

CONTRIBUTION OF CELL TYPE SPECIFIC CHANGES OF MONOCARBOXYLATE
TRANSPORTERS TO AMYOTROPHIC LATERAL SCLEROSIS (ALS) DISEASE
AND APPLICATION OF BORON DERIVATIVES AS AN ALTERNATIVE
TREATMENT

by
Nermin Başak Şentürk

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Doctor Philosophy in
Biotechnology

Yeditepe University

2022

CONTRIBUTION OF CELL TYPE SPECIFIC CHANGES OF MONOCARBOXYLATE
TRANSPORTERS TO AMYOTROPHIC LATERAL SCLEROSIS (ALS) DISEASE
AND APPLICATION OF BORON DERIVATIVES AS AN ALTERNATIVE
TREATMENT

APPROVED BY:

Prof. Dr. Fikrettin Şahin
(Thesis Supervisor)
(Yeditepe University)

.....

Prof. Dr. Ece Genç
(Yeditepe University)

.....

Assoc. Prof. Dr. Ayşegül Doğan
(Yeditepe University)

.....

Prof. Dr. Tunç Akkoç
(Marmara University)

.....

Assist. Prof. Dr. Yasemin Yılmaz
(İstanbul Sabahattin Zaim University)

.....

DATE OF APPROVAL: / / 2021

I hereby declare that this thesis is my own work and that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I have fully cited and referenced all material and results as required by these rules and conduct, and this thesis study does not contain any plagiarism. If any material used in the thesis requires copyright, the necessary permissions have been obtained. No material from this thesis has been used for the award of another degree.

I accept all kinds of legal liability that may arise in case contrary to these situations.

Name, Last name Nermin Başak Şentürk.....

Signature

ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the support and help of my thesis advisor, Prof. Dr. Fikretin Şahin, for his continued support and encouragement. He didn't just mentor me; he was there for me like a family member throughout my academic life and showed me great understanding in difficult times and motivated me to accomplish this research.

I am most grateful to Dr. Burcu Kasapoğlu Usta whom I mostly benefited from during this project for being there for me at all times. Her guidance helped me in all the time of research and writing of this thesis.

I would like to thank my co-advisor Assist. Prof. Dr. Mehmet Ozansoy and the rest of my thesis committee members including Prof. Dr. Ece Genç and Prof. Dr. Tunç Akkoç for their insightful comments and encouragement during this project.

Furthermore, I would like to express my sincere appreciation for the financial support received from Suna and İnan Kırac Foundation during my thesis study.

My heartfelt gratitude to dear Ceren Ertan for her support and great help in this project, especially in the endless *in-vivo* experiments.

I am indebted to my fellow labmates and dear friends, Batuhan Bozkurt, Emre Cebeci, Eslam Essam, Ezgi Taşkan, Hatice Burcu Şişli, Oğuz Kaan Kırbaş, and Zeynep İşlek for motivating me during the sleepless nights that we worked together. Special thanks to Selim Doğan for his help during animal studies, Dr. Zehra Çobanoğlu for the guidance in bioinformatics analysis, Dr. Volkan Can for providing the essential chemicals, and Dr. Şaban Kalay for the technical support in HPLC experiments. I am particularly grateful to my dear friends Merve Erdem, Sevcan Şahin and Binnur Kıratlı for their supports and friendships.

Finally, I would like to acknowledge with gratitude the support and the love of my family—my parents Sezgin and Mehmet Muhteşem, and my aunts, Çiğdem and Rukiye. It would not have been possible to complete my Ph.D study without their unlimited contribution and support.

ABSTRACT

CONTRIBUTION OF CELL TYPE SPECIFIC CHANGES OF MONOCARBOXYLATE TRANSPORTERS TO AMYOTROPHIC LATERAL SCLEROSIS (ALS) DISEASE AND APPLICATION OF BORON DERIVATIVES AS AN ALTERNATIVE TREATMENT

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal disease characterized by chronic degeneration of upper and lower motor neurons. Various mechanisms have been associated with degeneration of motor neurons in ALS up to date; however, unlike the classical findings, recently lactate transporters; MCT1 and MCT4 expressions were found to be decreased, suggesting energy deprivation may play a role in ALS pathogenesis. In the current study, in order to understand the contribution of MCTs to ALS pathogenesis, the changes in expression levels of MCT1, and MCT4 in the CNS of transgenic SOD1-G93A and C9ORF72 mice were examined in a cell-type-specific manner. According to the findings, a significant increase in MCT1 expressions was determined in both whole-spinal cord materials and brain-derived oligodendrocytes, while a significant decrease in MCT4 expressions was detected in brain-derived astrocytes, and no significant change was observed in MCT2 expressions in transgenic mice, suggesting a compensatory role for MCT1 and a causal role for MCT4 in ALS. *In-vitro* experiments using NSC-34 motor neuron-like cells revealed that lactate at appropriate doses can be used as an energy source, as an alternative to glucose, and when MCTs were chemically blocked with AR-C155858, lactate trafficking was partially interrupted, negatively affecting cell survival. Additionally, inspired by the introduction of some boron derivatives for the treatment of neurodegenerative diseases, the effect of various boron derivatives on locomotor behaviors and molecular mechanisms were tested in the SOD1-G93A mice model. It was observed that especially sodium pentaborate pentahydrate (NaB) and sodium perborate tetrahydrate (SPT) promote a considerable improvement in motor activities of transgenic mice, and moreover, according to microarray data, they positively changed the expression of genes involved in numerous metabolic pathways associated with neurodegenerative diseases. This study provided further evidence for the role of MCTs in ALS and was the first of the related studies to elicit the possible neuroprotective effect of NaB and SPT.

ÖZET

MONOKARBOKSİLAT TAŞIYICILARIN HÜCRE SPESİFİK DEĞİŞİMLERİNİN AMYOTROFİK LATERAL SKTEROZ (ALS) HASTALIĞINA OLASI ETKİSİ VE BOR TÜREVLERİNİN ALTERNATİF TEDAVİ OLARAK UYGULANMASI

Amyotrofik lateral skleroz (ALS), aşağı ve yukarı motor nöronların kronik dejenerasyonu ile karakterize, progresif, ölümcül bir hastalıktır. Bugüne kadar ALS'de motor nöronların dejenerasyonu çeşitli mekanizmalarla ilişkilendirilmiştir, ancak klasik bulgulardan farklı olarak son zamanlarda, laktat taşıyıcıları MCT1 ve MCT4 ekspresyonlarının azaldığının bulunması, enerji yoksunluğunun ALS patogeneğinde etkili olabileceğini düşündürmektedir. Mevcut çalışmada, MCT'lerin ALS'nin gelişimine bireysel katkısını anlamak için, transgenik SOD1-G93A ve C9ORF72 fare modellerinde merkezi sinir sistemine ait MCT1, MCT2 ve MCT4 ekspresyonlarındaki değişiklikler hücre tipine özgü bir şekilde incelenmiştir. Sonuçlara göre hem tam omurilik materyallerinde hem de beyin kaynaklı oligodendrositlerde MCT1 ifadelerinde anlamlı bir artış tespit edilirken, beyin kaynaklı astrositlerde ise MCT4 ifadelerinde anlamlı bir azalma tespit edilmiştir. MCT2 ifadelerinde herhangi anlamlı bir değişiklik gözlenmemiştir. Bu bulgular, ALS'de MCT1'in kompanse edici bir rol, MCT4'ün ise nedensel bir rol üstlenme ihtimalini güçlendirmektedir. Laktatın kullanımını araştırmak amacıyla NSC-34 motor nöron benzeri hücreler kullanılarak yapılan *in-vitro* deneylerde, uygun dozlarda laktatın glikoza alternatif bir enerji kaynağı olarak kullanılabileceği ve MCT'lerin AR-C155858 ile bloke edildiğinde, laktat trafiğinin kısmen kesintiye uğrayarak hücre sağkalımını azalttığı gözlenmiştir. Ayrıca nörodejeneratif hastalıkların tedavisi için bazı bor türevlerinin kullanılmaya başlamasından esinlenerek, çeşitli bor türevlerinin lokomotor davranışlar ve moleküler mekanizmalar üzerindeki etkisi SOD1-G93A fare modelinde test edilmiştir. Özellikle NaB (sodyum penta borat) ve SPT (sodyum perborat tetrahidrat)'nin transgenik farelerin motor aktivitelerinde anlamlı bir iyileşme sağladığı ve mikrodizi verilerine göre çok sayıda nörodejeneratif hastalıkla ilişkilendirilmiş metabolik yollarda yer alan genlerin ekspresyonunu olumlu yönde değiştirdiği gözlemlenmiştir. Bu çalışma, MCT'lerin ALS'deki rolünü güçlendirmiştir ve ALS ve diğer ilgili nörodejeneratif hastalıkların tedavisi için NaB ve SPT'nin olası nöroprotektif etkilerini ortaya koyan ilk araştırmadır.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
ÖZET	vi
LIST OF FIGURES	xi
LIST OF TABLES.....	xvii
LIST OF SYMBOLS/ABBREVIATIONS.....	xviii
1. INTRODUCTION.....	1
1.1. WHAT IS ALS DISEASE?	1
1.2. ALS ETIOLOGY	3
1.3. GENES INVOLVED IN ALS PATHOGENESIS.....	4
1.3.1. <i>SOD1</i>	5
1.3.2. <i>C9ORF72</i>	6
1.3.3. <i>NEK1</i>	7
1.3.4. <i>TARDBP</i>	7
1.3.5. <i>FUS</i>	9
1.3.6. <i>TP73</i>	10
1.3.7. Other ALS Related Genes.....	11
1.4. NEW INSIGHTS: ROLE OF MCT EXPRESSION IN ALS PATHOGENESIS	11
1.4.1. The Metabolism of Lactate	11
1.4.2. Astrocyte-Neuron Lactate Shuttle Hypothesis	13
1.4.3. Monocarboxylate Transporters	15
1.4.4. MCT Expression in CNS and Their Involvement in ALS Development	17
1.4.4.1. <i>MCT1 in Oligodendrocytes</i>	18
1.4.4.2. <i>MCT2 in Motor Neurons</i>	20
1.4.4.3. <i>MCT4 in Astrocytes</i>	20
1.5. NEW INSIGHTS-2: APPLICATION OF BORON AS A NEW APPROACH IN THE TREATMENT OF ALS AND OTHER NEURODEGENERATIVE DISEASES	24
1.5.1. Overview of Boron	24
1.5.2. Boron Treatment in Neurodegenerative Diseases	26

1.5.2.1.	<i>Boron and Brain Activity</i>	26
1.5.2.2.	<i>Neuroprotective Effect of Boron</i>	27
1.5.2.3.	<i>Boron Treatments in Alzheimer's Disease</i>	29
1.5.2.4.	<i>Boron Treatment in Parkinson's Disease</i>	32
1.5.2.5.	<i>Boron Treatment in Amyotrophic Lateral Sclerosis (ALS)</i>	33
1.6.	AIM OF STUDY	34
2.	MATERIALS AND METHODS	35
2.1.	ANALYSIS OF THE CELL-TYPE SPECIFIC GENE AND PROTEIN EXPRESSION OF MCTS IN CNS OF ADULT MICE CARRYING ALS-CAUSING MUTATIONS.	35
2.1.1.	Ethical Approval	35
2.1.2.	Mouse Model Handling and Genotyping.....	35
2.1.3.	Behavioural Characterization for SOD1-G93A Mice	37
2.1.3.1.	<i>Neurological Scoring System (NeuroScore)</i>	37
2.1.4.	Behavioural Characterization of Tg(C9orf72_3) Line 112 Mice	40
2.1.4.1.	<i>Rotarod Test</i>	40
2.1.5.	Papain Disassociation	41
2.1.6.	Magnetic Cell Sorting (MCS).....	42
2.1.6.1.	<i>Removal of Myelin and Separation of Oligodendrocytes</i>	43
2.1.6.2.	<i>Separation of Astrocytes</i>	44
2.1.6.3.	<i>Separation of Neurons</i>	44
2.1.7.	RNA Isolation and RT-PCR	45
2.1.8.	qPCR for Relative mRNA Expression Analysis of Cell Markers and MCTs	45
2.1.9.	Western Blotting for Protein Expression Analysis of Cell Markers and MCTs	47
2.1.10.	Protein Detection by Simple Western Analysis	49
2.1.11.	Statistical Analysis	50
2.2.	INVESTIGATION OF THE INHIBITION OF MCT FUNCTION AND THE EFFECT OF LACTATE TRANSFER ON CELL SURVIVAL IN AN <i>IN-VITRO</i> CELL MODEL.....	51
2.2.1.	NSC-34 Cell Line and Culture.....	51
2.2.1.1.	<i>Cell Thawing</i>	51

