. We did not observe any significant alteration between groups for the examined proteins (Fig. 5A). In order to find correlations across the alteration of TCA cycle enzyme expression between cortical and subcortical regions, we compared the protein levels of the examined subunits in the caudate nucleus (CN) between control and suicide groups. We found that the protein expression levels of all the 11 examined proteins were not altered in the CN between the two groups (Fig. 5B).

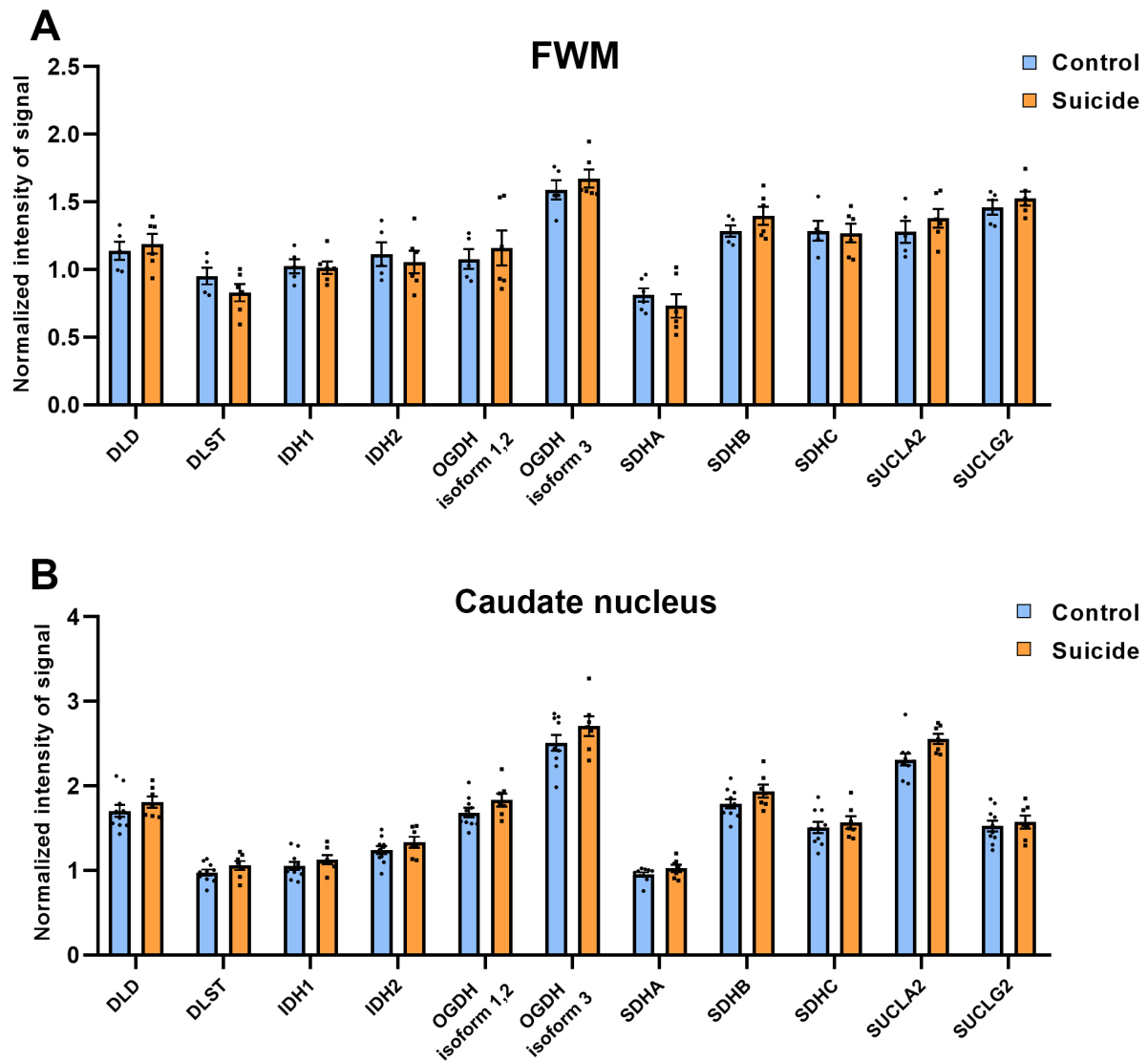


Fig. 5. Protein expression of the examined TCA cycle enzyme subunits in frontal white matter (FWM) and caudate nucleus (CN) related to suicidal behavior. (A) Bar graphs show that the expression levels of the investigated proteins did not change in the FWM of suicide individuals compared to the control subjects. (B) The Fig. represents that - similarly in the FWM - there were no differences in expression levels of the examined enzymes in the CN between the suicide and control groups. Error bars represent the SEM (control group for FWM: n=5; suicide group for FWM: n=6; control group for CN: n=10; suicide group for CN: n=7).