2.2.1.2.	<i>Cell Lines and Culturing Conditions</i>	51
2.2.1.3.	<i>Cell Subculturing</i>	52
2.2.1.4.	<i>Calculation of Cell Number</i>	52
2.2.1.5.	<i>Cryopreservation of Cells</i>	52
2.2.2.	Glucose Starvation	52
2.2.3.	Cell Viability Assays	53
2.2.4.	MCT Inhibition	54
2.2.4.1.	<i>RNA Isolation and RT-PCR</i>	54
2.2.4.2.	<i>qPCR</i>	54
2.2.4.3.	<i>Cell Culture</i>	55
2.2.4.4.	<i>HPLC Method (LC-MS/MS)</i>	55
2.2.4.5.	<i>Cell viability Assay</i>	56
2.2.5.	Statistical Analysis.....	56
2.3.	INVESTIGATING THE EFFECT OF BORON DERIVATIVES ON MCTs AND EVALUATING IT AS AN ALTERNATIVE TREATMENT METHOD FOR ALS.....	56
2.3.1.	Ethical Approval	56
2.3.2.	Mouse Model Handling and Boron Application.....	57
2.3.3.	Motor Function Tests	58
2.3.4.	CNS material collection.....	59
2.3.5.	RNA Isolation	59
2.3.6.	Microarray Preparation and Data Pre-processing	59
2.3.7.	Statistical Analysis.....	63
2.3.8.	PANTHER Gene Ontology (GO) Classification and Pathway System Analysis	63
3.	RESULTS.....	64
3.1.	GENOTYPING OF TRANSGENIC SOD1-G93A AND C9ORF72 MICE	64
3.2.	BEHAVIORAL TESTS	65
3.2.1.	NeuroScore Evaluation Table of Transgenic SOD1-G93A Mice	65
3.2.2.	Behavioural Characterization for Transgenic C9ORF72 mice – Rotarod test	71
3.3.	MAGNETIC CELL SORTING (mcs) confirmation.....	72
3.3.1.	qPCR Analysis.....	72

3.3.2.	Western Blot and Simple Western (WES) Analysis.....	81
3.3.2.1.	<i>Protein Expression Analysis of Neuronal and Glial Markers</i>	81
3.3.2.2.	<i>Protein Expression Analysis of MCTs</i>	84
3.4.	INVESTIGATION OF THE INHIBITION OF MCT FUNCTION AND THE EFFECT OF LACTATE TRANSFER ON CELL SURVIVAL IN AN <i>IN-VITRO</i> CELL MODEL.....	89
3.4.1.	Cell Viability Assays	89
3.4.2.	MCTs Expressions in NSC-34 Cell Lines and MCTs Inhibitions.....	92
3.4.2.1.	<i>qPCR Analysis</i>	92
3.4.2.2.	<i>MCT Inhibition by AR-C155858 and HPLC (LC-MS/MS) Analysis</i>	93
3.4.2.3.	<i>Effect of MCT Inhibition on Cell Survival</i>	97
3.5.	INVESTIGATING THE EFFECT OF BORON DERIVATIVES ON MCTs AND EVALUATING IT AS AN ALTERNATIVE TREATMENT METHOD FOR ALS.....	98
3.5.1.	Motor Activity Tests.....	99
3.5.1.1.	<i>Neurological Scoring System (NS)</i>	99
3.5.1.2.	<i>Rotarod Test</i>	100
3.5.1.3.	<i>Hanging Wire Grip Test</i>	101
3.5.2.	Microarray Analysis	102
3.5.2.1.	<i>Spinal Cord Materials</i>	103
3.5.2.2.	<i>Brain Materials</i>	120
4.	DISCUSSION.....	133
5.	CONCLUSION	152
	REFERENCES	154
	APPENDIX A.....	181

LIST OF FIGURES

Figure 1.1. Muscle atrophy and limitation of movements as a result of degeneration of motor neurons in ALS disease.	2
Figure 1.2. Schematic representation of the central nervous system.	3
Figure 1.3. Schematic representation of the mechanism for glutamate-induced glycolysis in astrocytes through physiological activation. Pyr: pyruvate, Lac: Lactate, Gln: glutamine, G: guanine nucleotide-binding protein.	15
Figure 1.4. The suggested topology of the MCT family members. (A) Predicted structure of MCT1, the N and C termini of MCT1 and, the ancillary protein CD147 that associates with MCT1 and MCT4 are demonstrated as well, (B) predicted structure of MCT4	16
Figure 1.5. Tissue distribution of monocarboxylate transporter (MCT) isoforms	17
Figure 1.6. Distribution of MCTs in CNS.	18
Figure 1.7. Schematic representation of lactate provide to axons.	22
Figure 1.8. Representative image of L-lactate generation and action at the tripartite synapse structure of axon, dendrite, astrocyte, and at the glia-vascular junction. Cx: connexin gap junctions, orange oval	24
Figure 1.9. Structure of Boron atom	25
Figure 1.10. The progression of Alzheimer's disease and amyloid plaques and neurofibrillary tangles in AD	31
Figure 1.11. Representative image of two MD runs (MD1 and MD2) for both (a, b) A β -trimer and (c, d) A β -trimer with BNNT ($t=1\ \mu\text{s}$). The percentage of helix and β -sheet are indicated in parentheses, respectively	33
Figure 2.1. Schematic representation of cell separation techniques. A) Positive selection of astrocytes. B) Negative selection of neurons. C) Customizable positive selection of target cells	45

Figure 2.2. Simple Western automated procedure.....	52
Figure 2.3. Workflow for sample preparation and array processing.	64
Figure 2.4. Schematic representation of amplified cRNA procedure. Generation of cRNA for a two-color microarray experiment is shown here.	65
Figure 3.1. Gel images of mouse -type, T: colony genotyping after maternal separation. (A) SOD1-G93A transgenic mouse line genotyping image. (B) C9ORF72 transgenic mouse line genotyping image. Wt: wild transgenic.	69
Figure 3.2. Locomotor activity tests applied for NeuroScore evaluation. (A), (B), and (C); tail suspension test, representing severe and mild hindlimb retraction in transgenic SOD1-G93A mouse, and no hindlimb retraction in wild-type counterpart, respectively. (D); rightening reflex measurement (duration: 10 seconds.) and (E) gait analysis; mice were allowed to walk across for 70 cm.	70
Figure 3.3. Phenotypical aspects of NeuroScore stages. (A) NS1: Slight hindlimb retraction, (B), (C) NS2 and NS3: ataxia and tremor during walking, toes are bent backward in NS3, (D) NS4: complete paralysis.	71
Figure 3.4. Rotarod test results of 7, 17, and 19 months old C9ORF72 mice and wild-type counterparts.....	75
Figure 3.5. Relative mRNA levels of marker genes in magnetically sorted (A) oligodendrocytes, (B) astrocytes, and (C) neurons in PND 90 wild-type mice.....	78
Figure 3.6. Relative mRNA levels of marker genes in magnetically sorted (A) oligodendrocytes, (B) astrocytes, and (C) neurons comparing whole-brain samples in PND 90 wild-type mice.	79
Figure 3.7. Gene expressions of MCT1 in sorted oligodendrocytes (A) and MCT4 in sorted astrocytes (B) in PND 90 SOD1-G93A mice.	80
Figure 3.8. MCT1 (A) and MCT2 (B) gene expressions in whole-spinal cord (s.c.) material of PND 90 SOD1-G93A mice.	81

Figure 3.9. MCT4 (A) and COX10 (B) gene expressions in whole-spinal cord (s.c.) material of PND 90 SOD1-G93A mice.	82
Figure 3.10. Gene expressions of <i>MCT1</i> in sorted oligodendrocytes (A) and <i>MCT4</i> in sorted astrocytes (B) in PND 60 SOD1-G93A mice.	83
Figure 3.11. Gene expressions of <i>MCT1</i> in sorted oligodendrocytes (A) and <i>MCT4</i> in sorted astrocytes (B) in 17 MO C9ORF72 mice.	84
Figure 3.12. Protein expressions of MBP, GLT-1, and NeuN markers in sorted oligodendrocytes in PND 90 SOD1-G93A mice.	86
Figure 3.13. Protein expressions of GLT-1, NeuN, and MBP markers in sorted astrocytes in PND 90 SOD1-G93A mice.	87
Figure 3.14. Protein expressions of NeuN, MBP, and GLT-1 markers in sorted neurons in PND 90 SOD1-G93A mice.	88
Figure 3.15. Protein expressions of MCT1 in magnetically sorted oligodendrocytes in PND 90 SOD1-G93A mice.	89
Figure 3.16. Protein expressions of MCT2 in magnetically sorted neurons in PND 90 SOD1-G93A mice.	90
Figure 3.17. Protein expressions of MCT4 in magnetically sorted astrocytes in PND 90 SOD1-G93A mice.	91
Figure 3.18. Protein expressions of (A) MCT1 in magnetically sorted oligodendrocytes, (B) MCT2 in magnetically sorted neurons, and (C) MCT4 in magnetically sorted astrocytes in PND 60 SOD1-G93A mice comparing wild-type controls.	92
Figure 3.19. The effect of lactate treatment on NSC-34 cell line's survival. The line graph (A) shows the effect of selected Na L-Lactate doses on cell viability by percentage, measured by MTS assay. The line graph (B) represents cell viability comparison of the same line and same lactate treatment with nuclear (Hoechst/ PI) staining.	95
Figure 3.20. Relative mRNA levels of marker genes in NSC-34 cells.	96

Figure 3.21. Relative mRNA levels of <i>MCT1</i> , <i>MCT2</i> , and <i>MCT4</i> genes in NSC-34 cells..	97
Figure 3.22. Area graphics of 0-40 mM L-lactate standards and L-lactate representation collected from media at t=0 min, which show the amount of lactate obtained by processing the data of HPLC (LC-MS/MS) assay via Masslynx Version 4.1.....	98
Figure 3.23. Area graphics of negative and positive controls of t=2, 4, and 8 min, which show the amount of L-lactate in media obtained by processing the data of HPLC (LC-MS/MS) assay via Masslynx Version 4.1.....	99
Figure 3.24. Area graphics of negative and positive controls of t=16 and 32 min, which show the amount of L-lactate in media obtained by processing the data of HPLC (LC-MS/MS) assay via Masslynx Version 4.1.....	100
Figure 3.25. The time-dependent inhibition of L-lactate uptake by AR-C155858.....	101
Figure 3.26. The effect of inhibition of L-lactate uptake by AR-C155858 for various time points on cell viability.....	102
Figure 3.27. Rotarod test results of mice before and after SPT, NaB and BA applications. (A) Rotarod test apparatus, (B) Bar graphic of the motor performances including before and after boron applications.....	105
Figure 3.28. Hanging wire grip test results of mice before and after SPT, NaB, and BA applications. (A) Hanging wire test apparatus (B) Bar graphic of the hanging performance of mice including before and after boron applications	106
Figure 3.29 Heatmap image of microarray data obtained from spinal cord materials of NaB, SPT (transgenic) and water treated (transgenic and wild-type) groups.	108
Figure 3.30. Scatter plot demonstration of the up-regulated and down-regulated genes in the (a) transgenic NaB, (b) SPT group, and (c) water treated wild-type group compared to the transgenic water group. The colour range represents $\pm$ fold change is indicated. SC: Spinal cord material	109

Figure 3.31. First part of heatmap representation of microarray data of selected genes belonging to spinal cord materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups	115
Figure 3.32. Second part of heatmap representation of microarray data of selected genes belonging to spinal cord materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups	116
Figure 3.33. Molecular function, biological processes and protein classes of the genes, which are up and down-regulated as a result of NaB treatment in spinal cord	117
Figure 3.34. Molecular function, biological processes and protein classes of the genes, which are up and down-regulated as a result of SPT treatment in spinal cord.....	118
Figure 3.35. Related pathways of the genes, which are down-regulated as a result of NaB treatment in spinal cord.....	119
Figure 3.36. Related pathways of the genes, which are up-regulated as a result of NaB treatment in spinal cord.....	120
Figure 3.37. Related pathways of the genes, which are down-regulated as a result of SPT treatment in spinal cord.....	121
Figure 3.38. Related pathways of the genes, which are up-regulated as a result of SPT treatment in spinal cord.....	122
Figure 3.39. Heatmap image of microarray data obtained from brain materials of NaB, SPT (transgenic) and water treated (transgenic and wild-type) groups.....	124
Figure 3.40. Scatter plot demonstration of the up-regulated and down-regulated genes in the (a) transgenic NaB, (b) SPT group and (c) water treated wild-type group compared to the transgenic water group. The colour range represents $\pm$ fold change is indicated. B: Brain material.	125
Figure 3.41. Heatmap representation of microarray data of selected genes belonging to brain materials of NaB, SPT (transgenic) and water treated (transgenic and wild-type) groups.....	128

Figure 3.42. Molecular Function, biological processes and protein classes of the genes, which are up and down-regulated as a result of NaB treatment in brain.....	129
Figure 3.43. Molecular Function, biological processes and protein classes of the genes, which are up and down-regulated as a result of SPT treatment in brain	130
Figure 3.44. Related pathways of the genes, which are down-regulated as a result of NaB treatment in brain	131
Figure 3.45. Related pathways of the genes, which are up-regulated as a result of NaB treatment in brain	132
Figure 3.46. Related pathways of the genes, which are down-regulated as a result of SPT treatment in brain	133
Figure 3.47. Related pathways of the genes, which are up-regulated as a result of SPT treatment in brain	134

LIST OF TABLES

Table 2.1. Primers used in transgenic mice genotyping.	37
Table 2.2. The content of PCR mixture.	37
Table 2.3. The experimental protocol for standard PCR.	38
Table 2.4. Number of transgenic and wild-type mice used in the experiments (WO: weeks old).	42
Table 2.5. Primers used in cell characterization qPCR.....	48
Table 2.6. The content of PCR mixture.	49
Table 2.7. Experimental protocol for qPCR.	49
Table 2.8. Solutions used in western blotting.	51
Table 2.9. Information about the laboratory animals used for boron application.	59
Table 2.10. Information about the animal groups used for boron application.....	60
Table 2.11. Information about the animal groups used for microarray analysis.	63
Table 3.1. Detailed comparison of the phenotypic characteristics of the NS1 stage in the experimental groups (wt: wild-type, tg.: transgenic, s: symptom, s1: hindlimb retraction, s2: tremor, s3: ataxia).....	104
Table 3.2. Regulation of genes in the treatment groups belonging to certain important pathways in samples collected from spinal cord materials.....	110
Table 3.3. Regulation of genes in the treatment groups belonging to certain important pathways in samples collected from brain materials. Tg: transgenic, wt: wild-type.....	127

LIST OF SYMBOLS/ABBREVIATIONS

ACSA-2	Astrocyte specific cell surface antigen
AD	Alzheimer's disease
AlCl ₃	Aluminum chloride
ALS	Amyotrophic lateral sclerosis
ALS2	Alsin
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANG	Angiogenin
ANLSH	Astrocyte-neuron lactate shuttle hypothesis
AP5	D-2-Amino-5-phosphonopentanoic acid
ATP	Adenosine tri-phosphate
A β	Amyloid beta
BA (B(OH) ₃)	Boric acid
BAC	Bacterial artificial chromosome
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BNNTs	Boron nitride nanotubes
BSA	Bovine serum albumin
Ca	Calcium
CaCl ₂ .	Calcium chloride
CAT	Catalase
CD11	Integrin α_M
CD147	Basigin
cDNA	Complementary DNA
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10
CNP	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNQX	Cyanquinoxaline (6-cyano-7-nitroquinoxaline-2,3-dione)
CNS.	Central nervous system
CNTs	Carbon nanotubes
COPD	Chronic obstructive respirator disorder
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats

C9ORF72.	Chromosome 9 open reading frame 72
DH ₂ O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo nucleic acid
DNase	Deoxyribo nuclease
ER stress	Endoplasmic reticulum stress
fALS	Familial amyotrophic lateral sclerosis
FDA	Food and drug administration
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GABA	Gamma aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein.
GLAST	L-Glutamate /L-Aspartate transporter
GLT-1	Glutamate transporter 1
GSH-Px	Glutathione peroxidase
Gp70	Embigin
ASN	Hexagonal boron nitrides
hBNs	Independently and identically distributed
HCAR	Hydroxycarboxylic acid receptor 1
HDFs	Human dermal fibroblasts
HUGO	Human genome organization
HUVEC	Human umbilical vein endothelial cell
H ₂ O ₂	Hydrogen peroxide
IGF1	Insulin-like growth factor 1
iPSC	Induced pluripotent stem cell
INs	Interneurons
K	Potassium
KCl	Potassium chloride
kDa	Kilodalton
K _m	Michaelis constant

K ₂ Cr ₂ O ₇	Potassium dichromate
LDH	Lactate dehydrogenase enzyme
MAP2	Microtubule-associated protein 2
MATR3	Matrin-3
MBP	Myelin basic protein
MCS	Magnetic cell sorting
MCTs	Monocarboxylate transporters
MCT1	Monocarboxylate transporter 1
MCT2	Monocarboxylate transporters 2
MCT4	Monocarboxylate transporters 4
MDA	Malondialdehyde
MgCl ₂	Magnesium chloride
mM	Millimolar
MN	Motor neuron
MPP ⁺	1-methyl-4phenylpyridinium
MS	Multiple sclerosis
MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4 sulfo-phenyl)-2H-tetrazolium
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaHCO ₃	Sodium bicarbonate
Na/K ATPase	Sodium/ potassium adenosine triphosphatase
NBa	Sodium pentaborate pentahydrate
NEK1	NIMA-related kinase 1
NeuN	Neuronal nuclei protein
NMDA	N-methyl-D-aspartate receptor
NPCs	Neural progenitor cells
NS	Neuro score
OLIG-2	Oligodendrocyte transcription factor
OPCs	Oligodendrocyte progenitor cells
OPTN	Optineurin

PBA	Phenylboronic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PEN1	Profilin-1
PMSF	Phenylmethylsulfonyl fluoride
PND	Postnatal day
Ppm	Parts per million
PSA	Penicillin/streptomycin/amphotericin
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RAN	Repeat associated non-ATG translation
RIN	RNA integrity number
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNPs	Ribonuclear proteins
ROS	Reactive oxygen species
Rpm	Revolutions per minute
sALS	Sporadic amyotrophic lateral sclerosis
SETX	Senataxin
SDS	Sodium dodecyl sulfate
shRNAs	Short-hairpin RNAs
Sirt1	Sirtuin (silent mating type information regulation 2 homolog) 1
SNs	Sensory neurons
SOD1	Copper/zinc superoxide dismutase
SpMNs	Spinal motor neurons
SPT	Sodium perborate tetrahydrate
TAE	Tris acetate-EDTA
TAS	Total antioxidant status
TBI	Traumatic brain injury
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline

TCA	Tricarboxylic acid cycle
TDP43/TARDBP	Transactive response DNA binding protein 43
TOS	Total oxidant status
TUBA4A	Tubulin, alpha 4A
UBQLN2	Ubiquilin-2
VCP	Valosin-containing protein
WT	Wild-type
μs	Microsecond



1. INTRODUCTION

1.1. WHAT IS ALS DISEASE?

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is one of the most lethal neurodegenerative diseases affecting the motor neurons (MNs) [1]. The disease is characterized by progressive degeneration of MNs in the brain stem, motor cortex, and spinal cord, causing paralysis of muscles and eventually death [2]. The death of MNs in the spinal cord severely impair basic and vital movements, including breathing, and patients usually die due to respiratory failure [1].

The National ALS Registry reports that more than 12,000 people are diagnosed with ALS, with a prevalence of 3.9 cases per 100,000 people in the U.S. The ALS Association also reported that almost 6,000 people have a diagnosis of ALS each year [3]. The disease onset lies between the age of 51 and 66 [4].

Roughly estimated, it can be said that approximately 1,500 to 4,500 patients are diagnosed with ALS each year in Turkey, and a total of 8000-10000 patients are living with ALS. Today, unfortunately, there is no epidemiological study carried out on ALS disease in Turkey. The majority of ALS patients (90 percent) die within 5-7 years following the disease onset. The remaining 10 percent consists of those who live up to 15-20 years and the world's longest-living ALS patient, Stephen Hawking, is the first one with 50 years [5]. Patients who lose their breathing ability require breathing aid equipment, which is crucial for the survival of patients [1].

ALS is a fatal neuromuscular illness described as the degeneration of both upper and lower MNs. Upper MN cell bodies are presented in the primary and pre-motor sites of the cerebral cortex. Since there are glutamatergic connections between upper MNs and lower MNs localizing in the central nervous system (CNS), commands are transferred toward the lower MNs from upper MNs [6]. Upper motor neurons constitute about 150,000 of two billion cells in the brain, which means, mathematically, they could be considered insignificant, however, their function is quite important [7]. Lower MN cell bodies are presented both in the ventral horn of the spinal cord and specific nuclei in the brainstem and thus, similar to upper MNs, are settling within the CNS. Their axonal projections and

connection exceeding CNS (Figure 1.1.) is one of the important properties of lower MNs. MNs are cholinergic and transfer signals from upper MNs, interneurons (INs), and from sensory neurons (SNs) [6].

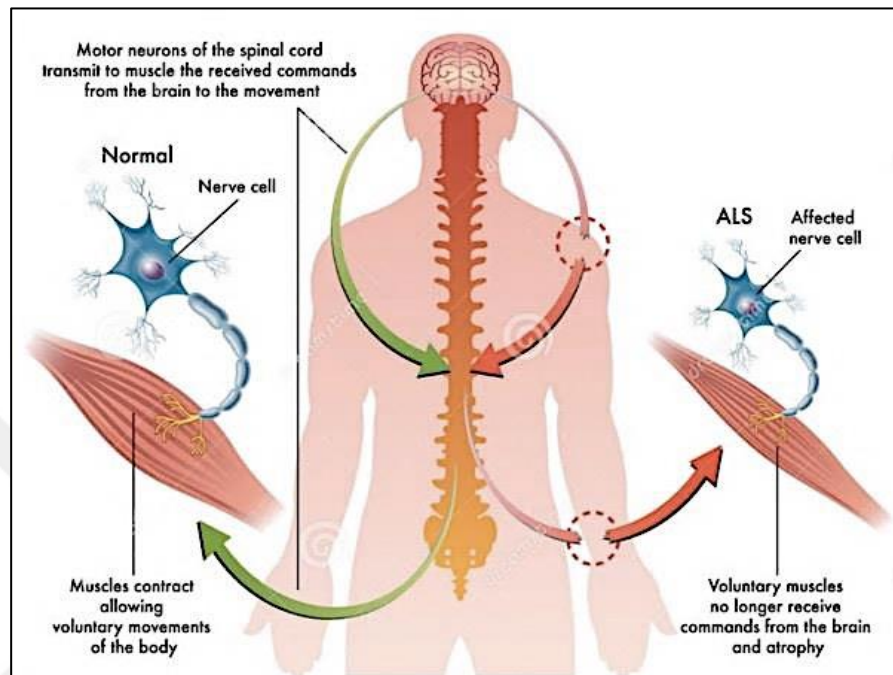


Figure 1.1. Muscle atrophy and limitation of movements as a consequence of motor neuron degeneration in ALS disease [8]

Cell bodies of lower MN are primarily localized in ventral horn of the spinal cord and also specific nuclei in the brainstem, thus they are located in the CNS, similar to upper MNs. The important properties of lower MNs are their long axonal projections and connections exceeding CNS (Figure 1.2.). Lower MNs are cholinergic and provide signal transduction throughout upper MNs, sensory neurons (SNs), and interneurons (INs) [6].

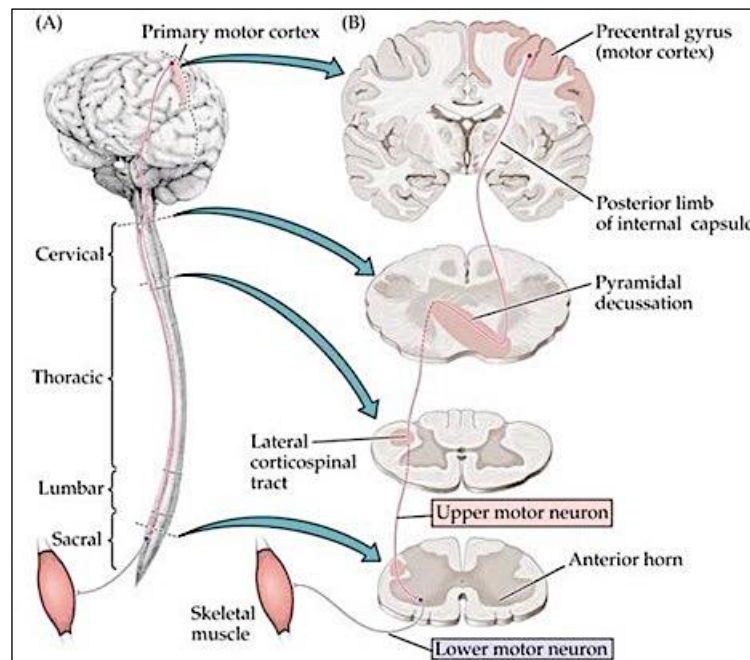


Figure 1.2. Schematic representation of the central nervous system [9]

As mentioned above, although the physiological aspects of ALS disease were demonstrated many years ago, the laying mechanisms behind its etiology are still not elicited completely and still under investigation.

1.2. ALS ETIOLOGY

Although the disorder exhibits a dominant inheritance in around 10 percent of ALS sufferers (familial), most of the cases are sporadic having an unclear genetic background [10]. In cases where there are two or more members with ALS in the family, the pathology is evaluated as familial [11].

The particular vulnerability of motor neurons in ALS has been subject to many studies. Several mechanisms, such as oxidative stress, glutamate excitotoxicity, mitochondrial malfunction, declined neurotrophic support, changed glial function, perturbations of RNA processing, the toxicity of misfolded proteins, declined axonal transport, and neuroinflammation has been associated with the degeneration of MNs in ALS up to now [12]. Moreover, their specific intrinsic properties, including their long axonal processes and size (the most massive neurons in overall CNS), high metabolic levels, and weak

calcium-buffering capacities, can be the key elements for a better understanding of their susceptibility. Nevertheless, according to the latest data, glial cells also have a role in the disease development [13]. In spite of important progress providing a better perception of pathological formation related to the disease, there is still a lack of knowledge of MN degeneration in ALS, which obstructs finding out therapeutic strategies such as drug development [14].

As of today, U.S. Food and Drug Administration (FDA) has approved five drugs to treat ALS including Radicava, Rilutek, Tiglutik, Exservan, and Nuedexta. Rilutek, Tiglutik, and Exservan drugs contain different forms of Riluzole which suppresses the glutamate response to decrease the emerging pathology, approved in 1995 [15]. On the other hand, Radicava (edaravone, previously MCI-186), approved in 2017, is the first treatment for ALS that FDA approved in more than 20 years, which mitigates the effects of oxidative stress acting as a free-radical scavenger [15,16].