4. DISCUSSION

The major finding of the study is the increase in the level of some mitochondrial proteins in the parahippocampal cortex (PHC) of suicide victims. RPPA data in the PHC were obtained from 10 control and 10 suicide patients (Table 3). This is a high enough number of independent samples to draw conclusions. Groups of samples in control brain regions contained a smaller number of samples, however, a minimum of 5 was the sample number per group. In these brain regions, we found no changes in the level of the same mitochondrial proteins, moreover, tendencies were also not present suggesting that the alterations are specific to the PHC. Any potential methodological bias caused by the brains themselves is also not likely because the majority of the samples in the control brain areas are derived from the same brains as the PHC samples. Even local events (e.g. acidification, freezing time, etc.) affecting the results are not likely because the EC, which did not exhibit alterations, is situated immediately adjacent to the PHC. Therefore, we can conclude that changes in mitochondrial proteins are specific to the PHC.

Sex and age are also not likely to affect the results (Table 3). There was, however, a postmortem time (PMI) delay, which differed between the groups as it was higher in the suicide group. While previous analysis using postmortem samples stored at 4°C did not find decay in the amount of proteins within 24 hours [40], however, the maximum PMI in our study is 10 hours, it cannot be ruled out that this factor affected the results. Therefore, we addressed this question statistically. A PCA plot showed clusters of the PHC samples based on which group they belong to but not based on PMI (Fig. 1). In addition, a linear regression analysis of samples of the parahippocampal and entorhinal cortices did not find correlation with PMI (Table 4) arguing against a role of PMI in protein level differences between the 2 groups.

Protein levels of 5 subunits changed among the 11 examined mitochondrial TCA cycle enzyme subunits in the PHC (Fig. 2). In an attempt to elucidate if transcriptional events could be responsible for the increased protein levels, the mRNA levels of these proteins were also investigated (Fig. 3). Three out of 5 altered level proteins demonstrated elevated mRNA levels suggesting the involvement of transcriptional regulation. The mRNA measurements represent an independent technique, thereby also validating the RPPA data. In this regard, it is also interesting to note that mRNA level changes were not found in the EC conforming to the spatial restriction of the elevation in mitochondrial function of the PHC. Mitochondrial alterations were also not found in other brain regions known to be involved in depression, such as the

caudate nucleus (CN). We compared the protein levels of the examined subunits in the CN and found no alteration in protein expression for the examined proteins between the control and suicide groups suggesting that mitochondrial alterations in PHC may have a more prominent role in the development of suicidal behavior.

While a relatively large percentage of mitochondrial proteins changed in the PHC, none of the examined non-mitochondrial proteins, EGFR, NOTCH1 and GNAQ showed alteration in its level in the PHC (Fig. 4). Since the non-mitochondrial proteins we examined are involved in common metabolic signaling processes [37-39], their unaltered level between the control and suicide groups suggests that dysregulations of processes involving mitochondrial rather than cytosolic metabolism are more prominent in the PHC related to the pathophysiology of suicidal behavior. A growing body of evidence from animal models and human studies suggests that impaired mitochondrial function might affect synaptic functioning and thus contribute to mental disorders [26, 44-48]. However, our data allowed more specific conclusions because functionally, the altered proteins all catalyse reactions belonging to the TCA cycle. Among them, the ketoglutarate dehydrogenase complex (KGDHC) containing OGDH and DLD, catalyzes the irreversible decarboxylation of α KG to succinyl-CoA while the succinyl-CoA ligase ADP-forming (SUCLA2) and GDP-forming subunit beta (SUCLG2) are crucial for converting succinyl-CoA to succinate (Fig. 6.). Within the TCA cycle, they are all involved in the conversion of α -ketoglutarate to fumarate (Fig. 6), the major pathway of glutaminolysis. Glutaminolysis is an alternative pathway for energy production that converts glutamine to glutamate and then to α -ketoglutarate [49]. In fact, subunits of all major TCA enzymes belonging to glutaminolysis were upregulated in the PHC of suicide victims suggesting the potentiation of glutaminolysis and a prominent role of this process in suicidal behavior. While our data represent new discoveries as far as the alteration of glutaminolysis in suicide victims, the pyruvate dehydrogenase complex (PDC) has been reported downregulated in depression, preventing the pyruvate conversion to acetyl-CoA which facilitates the dysregulation of the TCA cycle and mitochondrial metabolism [50, 51].