It has been reported, however, that Riluzole extends the life span of ALS patients by barely three months, which is only if the patients are in the early stages. For ALS sufferers in the late stages, no decline in the symptoms is observed, pointing out the inadequacy of therapeutic alternatives for the disease [17]. Therefore, cell transplantation therapy stands out among many alternative therapeutic approaches for its potential to allow the amelioration of MNs. The latest application of non-autologous fetal spinal stem cell transplantation for patients started a new era in the treatment of the disease and paved the way for more human trials. As one of those trials, human autologous somatic stem cell-based treatment trials are currently progressing at a rapid pace. Besides autologous and non-autologous cell transplantation options, induced pluripotent stem cell (iPSC)-based drug screening is also one of the promising new cell-based treatment methods for ALS [1].

1.3. GENES INVOLVED IN ALS PATHOGENESIS

Nowadays, clinicians have understood that the sporadic and familial ALS cases classification methods are no longer strictly valid, and genetics still seems to be an important player in both sporadic ALS (sALS) and familial ALS (fALS). Approximately 20 genes within the gene pool, which accounts for 60 percent of the genes associated with familial ALS, were determined to be associated with ALS pathology [16]. The most

prevalent fALS mutations are found in genes copper-zinc superoxide dismutase 1 (*SOD1*) and *TARDBP* [18,19] (responsible for the synthesis of transactive response DNA binding protein 43, which is also called TAR DNA binding protein [20]) and also GGGGCC repeat in the non-coding region of *C9ORF72* [1].

Together with findings of novel genes linked with fALS, relevant animal models were generated in order to analyse and explore the mechanisms of cell death, moreover to develop more advanced therapeutic strategies against the disease [17]. In addition to *in-vitro* studies, employment of the transgenic animals highlighted that the mutations present in those genes lead to excessive endoplasmic reticulum (ER) stress, mitochondrial disruption and production of reactive oxygen species (ROS), insulin-like growth factor (IGF1) dependence for survival, glutamate excitotoxicity, and accumulation of neurofilaments, especially in MNs. Unfortunately, researchers studying these pathologies have not achieved a clear success in the treatment of ALS yet [1].

1.3.1. *SOD1*

Copper/zinc superoxide dismutase (*SOD1*) is a member of the superoxide dismutase family and is in charge of superoxides conversions in order to prevent cell damage [20]. The missense mutations found within the gene encoding *SOD1* account for 12-13 percent of the autosomal dominant inheriting fALS cases [18]. The first genetic link to ALS has been discovered by studies, which report mutations in Cu/Zn superoxide dismutase-1, the *SOD1* gene encoding the antioxidant enzyme *SOD1* [22]. Up to now, approximately 160 missense point mutations have been defined in the *SOD1* gene, and at least 75 of *SOD1*'s 154 amino acids have been determined to undergo mutation in ALS and their respective locations are distributed throughout the five exons of the gene [22,23]. Interestingly, the loss of *SOD1* gene expression does not result in neurodegeneration in mice, instead, the ALS pathologies were observed specifically in tissues belonging to the transgenic animals characterized by expression of mutant *SOD1* in high levels [21,23]. Up to date, the most widely used *SOD1* mutation is the base change corresponding to the conversion of glycine 93 to alanine (G93A). The studies carried on transgenic lines having this mutation revealed that impairment in this line, takes place among fast-twitch fatigable motor units, followed by fast-twitch fatigue resistance, and eventually a late phase deprivation of the slow-twitch

fatigue-resistant subtype of motor neurons [24,25]. In humans, however, this kind of progress in motor neuron degeneration could not be precisely put forward by any research. The distal neuromuscular junctions are suggested as the initial sides for the degeneration according to the results obtained from studies performed in SOD1-G93A mouse model and human autopsy material [27,28]; nevertheless, further evidence acquired from both animal models and humans is required. Generally, previous studies have focused on clarifying either cell or non-cell-autonomous response to the toxicity arising from mutated SOD1 activity [29]. In one of those studies, motor neurons gave decreased survival rates when they were co-cultured with astrocytes expressing mutant SOD1. Interestingly, their viabilities seemed to be affected when they were exposed to the conditioned media derived from mutant astrocytes. Astrocytes obtained from sALS neural progenitor cells (NPCs) of post-mortem spinal cord in co-culture systems with motor neurons also lead to motor neuron death specifically [2,30]. In transgenic fALS mice, even in the presence of endogenous, intact SOD1, mutant SOD1 leads to the development of an ALS-like phenotype, pointing out the role of SOD1's toxic gain of functions in ALS disease. In concordance with that, in astrocytes, shRNA-mediated knockdown of mutant SOD1 was reported to completely ameliorate motor neuron deterioration [29]. In addition to the toxicity generated by mutant SOD1 derived from fALS, there had been an ongoing debate in recent years that wild-type (wt) SOD1 might accumulate by undergoing conformational change, presumably due to abnormal posttranslational modifications, thus takes part in also sALS pathology [31,32]. Re et al. in 2014, [34] knocked down wild-type SOD1 in sALS-derived astrocytes utilizing shRNA constructs to investigate whether wt SOD1 take a part in motor neuron loss. Notably, it is observed the knockdown of wt SOD1 did not appear to have a supportive role in motor neuron loss in the experiment. All this evidence suggests a common astrocyte-dependent disease mechanism in sporadic and familial ALS. Even though these studies prove that astrocytes boost motor neuron degeneration, the mechanism lying behind the astrocyte-mediated toxicity is still not completely resolved [2].

1.3.2. *C9ORF72*

In 2011, *C9ORF72* gene variants were discovered, which are known as the most frequently encountered genetic reason for ALS disease as up today. Chromosome 9p21 has been

linked with ALS and the repeated expansion of hexanucleotide GGGGCC in the chromosome 9 open reading frame 72 (*C9ORF72*) gene has been defined as one of the hereditary reasons for the disease development [32]. These expansions account for approximately 25.9 percent of the familial [33] and around 7 percent of the sporadic cases as well. The mutation in this gene exhibits a dominant inheritance. This gene also plays a triggering role in 25 percent of other neurodegenerative disorders, including frontotemporal dementia (FTD). How *C9ORF72* leads to disease development is still the main subject of a large number of intense studies. Current studies highlighted that *C9ORF72* hexanucleotide repeated expansion may produce insoluble polypeptides specific to C9RANT antibody immunoreactivity via repeat-associated non-ATG (RAN)–initiated translation [35], which is not observed among other neurological disorders disease, such as CAG repeat disorders [36,37]. Results acquired from these studies could help to understand the ALS mechanisms more comprehensively and to develop therapeutic strategies targeting RAN-translated peptides [36–38].

1.3.3. *NEK1*

NEK1 (NIMA-related kinase 1) mutations discovered by the contribution of Project MinE, in 2016 are found in both sporadic and familial cases. Totally, 3 percent of the ALS cases are related to the *NEK1* gene. sALS cases and independent controls revealed a remarkable link with the disease in both p.Arg261His mutation and *NEK1* loss of function variants via genetic analyses [39,40]. *NEK1* protein has been linked with a number of functions in cellular metabolism, including microtubule stability, cilia formation, neuronal morphology, axonal polarity, and DNA-damage response. These findings help to develop novel and promising approaches for enlightening ALS pathogenesis and genetic etiology. However, the role of *NEK1* in ALS disease development, its inheritance, and other necessary questions are still the subject of intensive investigations [40].

1.3.4. *TARDBP*

The *TARDBP* gene provides instructions for the synthesis of a protein [41] known as transactive response DNA binding protein 43, which is also called as TAR DNA binding

protein (TDP-43) [19], and was linked with the pathology of ALS in 2008. *TARDBP* gene variants lead to a dominant form of ALS and constitute approximately 4 percent of fALS and approximately 1 percent of sALS [3]. As a member of the ribonucleoprotein family, TDP-43 is a DNA/RNA-binding protein. It is involved in many processes, including gene transcription, microRNA processing, RNA splicing, and maintenance as well as the transfer of mRNA [43]. In ALS, ubiquitinated TDP-43 is one of the most prevalent elements of inclusions in cytoplasm [42]. *TARDBP* gene mutations cause the misplacing of TDP-43 protein in MNs, at a distance from the nucleus where it is typically located, and into the cytoplasm, where it forms clumps by aggregating, which can be observed via microscope. In approximately all cases of ALS (aside from that of SOD1), although *TARDBP* mutations are not present, abnormally accumulated TDP-43 protein can be seen implying that TDP-43 presumably displays an agitating effect in many types of ALS [44].

TDP-43 mutations in the C-terminal glycine-rich region are in charge of protein-protein interactions between TDP-43 and other ribonuclear proteins (RNPs) [45]. Moreover, TDP-43's C-terminal fragments are found to have a particular aggregation propensity, proposing that TDP-43 protein's proteolytic cleavage causes insoluble protein aggregates, dysfunction in protein binding, or another toxic degeneration in ALS [46]. In 2009, Wegorzewska et al. reported a TDP-43 induced neurodegeneration that contains characteristics of both ALS and FTD, such as aggregation of ubiquitin, co-occurring with a specific SpMNs, and cortical neuron vulnerability, without forming TDP-43 inclusions in the mouse model [47].

Collected data from the transgenic TDP43^{A315T} mice (a transgenic mice model carrying a human TDP-43 construct possessing the A315T mutation observed in fALS [48]) studies show that the mutant protein may cause toxicity regardless of TDP-43 aggregates development. On the contrary, it is proposed that soluble TDP43^{A315T} both directly and indirectly (by damaging stability/splicing of various mRNA species) changes pathways associated with protein degradation in various neuronal sites via enhancing ubiquitinated protein aggregation and ultimately causes neurodegeneration [47].

Interestingly, TDP-43 aggregation along with its nuclear reduction is involved in more than 95 percent of ALS and 40 percent of FTD cases. However, nuclear reduction and Proteinopathy of TDP-43 resulting from mutations in the *TARDBP* gene are rare in ALS cases as mentioned previously. Studies have indicated that the aggregation of TDP-43

tends to generate cellular toxicity, and could result in a partial loss of the protein function when accompanied by its nuclear reduction. In that case, the possible role of partial loss of function in TDP-43 in degeneration of MNs and development of ALS should be investigated. As a response to this question, in 2014, Yang et al. knocked down the *TARDBP* gene utilizing RNAi in the amiR–TDP43i Tg mouse model (a transgenic mouse model which carries an artificial miRNA (amiR-TDP43), targeting the mouse TDP-43 protein mRNA for knockdown [48]). The study elicited that in this model, ALS symptoms emerged in a short time, and a partial loss of function in TDP-43 and MN degeneration were observed [49].

1.3.5. *FUS*

In 2008, fused in sarcoma (*FUS*) gene variants were found out to take part in ALS. It accounts for approximately 4 percent of fALS cases and 1 percent of sALS cases. *FUS* is a RNA binding protein and shows a quite resemblance with TDP43 structurally. In ALS, approximately 50 gene variants of the *FUS* gene have been identified up until now, which are majorly dominant, missense changes in and nearby C-terminal nuclear localization signal [51]. The discovery of mutations in the *TARDBP* that causes ALS enabled the subsequent detections of variants for the gene responsible for the generation of fused in sarcoma (*FUS*) protein [52]. Along with this, researchers proposed that common pathways of neurodegeneration in ALS may base on abnormal RNA mechanisms and started to examine more intensively the normal function of *FUS* and TDP-43, together with other ALS-related RNA-binding proteins [53].

In 2016, Sharma et al. revealed that they produced a transgenic mice line consisting of a structure expressing either wild-type (hFUS^{WT}) or ALS-associated human *FUS* mutant variants; hFUS^{R521C} and hFUS^{P525L} in order to understand which ALS-causing mutations in *FUS* associate with motor neuron degeneration. Results showed that mutant hFUS impair MNs and that the pathogenic allele in ALS sufferers; hFUS^{P525L} also leads to earlier and more evident motor neuron degeneration compared to hFUS^{R521C}. Notably, similar to SOD1, the normal activity of *FUS* alone, is found not to be crucial for the long-term survival of MNs [54].

1.3.6. *TP73*

TP73 (tumour protein 73 [54]), a member of the tumour suppressor *TP53* gene family, induces the p57 protein generation, which is found as two different main forms, TA-p73 and delta-N-p73 (Δ N-p73). While transcriptionally active TA-p73's primary duty is to trigger target genes that will later induce cell death, Δ N-p73, in an opposite manner, hinder both TA-p73 and p53 (encoded by *TP53*) activity, taking part in controlling cell division and apoptosis [56]. In CNS, p73 is mostly found in the form of Δ N-p73 and ensures nerve cell viability by guarding against apoptosis that can be promoted by TA-p73 and p53 proteins [57].

In this context; an exome sequencing study published in 2021 [57] has defined a number of mutations in the tumour suppressor gene *TP73* in patients sALS patients [58,59]. 87 patients' exome sequences and 324 controls were analysed using the sequencing data of an independent ALS cohort of more than 2800 patients for confirmatory purposes. While four heterozygous, nonsynonymous, and rare mutations in *TP73* were detected in the sALS cohort of the study, additional 19 rare, deleterious variants in *TP73* were defined in the independent, confirmatory ALS cohort. Moreover, the myoblast differentiation assay results also showed that *TP73* mutations cause abnormal myotube formation and abnormal differentiation, and also possess an apoptosis-activating effect in patients.

In a zebrafish model in which the *TP73* was knocked out using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technique, inconsistent with ALS pathology characteristics, degenerated motor neuron formation and deterioration in axonal morphology have been observed. In addition, the immunoprecipitation assay performed with mutant Δ N-p73 protein demonstrated that these mutations in patients restricted the capability of Δ N-p73 to interact with P53 [58]. Considering all these findings, it is understood that apoptosis caused by mutations in the *TP73* gene presumably take part in the ALS pathology, revealing that the concept of apoptosis may also exist in ALS disease and lead to the exploration of new therapeutic agents that could prevent the apoptosis caused by mutant *TP73* [58,59].

1.3.7. Other ALS Related Genes

There are other mutated genes of ALS affecting comparatively fewer people. However, understanding their underlying role in disease development may provide a higher awareness of disease progression, which could contribute to the discovery of new therapeutic agents. VCP (valosin containing protein), UBQLN2 (Ubiquilin-2), *ALS2* (alsin), *OPTN* (optineurin), *ANG* (angiogenin), *MATR3* (matrin-3), *CHCHD10* (coiled-coil-helix-coiled-coil-helix domain containing 10), *PFN1* (profilin-1), *SETX* (senataxin), *TUBA4A* (tubulin, alpha 4A), *TBK1* (TANK-binding kinase 1) [60], and *C21ORF2* (chromosome 21 open reading frame 2 [60]) are among those genes. Remarkably, almost all of these gene mutations are found in proteins with resembling functions. Mutations can be divided into two groups, those related to cell-axon association and those related to the cell's clearance mechanism [3].

1.4. NEW INSIGHTS: ROLE OF MCT EXPRESSION IN ALS PATHOGENESIS

Latest studies asserted that oligodendroglia and/or their precursors might be defected in the SOD1 transgenic mouse model [62,63] In 2012, Lee et al. suggested that the decreased capacity of oligodendroglia in white and grey matter to sustain motor neuron stability, resulted from by down-regulated MCT1 expression, could promote ALS development [14]. However, before discussing the role of MCTs in ALS pathogenesis, lactate metabolism and how MCTs function should be examined more extensively.

1.4.1. The Metabolism of Lactate

Lactate, pyruvate, and the ketone bodies (acetoacetate and β -hydroxybutyrate) are monocarboxylates that have important roles in carbohydrate, amino acid, and fat metabolism. Thus, they have to be quickly transported across the cell membrane [64]. L-lactate, pyruvate, and ketone bodies (e.g., β -hydroxybutyrate and acetoacetate) are monocarboxylates, which are migrated through various brain regions by monocarboxylate transporters (MCTs) [64]. The most abundant monocarboxylate in the nervous system; L-lactate is transferred according to a concentration grade in the CNS metabolism. Mediating

facilitative transport, MCTs play a role in equilibrating substrate concentrations in plasma membranes, depending on the concentration gradients, that is, substrates including L-lactate proceeds generation regions to catabolism regions, between cells or organs via the bloodstream [66]. An apparent efflux of lactate from the brain into the bloodstream occurs at rest. However, as levels of blood lactate increase, for instance, in physical activity, a net lactate influx occurs from blood toward the various brain regions [66].

The adult brain is mainly fuelled by glucose [68]. While the cells are able to reserve glucose as glycogen form, they can also convert it into pyruvate in the glycolysis phase. Under specific conditions, other substrates can also contribute to brain energy demands [69]. ATP is converted into energy in the mitochondria via pyruvate metabolism in two steps; TCA cycle and the following oxidative phosphorylation. The cells convert pyruvate into lactate by lactate dehydrogenase enzyme (LDH) when the rates of glycolysis exceed that of oxidative phosphorylation. The lactate can be released from the cells through MCTs and be transferred to various cells carrying MCTs as well. In the light of these findings, lactate is suggested to serve as an even more preferred energetic fuel in the brain metabolism [70].

Lactate receptor, HCAR1 (hydroxycarboxylic acid receptor 1 [70]), similar to MCT2, is found most abundantly in neurons. HCAR1, [71] are localized on the synaptic membrane, as is MCT2 (Figure 1.7.) and intra-vesicular organelles, presumably showing protein trafficking. As a novel principal, co-localizing of HRC1 with lactate transporter MCT2 on intracellular vesicular compartments contributes to the fact that intravesicular lactate possibly promotes lactate signalling. Most importantly, two proteins together with glutamate receptors at postsynaptic membranes of glutamatergic synapses can be an indication of a close link of lactate with glutamatergic neurotransmission. This association has previously been defined within the scope of the astrocyte-to-neuron lactate shuttle (ANLS) hypothesis [72].

Various studies have repeatedly documented a lactate accumulation, implying the lactic acidosis hypothesis of ischemic damage during cerebral ischemia [73]. The vast majority of these studies also demonstrated a puzzling increase of glucose levels in the brain, which generally exceeded the concentration measured prior to ischemia, as well as the dramatic decrease in lactate concentration following re-oxygenation. This inverse correlation between lactate and glucose levels could very likely point out that lactate might be used

aerobically, causing glucose accumulation since it remains unutilized in the post-ischemic, reoxygenated brain. Pellerin and Magistretti [75] proved the capacity of the astrocyte-derived excitatory neurotransmitter glutamate to induce aerobic glycolytic lactate formation and its neuronal consumption [73].

In 2011, Suzuki and his colleagues including P. Magistretti also reported that, in the hippocampus of rat brain, memory formation triggered a remarkable rise in extracellular lactate concentration generated through glycogen via glycolytic astrocytes. Deterioration in the glial lactate transporters' expression (MCT4 or MCT1) leads to long-term potentiation disruption and amnesia, which is recovered by not glucose, but notably L-lactate [73]. In 2012, Lee et al. investigated how oligodendrocytes metabolically support axons by rather focusing on lactate, released by monocarboxylate transporter-1 (MCT1) [14]. According to the evidence, MCT1 expression decreased in ALS and, therefore, axons are shown to be incompetent to meet their energetic demands during ALS progression [14,75].

These findings reinforce the ideas that lactate has a crucial role in synaptic plasticity maintenance and is also involved in the rescue mechanism in hypoxic brain injuries or diseases arising from impairment in the energy supplementation of the neuronal cells.

1.4.2. Astrocyte-Neuron Lactate Shuttle Hypothesis

In the CNS and peripheral nervous systems, mainly found cells are glia (astrocytes, oligodendrocytes, and microglia) and neuron cells. Among these cells, glia is the most excessively found cell type in the brain with a rate of 90 percent.

Even though the brain only accounts for two percent of the total body weight, it is responsible for 20 percent of the total oxygen consumption at rest in the body. A decrease in the oxygen level in the blood (hypoxia) stimulates neurons and glia and initiates a series of intracellular regulations [77]. Until recently, it has been argued that glucose is the only energy source for the brain. However, only one percent of total body glycogen is found in the brain, and this amount is not sufficient to be evaluated as a carbohydrate reserve by neuronal cells [78]. Therefore, brain cells require exogenous glucose to maintain their vital metabolic activities.

Glucose deprivation, caused by the decrease in its transfer from the blood to the brain, may cause damages in cerebral functions and respiration. Brain regions are particularly vulnerable to hypoglycemia compared to the other organs and tissues. While brain cells take up glucose from the blood and converted it to pyruvate and lactate in the cytoplasm, oxidative respiration takes place in the mitochondria [75,79].

Neuro-glial interactions have significant importance for the endurance of the brain's energy metabolism. According to the conventional suggestions, together with astrocytes, neurons use glucose obtained through oxidative metabolism as a carbon source. Lately, however, evidence implies that this compartmentalization of carbon source utilization should not be limited to cytoplasm and mitochondrion only, but could also include the overall cell metabolism. According to Astrocyte-Neuron Lactate Shuttle Hypothesis (ANLSH), initially suggested by Luc Pellerin and Pierre j. Magistretti in 1994, glutamate; besides its receptor-mediated activity on neuronal excitability, induces glycolysis and lactate production in astrocytes, and the generated lactate is transferred to neurons, especially during neurotransmission (Figure 1.3.) [74,79].

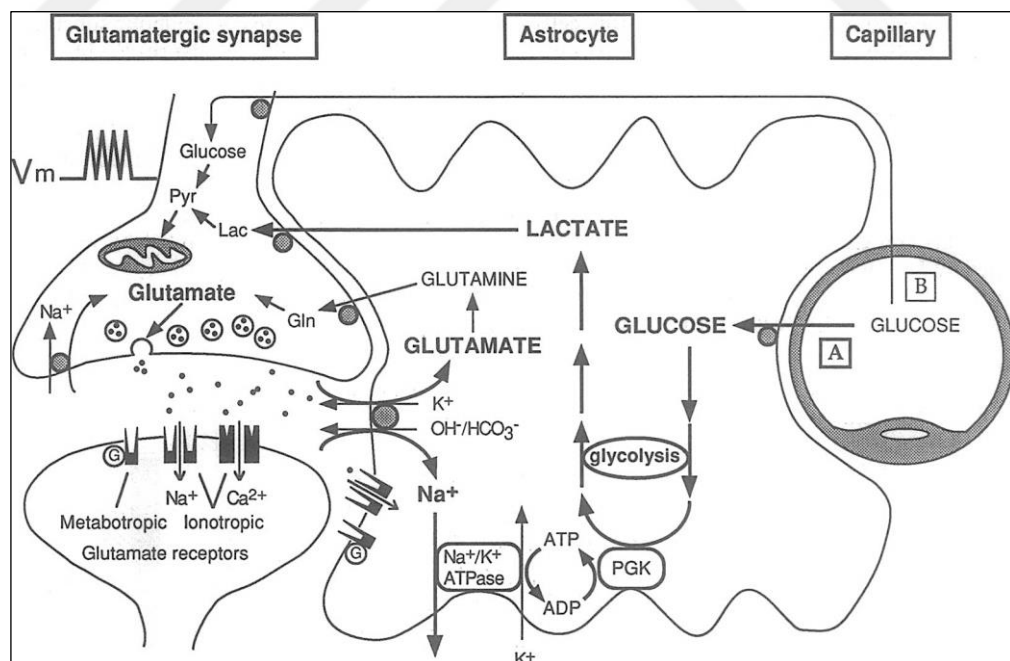


Figure 1.3. Schematic representation of the mechanism for glutamate-induced glycolysis in astrocytes through physiological activation. Pyr: pyruvate, Lac: Lactate, Gln: glutamine, G: guanine nucleotide-binding protein [75]

1.4.3. Monocarboxylate Transporters

The MCTs are the member of the solute carrier 16 (SLC16) gene family as indicated in the Human Genome Organization (HUGO) Nomenclature. Additionally, the Transporter Classification system for functional and phylogenetic classification of membrane transport proteins, approved by the International Union of Biochemistry and Molecular Biology reports that they belong to the monocarboxylate transporter family (2.A.1.13) [69,80]. Up until now, 14 members of the monocarboxylate cotransporter (MCT) family have been identified, of which only the first four (MCT1-MCT4) have been demonstrated to mediate proton-linked transport of metabolically important monocarboxylates including, pyruvate, ketone bodies, and most importantly lactate [82,83]. It is suggested that MCTs possess 12 transmembrane domains with the N- and C-terminals intracellularly localized (Figure 1.4.) [64,83].

α -Cyano-4-hydroxycinnamate is the first developed and the most widely used MCT inhibitor, however, its effect is not specific to MCTs only. After α -cyano-4-hydroxycinnamate AR-C155858 inhibitor (K_i 2 nmol/L for MCT1) was developed by AstraZeneca (Stockholm, Sweden) and reported to inhibit both activities of MCT1 and MCT2, but not that of MCT4 [84]. The inhibitor seems to interact with the cytosolic side collaborating with the C-terminal part of the protein. MCT1 (SLC16A1), MCT2 (SLC16A7), and MCT4 (SLC16A3) are extensively expressed in the brain, while MCT3 (SLC16A8) is limited to the basolateral membrane of the cells in the choroid plexus and the retinal pigment epithelium (Figure 1.5.) [83]. As members of the immunoglobulin superfamily, ancillary proteins are involved in cell recognition, and unlike MCTs, are heavily glycosylated, mediate the catalytic function and membrane localization of the transporters. Basigin (CD147, extracellular matrix metalloproteinase inducer), one of those ancillary proteins, builds a complex with MCT1, MCT3, or MCT4 (Figure 1.4.), on the other hand, embigin (gp70), only interacts with MCT2 [84].