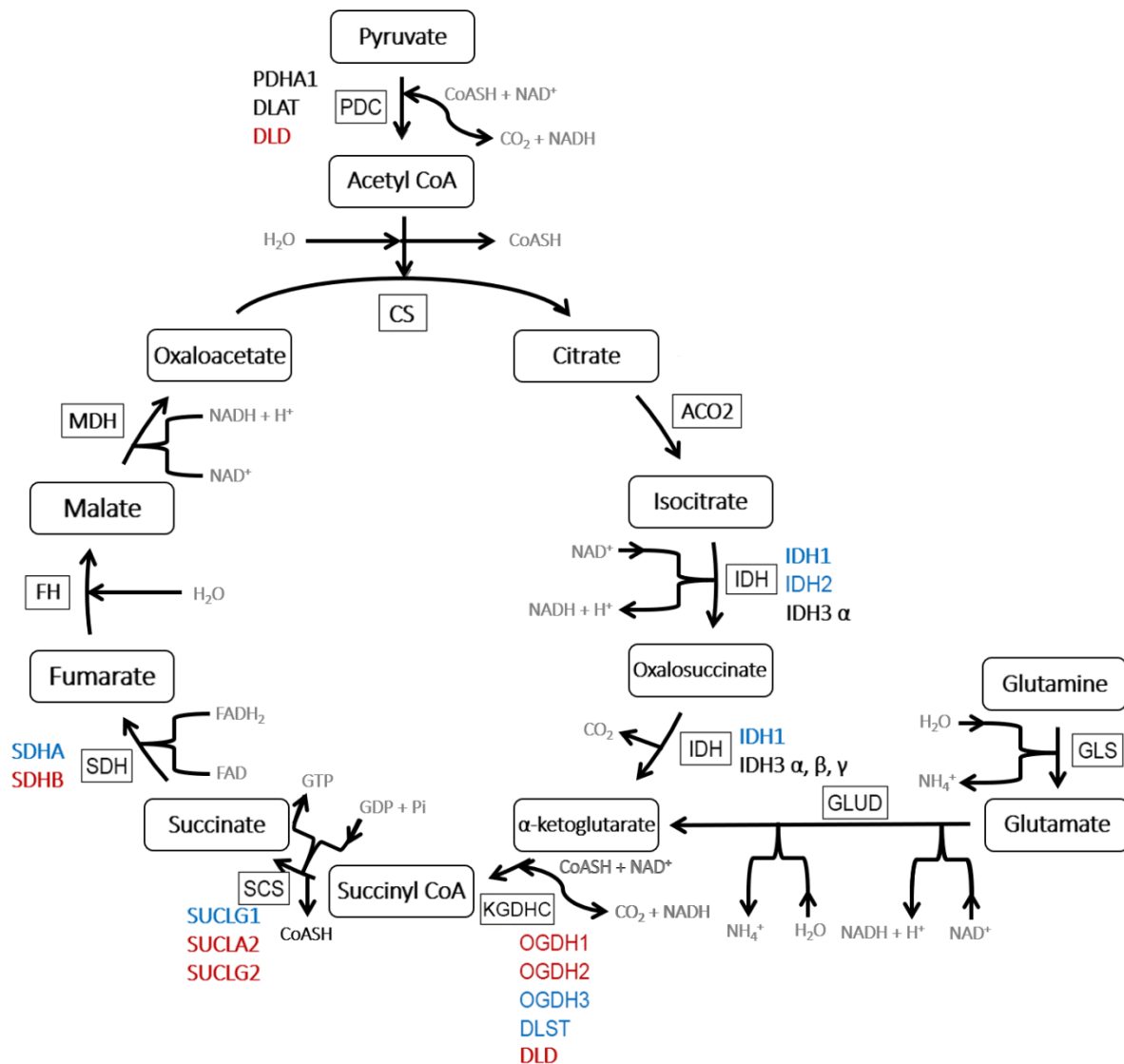


Fig. 6. Molecular link between TCA cycle and glutaminolysis in mammalian cells. The Fig. was adapted and modified from Fig. 1 of Dhimi, et al. [52]. The genes in colors under boxes are either isozymes part of the enzyme complex or are subunits catalyzing the TCA reaction. The significantly upregulated TCA cycle enzyme subunits are indicated with red color, while the significantly not altered subunits are indicated with blue color.

It is likely that increased glutaminolysis takes place in neurons and not glial cells in the PHC because, in the white matter where glial bodies are numerous as compared to neuronal cell bodies, no changes were found in the level of TCA enzyme subunits (Fig. 5). In addition, the TCA cycle is less active in glial cells than in neurons, some of the enzymes we found elevated in suicide victims, such as the OGDH, SUCLA2 and SUCLG2 subunits are not even present in glial cells to a detectable degree [53-55]. Glutaminolysis is not active in glial cells because they transport glutamine to the neurons instead of using it as an energy source. The astrocyte-derived

glutamine can be used by the neurons to produce energy via the TCA cycle or produce glutamate for neurotransmission. While our data provide no information about the amount of glutamate used as a neurotransmitter in suicidal individuals, the increased glutaminolysis implies increased energy production, which is used primarily to increase neuronal activity in neurons. The increased energy production requires more oxygen, which is consistent with the results of fMRI studies reporting elevated BOLD response to the PHC of suicide attempters [56-58].

In conclusion, the present study implies a specific role of the PHC in suicidal behavior. Furthermore, a potential molecular component of the increased neuronal activity in this brain area was identified. Molecular changes in the PHC provide the possibility that the cause of suicidal behavior originates in this brain region through mitochondrial dysfunctions. The PHC can then potentially affect other members of the DMN by its increased activity, which may contribute to the execution of suicidal behavior.

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