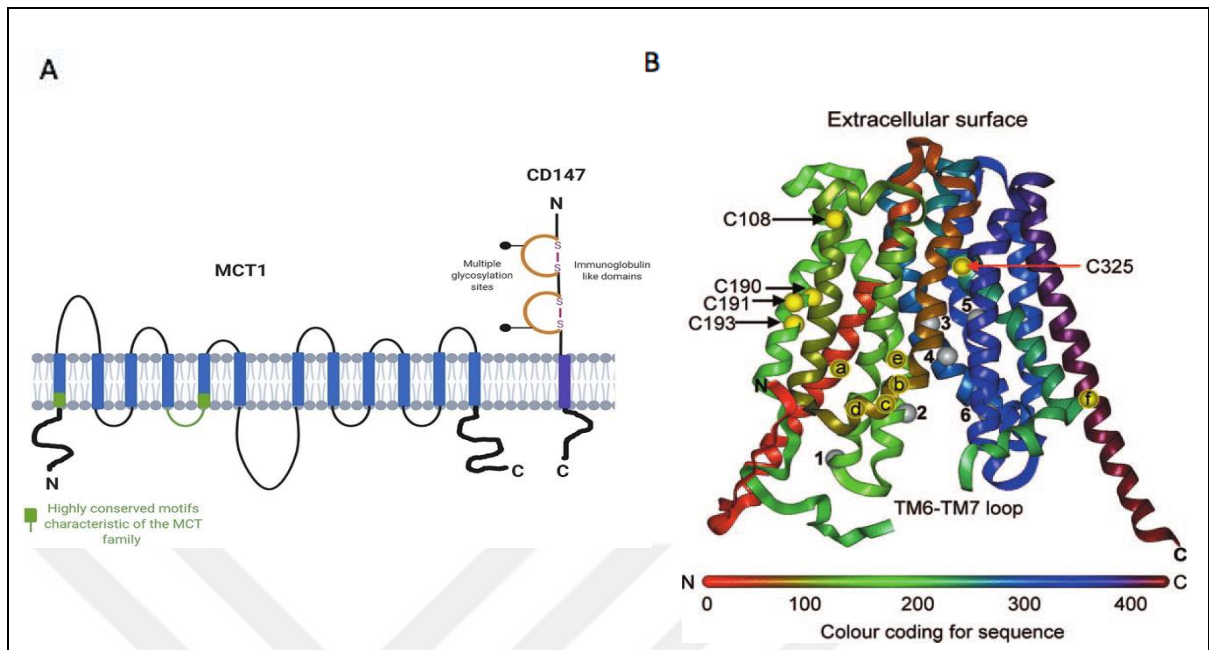


Figure 1.4. The suggested topology of the MCT family members. (A) Predicted structure of MCT1, the N and C termini of MCT1 and, the ancillary protein CD147 that associates with MCT1 and MCT4 are demonstrated as well [81], (B) predicted structure of MCT4 [87]

The MCT isoforms' affinities for lactate and other metabolites are different, in accordance with their tissue distributions: in cells that primarily import lactate, high-affinity isoforms (MCT1 and MCT2) are expressed, whereas in lactate exporting cells the low-affinity isoform (MCT4) is expressed. The transporters' expression on the cell surface appears to be controlled by trafficking between intracellular vesicular organelles and the cell membrane [70].

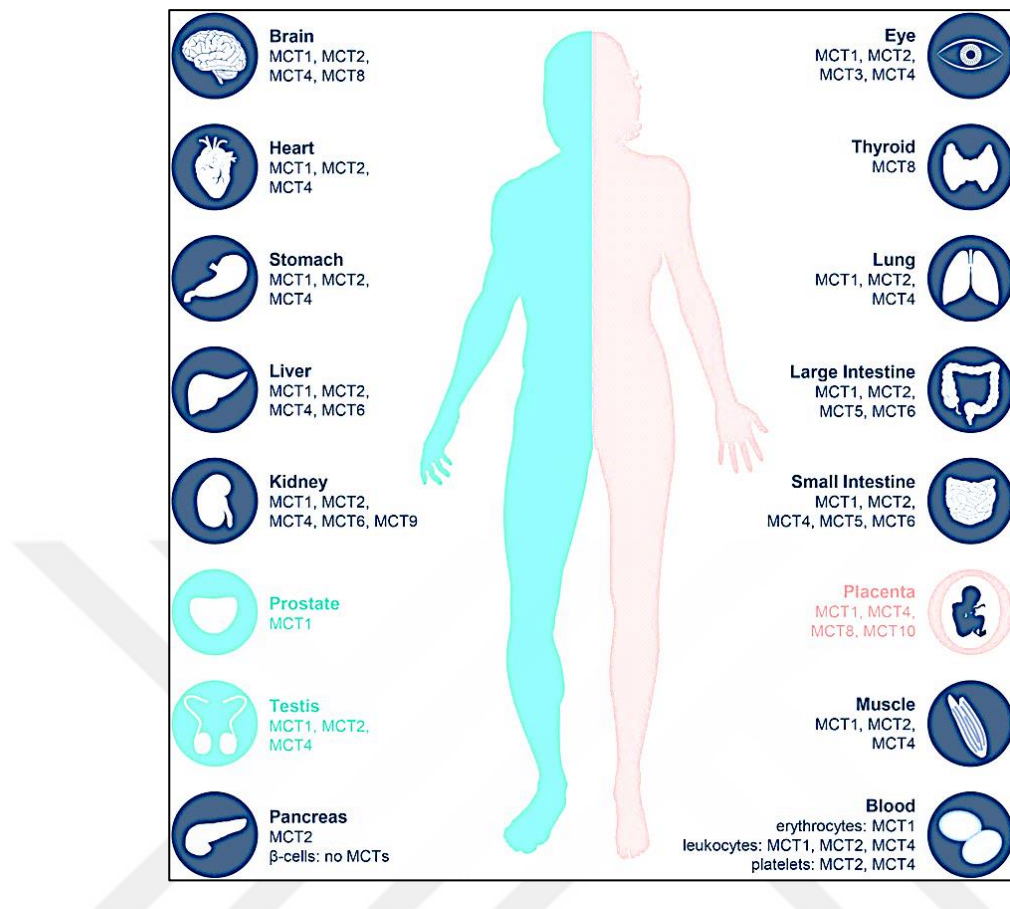


Figure 1.5. Tissue distribution of monocarboxylate transporter (MCT) isoforms [86]

1.4.4. MCT Expression in CNS and Their Involvement in ALS Development

Monocarboxylic acids including lactate, pyruvate, and ketone bodies are imported or exported by the transporters, MCT1, MCT2, and MCT4, located in the CNS. MCT2 is mainly expressed by neurons, and MCT1 and MCT4 are localized in the glial cells, even though MCT1 is the primary glial transporter in the nervous system (Figure 1.6.) [14,86]. Previously, MCT1 was found in and limited to endothelial cells found on the blood-brain barrier (BBB). However, recent immunohistochemistry studies show that MCT1 has also been found in oligodendroglia [89]. MCT4, the low-affinity transporter seems to be limited to astrocytes [88]. Lactate generation during aerobic glycolysis has been demonstrated in astrocytes *in-vitro*, and lactate has been suggested to support neurons in glucose deprivation, probably via MCT2 found in neurons [74]. This hypothetical energetic fuel migration from astrocytes toward neurons was called the astrocyte-neuron lactate shuttle [79] as mentioned before in Section 1.4.2.. *In-vitro* and *in vivo* assays have proved

neuronal support provided by lactate; nevertheless, in the CNS, the function of lactate under normal conditions is largely unclear. In 2012, Lee et al. reported that MCT1 expression has significantly been displayed in oligodendroglia, and suggested that, for axons, the spinal cord is the leading metabolic producer of lactate [14].

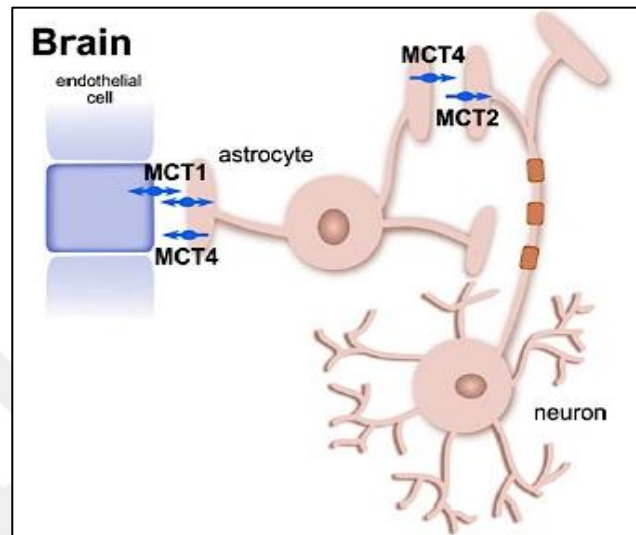


Figure 1.6. Distribution of MCTs in CNS [88]

1.4.4.1. *MCT1 in Oligodendrocytes*

The main function of oligodendrocytes is producing myelin in the CNS. In order to generate myelin sheath of axons eventually, precursor cell lineage undergoes a series of complicated and rigorously timed events including proliferation, migration, differentiation, and myelination and, mature into oligodendrocytes at the end. Oligodendrocytes are one of the most vulnerable cells considering their specific metabolism and the complex differentiation schedule [89]. It has been known that in primary culture, lactate is adopted and utilized by developing oligodendrocytes [90] and organotypic brain slices [93]. During the development period, the amount of substrate required to sustain lipid synthesis increases considerably. Especially at the peak of myelination, oligodendrocytes are obligated to produce lipids at least three times higher than their own cell body weight daily [92]. The myelinating oligodendrocytes, along with their undifferentiated cells are the most sensitive ones to energy deprivation among other brain cells. Glutamate transporters initiate to function in the opposite manner due to this energy deprivation, leading to the accumulation of extracellular glutamate. In addition to that, an impairment caused by

increased intracellular Ca^{+2} levels in oligodendrocyte lines occurs due to enhanced activity of AMPA/kainate and NMDA receptors induced by lack of energy [70].

Furthermore, the same mechanisms cause disruption in glutamate receptors, expressed in axons [95]. Energy deprivation blocks myelination by restricting the utilization of carbon sources required for the production of lipids forming myelin. Thus, for survival and to fulfill their biological needs, oligodendrocytes demand alternative energetic sources such as lactate. Correlatively, organotypic brain cultures in a low-glucose environment indicated a loss of oligodendrocytes and thereby myelin depletion, which could be alleviated by providing exogenous lactate [91]. After myelination formation, the lactate utilization by oligodendrocytes seemingly alters. Axons tend to utilize the lactate released from the myelin sheath in the adult mice model [96] confirming that lactate synthesized through glycolysis in oligodendrocytes may feed the axons, especially the long ones (Figure 1.7.) [95]. These results put forward that in the developmental stage, oligodendrocytes might require lactate for ATP production and lipid synthesis. Once myelination becomes stable, lactate is released triggering other metabolic pathways.

MCT1, in rodents [68] and humans is primarily found in cerebral microvessels and also endothelial cells located on the blood-brain barrier (BBB) (Figure 1.5. and 1.6.) [48]. Due to this critical localization, MCT1 provides a controlled transition of the molecules acting as a second blood-brain barrier, for instance, while lactate levels increases in the blood, during exercise [76]. It possesses an intermediate-to-high-affinity for lactate (about 4 mmol/L) [77]. In 2011, Rinholm et al. show that MCT1 was expressed in oligodendrocytes and myelin sheath as well [71].

The results obtained from MCT knockdown animal models revealed that feeding with lactate is essential for axon stability. In accordance with that, it is also demonstrated that neuronal degeneration and axon deficiency could be overcome by lactate support [14]. Similar damages were observed in patients with ALS, resulting from MCT1 knockdown. Additionally, MCT1 expression was reported to be reduced in oligodendrocytes derived from patients and animal models, as well. For this reason, inadequate lactate intake from oligodendrocytes (or impairment in the upstream mechanisms) may contribute to neurodegenerative disorders, including lactate. Thus, the function of lactate in CNS, particularly in oligodendrocytes and myelinated axons has been subject to intensive studies recently [94].

Recent studies suggest that oligodendroglia or/and their precursor cells could be damaged in transgenic SOD1 mice model [62,63]. According to Lee et al., the decreased capacity of oligodendroglia in grey and white matter to feed motor neurons, due to irregular MCT1 expression indicated in *in-vitro* and *in-vivo* studies, may cause ALS development. In the spinal cord of mutant SOD1-G93A transgenic mice; an extensively preferred model of ALS (Section 1.3.1.) that had been crossed with MCT1–tdTomato BAC reporter mice, MCT1 reporter activity was studied [14]. A reduction in MCT1 mRNA expression was observed in the early symptomatic and late-stage mice's spinal cords. Nevertheless, MCT1 reporter endured to specifically label oligodendroglia all over the brain, even if a significant down-regulation were seen in MCT1 expression in the ventral horn grey matter compared with wild-type counterparts. In the study utilizing human samples, the motor cortex of ALS sufferers' MCT1 and MCT4 expression displayed a reduction higher than 50 percent ($P \leq 0.01$ and $P \leq 0.001$, respectively) when comparing age- and gender-matched control patients. Notably, this decline was not observed in the intact frontal cortex or in the ancillary glycoprotein CD147, also mentioned in Section 1.4.3. [96]. Taken as a whole, these results imply that alterations in oligodendrocyte originated MCT1 may act as an agitating factor in the emerging degeneration of motor neurons in ALS [14,99].

1.4.4.2. MCT2 in Motor Neurons

Motor neurons (MNs) are types of neuronal cells, responsible for many downstream processes in the CNS. They are divided into two main groups, upper motor neurons originating from the cerebral cortex and lower motor neurons originating from the brainstem and spinal cord. As a type of lower MN, spinal MNs (SpMNs) have been largely examined in recent years and shed valuable insight into novel molecular studies. Localized in the ventral horn of the spinal cord, SpMNs regulate effector muscles in the peripheral nervous system. They constitute the ultimate and the most essential element of the neuronal circuitry, as no other direction exists to transfer impulses from CNS to the effector muscles in the periphery. Their axons form an extraordinary anatomical construct by projecting through several meters in mammals, forming the longest known cell type in human body [6]. Since the energy produced in the cell body may not spread adequately through such long cell projections, possible energy deprivations that occur in the long axons of SpMNs can be considered as one of the hypotheses for ALS development [14,100].

MCT2 is the major expressed MCT in the neurons (Figure 1.6.). Its abundance in the postsynaptic membranes of glutamatergic synapses in the spines of Purkinje cells in cerebellum [88] and hippocampal pyramidal cells is quite noteworthy. The lack of MCT2 at inhibitory synapses on the soma of pyramidal cells suggests an association with glutamatergic neurotransmission. Consistent with that, MCT2 is found to be co-localized with the AMPA glutamate receptor's GluR2/3 subunit (Figure 1.8.). As a component of activity-dependent synaptic plasticity, this receptor is seen to take part in trafficking between intracellular vesicular stores in the spines and the plasma membrane [99].

Similar to MCT2, HCAR1 was observed to be found most abundantly in neurons. HCAR1 and MCT2, [102] co-locating on the intravesicular organelles and synaptic membrane, mirror protein trafficking. The co-existence of the lactate receptor with a lactate transporter in intracellular vesicular organelles implies that intravesicular lactate may promote lactate signalling. In conclusion, the co-localization of two proteins with glutamate receptors on the postsynaptic membranes of glutamatergic synapses points out the fact that lactate may involve in glutamatergic neuronal transmission. This involvement has also been suggested in the astrocyte-to-neuron lactate shuttle (ANLS) hypothesis (Section 1.4.1.). Regardless of its origin (generated in astrocytes or adopted from blood), lactate entry into dendritic spines via MCT2 in the postsynaptic membrane contributes to aerobic energy synthesis, which is in accordance with that postsynaptic ion currents utilize the vast majority of the energetic fuel in the brain. Nevertheless, even though mitochondrial activity is essential for spine development and its functioning normally, the synaptic spines occasionally contain their own mitochondria, which means that the exogenous lactate would have to be adopted by the dendrite branch prior to its oxidation and following ATP production (Figure 1.8.) [70]. In concordance with that, the rapid ATP synthesis during neuronal activation seems to be provided primarily by glycolysis [101], instead of oxidative metabolism. The fact that cytosolic glycolysis occurs nearby the sites where ATP is utilized, although the mitochondria are far away, strongly supports this suggestion. Bergersen et al. proposed that in the spine, lactate generated through triggered glycolysis is released via the synaptic MCT2 to induce the co-localization of lactate receptor, behaving as an autocrine in order to limit spine glycolysis levels [70]. In addition to locating in dendritic spines, MCT2 was also detected in axon shafts, [102] reinforcing the hypothesis that astrocytic glycogen provides energy to nearby axons either in low glucose levels or increased neuronal activity

(Figure 1.8.).

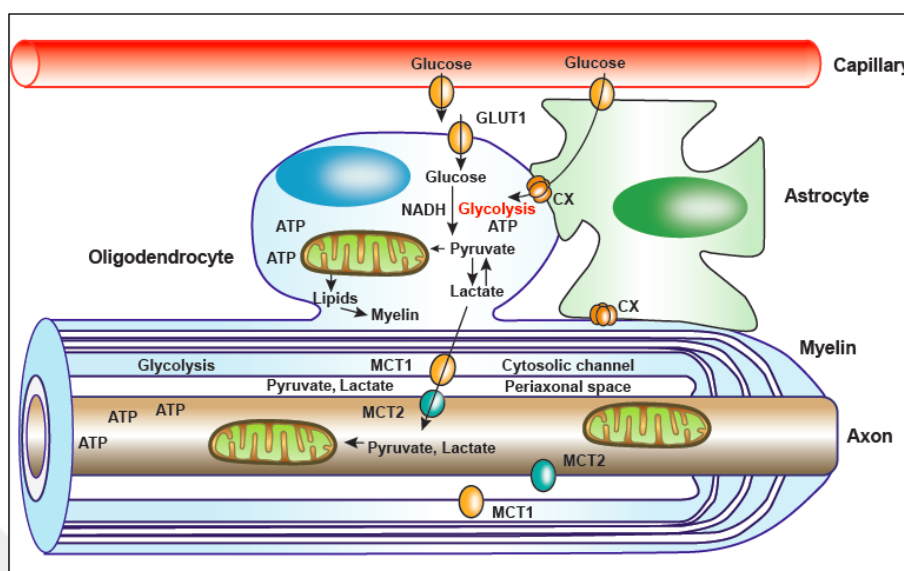


Figure 1.7. Representative image of lactate delivery to axons [103]

1.4.4.3. *MCT4 in Astrocytes*

Glial cells are one of the non-neuronal cell types in the CNS, and astrocytes are a subtype of this category. These highly active, star-shaped cells encompassing neurons in the brain and spinal cord, outnumbering neurons fifty to one, and contrary to previous belief, they are not only filler cells. They are employed in supporting, repairing, and maintaining nervous tissue, as well as in maintaining synaptic plasticity [104,105]. Astrocytes also maintain neurotransmission by providing neurotransmitter recycling, removal of extracellular K^+ ions, and diffusion of Ca^{2+} ions [108]. The other role of astrocytes, as mentioned before, is providing energy source for neurons, which is initially suggested by Pellerin and Magistretti in 1994 and called ANLSH [75].

Astrocytes respond to all forms of structural lesion and degeneration emerging in the CNS through a process called reactive astrogliosis, which serves as a pathological marker. Recently, a large amount of progress have been made in identifying key mechanisms and functions of reactive astrogliosis and in determining casual roles of astrocytes in CNS diseases [109]. Huntington's disease, Alzheimer's disease, stroke, concussions, and certain infections in CNS are some of the neurodegenerative diseases that astrocytes have been found to be involved in [110].

In addition to the aforementioned diseases, Re et al. (2014) found that sALS and fALS-originated adult human astrocytes release neurotoxic substances, which preferentially damage MNs through necroptosis *in-vitro*, asserting a novel treatment route. On the contrary, astrocytes derived from chronic obstructive respiratory disorder (COPD) or Alzheimer's disease patients did not affect the survival of these motor neurons. Moreover, GABAergic originated non-motor neuron cells were also not affected by sALS derived astrocytes. Hence, as suggested in the earlier studies, MNs are presumably possess a particular susceptibility to the toxicity caused by astrocytes in sALS [2].

Astrocytes possess the high-capacity and low-affinity transporter MCT4 (Figure 1.6.-1.8.), on cell membranes of perisynaptic processes and on perivascular end-feet in the brain [88]. MCT4 is generally not saturated owing to the high K_m (about 30 mmol/L), thereby, the level of transport enhances with the lactate concentration. Since the low-affinity term is found to be relatively more appropriate for efflux than influx, MCT4 existence in astrocytes is consistent with the fact that astrocytes are primarily glycolytic and actually the only brain cell type containing glycogen [109]. Initially, MCT1 was demonstrated in astrocytes cultures [112] and MCT2 has also been revealed to be expressed in low levels in astrocytes. The conflict between these findings can be resulted from species or experimental differences. MCT4 is found to express in limited amounts in astrocytes during development and reaches adult levels 14 days postnatally, [111], proceeding the time of synaptogenesis [114].

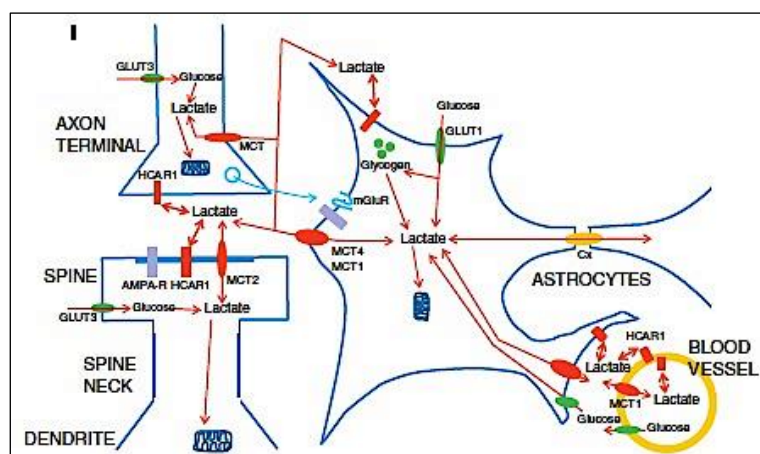


Figure 1.8. Representative image of L-lactate generation and action at the tripartite synapse structure of axon, dendrite, astrocyte, and at the glia-vascular junctions, orange oval [70]

As mentioned in Section 1.4.4.1., Lee et al. reported that in the motor cortex of ALS patients, a higher than 50 percent decrease in MCT4 and MCT1 expressions were observed ($P < 0.01$ and $P < 0.001$, respectively) in comparison with control patients. However, this decline was not shown in the intact frontal cortex, or CD147 [98]. In the light of these findings, it could be proposed that an interruption in MCT4 expression may contribute to the emerging deterioration of energy metabolism in ALS. Thus, MCT4 can be offered as a valuable target in the effective ALS treatment, as well as MCT1 and MCT2.

1.5. NEW INSIGHTS-2: APPLICATION OF BORON AS A NEW APPROACH IN THE TREATMENT OF ALS AND OTHER NEURODEGENERATIVE DISEASES

1.5.1. Overview of Boron

Boron is a type of naturally occurring trace element [115] with the symbol B and atomic number five (Figure 1.9.) [114]. It is not found as an elementary form in nature, it usually forms organoboron complexes with oxygen and sodium. Also, boron is an essential nutrient for many organisms. Especially, organoboron complexes are important for the biological activities of living organisms. Microorganisms need boron for their vital processes such as quorum sensing, antibiotic activity, and nitrogen fixation [117–119].

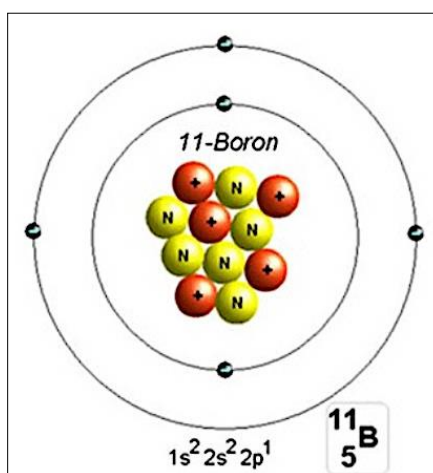


Figure 1.9. Structure of Boron atom [118]

In healthy tissues, boron exists in the form of boric acid or borate. The total amount of boron in the body is approximately 3-20 mg; however, the amount of boron varies according to the structure of the tissues. For instance, creatine-containing tissues such as bones and hair possess the maximum amounts of boron (56 ppm) in the body compared to other tissues, so that lack of boron leads to arthritic diseases in the bone. On the contrary, boron concentrations are quite low when it comes to body fluids. For example, while the boron concentration in the blood is 0.06 ppm, it drops further to only 0.02 ppm in the plasma and around 0.75 ppm in the urine [119].

The accumulated evidence demonstrated that boron shows diverse pleiotropic effects, ranging from antimicrobial, antiviral [120,121] anti-inflammatory [124], antioxidant [123,125] wound healing effects [124], to the modulation of different body systems. In the light of these developments, the designs of boron-based drugs have come to the fore [126,127].

Recent studies concluded that boron was employed in various metabolic pathways, such as wound healing, bone formation and sustenance [126], hormonal activity [129], and psychological functions [116]. It is recently revealed that boron supports short and long-term weight loss when consumed as a dietary supplement [98, 99].

In a recent study, it has been shown that the use of boric acid (BA; H_3BO_3) could be an alternative approach for trichomoniasis treatment due to its anti-microbial effect on *T.vaginalis* [122]. There is also another study proving the antibacterial activity of sodium pentaborate pentahydrate (NaB; $NaB_5O_8 \cdot 5H_2O$) which is another boron derivative, against *Streptococcus mutans* in dental composites [130]. When boron-containing thiosemicarbazones were examined in terms of a potential antifungal effect, acceptable anti-fungal activity was determined in *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Saccharomyces cerevisiae* species [114].

Boron derivatives are also known for their antioxidant effects. Antioxidant enzymes found in mammalian blood can be inhibited or induced by exposure to different substances. Cell survival depends on calcium influx into the cell. When NAD / NADP production terminates, the functional capacity of the cell is severely affected. Boric acid reduces the proliferation of cancer cells by 30 percent-97 percent by changing NAD production and

calcium influx in cancer cells [125]. In a study in which boric acid and borax derivatives were used, antioxidant enzyme activities changed in the direction of increasing antioxidant capacity [133].

Different boron compounds have been proven to be effective in various types of cancer [134–137]. Also, not only boron derivatives but also structures designed using boron, such as nanoparticles and nanotubes have proven themselves as promising anticancer agent candidates for many cancer types such as prostate cancer [136], oral cancers [136], breast cancer [139], endometrial cancer [140], colon cancer [141], and lung cancer [142–144]. In this context, by inhibition of cyclin proteins expressions; boric acid has been shown to restrain the tumour growth and metastasis [143,145]. In lung cancer, boron compounds show anti-oxidant and also anti-inflammatory effects due to increased levels of estrogen expression, as for hormone replacement therapy, which works with regulating the 17 β -estradiol hormone level [142].

Boric acid, the most known boron derivative, possesses a proven effect of healing chronic wounds at three percent concentration. In addition to that, the importance of sodium pentaborate pentahydrate (NaB) and its various combinations in wound healing has been demonstrated in studies. According to the results, NaB has been shown to be effective in both *in-vitro* and *in vivo* wound healing models by increasing superoxide dismutase activity in addition to significantly increased cell migration [146]. In addition to NaB, Hexagonal boron nitrides (hBNs) synthesized from boric acid were evaluated as a therapeutic approach to wound healing owing to their biodegradability and uniform and efficient distribution in aquatic environments. In the study using human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts (HDFs), it was observed that cell proliferation and migration, which are phases of wound healing, increased significantly compared to cells treated only with BA, and this improvement was considerably low in cells treated only with BA [147].

1.5.2. Boron Treatment in Neurodegenerative Diseases

1.5.2.1. Boron and Brain Activity

It has been known for many years that boron nutrition has effects on bone, mineral, and

lipid metabolism, energy use, and immune function. As evidence accumulated to support that boron is crucial for human health, in the early 90s, the possible relationship between boron nutrition and brain and psychological activity began to be explored.

The first study conducted with mature rats showed that a gradual increase of boron in a low boron-containing diet enhanced the electrical activity of the brain [148]. Inspired by this study, three separate human studies were conducted assessing the boron effect on the electrical activity of the brain and the various motor and mental tests performance. Two studies have shown that consuming low boron-containing dietary nutrients draw a similar picture of decreased brain activation, and brain electrical activity changes in a manner similar to that observed in common malnutrition and lead toxicity. In addition, a very low boron-containing diet was found to cause lower performance on motor speed and dexterity, attention, and short-term memory tasks; the last two tasks were performed poorly in all three studies. In one of the human research, while some of the subjects consumed 0.35 mg of boron per day, some of them consumed 3 mg of boron per day. As a result of the 63-day study, less activity at lower frequencies and more activity at higher levels were measured, reflecting a physiological status where malnutrition and heavy metal toxicity emerges. Boron supplementation has led to the improvement of psychomotor skills of elderly men and women, elimination of drowsiness, short-term memory development, and mental alertness [118]. Similar results were observed in rats [148].

All these findings strengthened the view that boron is an important nutrient for brain and psychological functions in humans and served as a basis for the investigation of the possible neuroprotective role of boron.

1.5.2.2. *Neuroprotective Effect of Boron*

In a study showing the neuroprotective effect of boron, a positive improvement was observed in the oxidative damage caused by potassium dichromate ($K_2Cr_2O_7$) in the brain tissue of Sprague Dawley rats as a result of boron application. Superoxide dismutase (SOD), Malondialdehyde (MDA), catalase (CAT), and glutathione peroxidase (GSH-Px) enzymes levels and activities were measured in the brain tissue of rats. GSH-Px, CAT, and SOD are some of the fundamental antioxidants that constitute the main defense mechanism against oxidative stress [149–151]. Malondialdehyde (MDA), on the other hand, is a stable end product of lipid peroxidation and consequently can be used as an indicator of

cumulative lipid peroxidation [152–154]. Although no statistically significant alteration was detected in SOD, CAT, and GSH-Px enzyme activities, it was stated that there was a significant enhancement in the brain tissue MDA level of the group, treated with only 10 mg/kg of potassium dichromate ($K_2Cr_2O_7$) comparing to the control groups. It was also examined that lipid peroxidation in brain materials of rats treated with $K_2Cr_2O_7$ was significantly reduced after 5 and 10 mg/kg of boron application [153].

Most of the secondary brain injury cases arise from lipid peroxidation formation as a result of an emerging imbalance between antioxidant mechanisms, reactive oxygen species (ROS) levels, and reactive nitrogen species secreted in the brain with trauma. Antioxidants have already been proven to have a curative effect on clinical and histopathological aspects of CNS [156–160]. In this context, a recent study evaluated the neuroprotective effect of boric acid (BA) in a rat model of traumatic brain injury (TBI) for the first time [160]. According to the results, it is stated that in the group with TBI, MDA levels were significantly higher than the control group and BA application significantly decreased the MDA level implying an anti-inflammatory effect via inhibiting vascular permeability [159]. It was also noted that the activity of CAT was similarly decreased. This significant decrease in CAT activity in the BA-treated trauma group comparing the saline-only TBI group could be explained as a decrease in CAT enzyme consumption due to reduced need. When examined histopathologically, in the BA-treated trauma group a considerably reduced levels of necrotic cell death and edema were observed in contrast with the animals with untreated TBI [158].

Fluoride toxicity can also create oxidative damage due to reactive oxygen just like potassium dichromate toxicity mentioned above. It activates cell death mechanisms by inducing lipid peroxidation and DNA damage. In a study, different doses of sodium fluoride (NaF) were administered to rat brains in an attempt to create neurotoxicity, and the effect of boric acid (BA) in neurodegeneration was investigated. Malondialdehyde (MDA), superoxide dismutase (SOD) levels as in the previous study, Na/K ATPase activity, and DNA fragmentation levels were measured in rat synaptosomes. When the results were examined, it is revealed that 10 mM and 25 mM of BA treatment provided a significant improvement in DNA fragmentation and MDA, SOD, and Na/K ATPase levels compared to the 80 mg/L NaF group. When the results are examined, with the administration of 10

mM and 25 mM of BA, a significant amelioration in MDA, SOD, Na/K ATPase, and DNA fragmentation levels were observed when compared to the 80 mg/L NaF group [160].

The intense toxicity of aluminium chloride (AlCl_3) and other aluminium compounds is highly prevalent in both humans and animals. As a result of various studies, these compounds with high neurotoxicity are known to cause multiple sclerosis (MS), ALS, and Alzheimer's disease (AD) [163–165]. Recent studies have shown that aluminium-based neurotoxicity causes blood-brain barrier distortion [164].

According to the results of experiments in which different doses of boric acid (BA) were applied against aluminum chloride (AlCl_3 , 5 mg/kg per day) neurotoxicity on rats, a neuroprotective effect was observed provided by a low dose of BA (3.25 mg/kg per day) application. It is revealed that low-dose BA could neutralize the negative effects of AlCl_3 on the cerebral cortex. The number of cells in the 3.25 mg/kg BA+ AlCl_3 group was also reckoned to be the closest to that of the control group, whereas, a significant decrease was observed in the estimated neuron number only in the AlCl_3 , 36 mg/kg BA+ AlCl_3 , and 58.5 mg/kg BA+ AlCl_3 groups comparing the control group [159].

1.5.2.3. Boron Treatments in Alzheimer's Disease

It has been known for years that boron and boron-containing compounds have regulatory effects on NADP^+ and NAD^+ metabolism; implying they could play key roles in cellular energy metabolism [115,165]. Hence, it is not surprising that they may also be effective in mechanisms involving neurodegeneration, where oxidative stress and related pathologies are the major cause [168].

In a study conducted by Ozansoy and his colleagues [166], the effects of boron derivatives, sodium borate decahydrate (borax decahydrate), and boric acid (BA) on amyloid beta ($\text{A}\beta$) toxicity were investigated in the SH-SY5Y neuroblastoma cell line. $\text{A}\beta_{1-42}$ was chosen to provide toxicity, as scientific literature has proven that amyloid-beta aggregates in the brain leading to neuronal toxicity and eventually death, increase with aging and various neurodegenerative disorders, including Alzheimer's disease (AD, Figure 1.10.) [167–169].

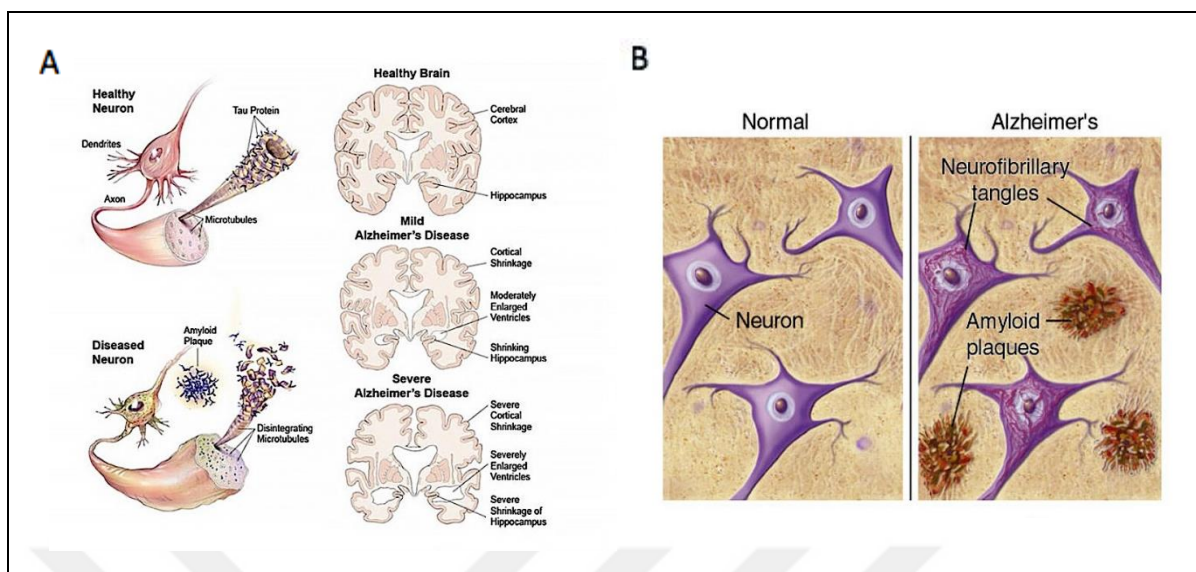


Figure 1.10. The progression of Alzheimer's disease (AD) (A) [170] and amyloid plaques and neurofibrillary tangles in AD (B) [171]

Sirtuin protein families are distinguished from the rest by playing essential roles in cell survival under various stress conditions, and Sirt1, which is a NAD^+ dependent histone deacetylase is one of the family members. It has been shown that Sirt1 guards against oxidative stress [174]. The results revealed that when $\text{A}\beta_{1-42}$ was administered to SH-S5Y5 cells, Sirt1 expression was increased, and more notably, boric acid (BA) treatment after $\text{A}\beta_{1-42}$ administration enhanced Sirt1 expression more in the $\text{A}\beta$ -only group (Figure 1.11., (a)). These findings suggest that BA provides protection against $\text{A}\beta_{1-42}$ toxicity, and besides its therapeutic nature, it can be considered as a preventive treatment due to its ability to increase Sirt1 expression.

Another treatment method developed for Alzheimer's disease was synthesizing various boron-containing compounds in an attempt to utilize them as $\text{A}\beta$ aggregation inhibitors, metal chelating agents, and antioxidants. The biological activity data indicated that these compounds have a considerable capacity to prevent self-stimulated $\text{A}\beta$ aggregation. Among the compounds prepared in different forms, especially 2- (3,4-dihydroxyphenyl)-3H-naphtho[1,2-e]oxaborinine-3,9-diol, decreased $\text{A}\beta$ aggregate inhibition by 82.8 percent comparing other forms. They also played a role as a potential antioxidant (oxygen radical absorbance capacity assay using fluorescein (ORAC-FL) values of 2.70–5.87). These

findings highlighted that boron-containing compounds may serve as novel treatment candidates for AD acting as a structural scaffold [175].

In addition to the knowledge that Alzheimer's disease results from the prefibrillar aggregation of amyloid β fibrils, the low-molecular-weight $A\beta_{1-42}$ trimer, and its higher-order oligomers cause cytotoxicity in the neuronal cells [174–177].

Boron nitride nanotubes (BNNTs) resemble carbon nanotubes (CNTs) structurally. The ionic B–N bonds formed by the boron and nitrogen atoms in their structure make them chemically stable and protect them from oxidation. In addition, their biocompatibility and noncytotoxicity properties make BNNTs promising candidates for therapeutic, nanomedical, diagnostic, and biomolecular administrations [178–183].

In line with this information, Sorout and their colleagues examined the effects of BNNTs on the secondary structure of $A\beta_{1-42}$ trimer recently. It was observed that the α -helix or β -sheet usually randomly transforms into a coil, twist, or turn [184–188]. In the study, it is confirmed that since BNNTs are not as hydrophobic as CNTs, they keep peptides of $A\beta_{1-42}$ trimer apart and appear to limit self-aggregation (Figure 1.11.). Analysis of hydrogen bonding characteristics performed by the group reveals that BNNTs assist to preserve the initial structure of the peptides via protecting the peptide hydrogen bonds. In association with this finding, an inspiring study regarding functionalized BNNTs in 2012 highlighted that they possess the capability to enter human umbilical vein endothelial cells recruited for use as a blood-brain barrier (BBB) endothelium model [191], suggesting that these structures could be used specifically for drug delivery in the CNS, in addition to their ability to act as therapeutic agents themselves for AD treatment as reported in this study.

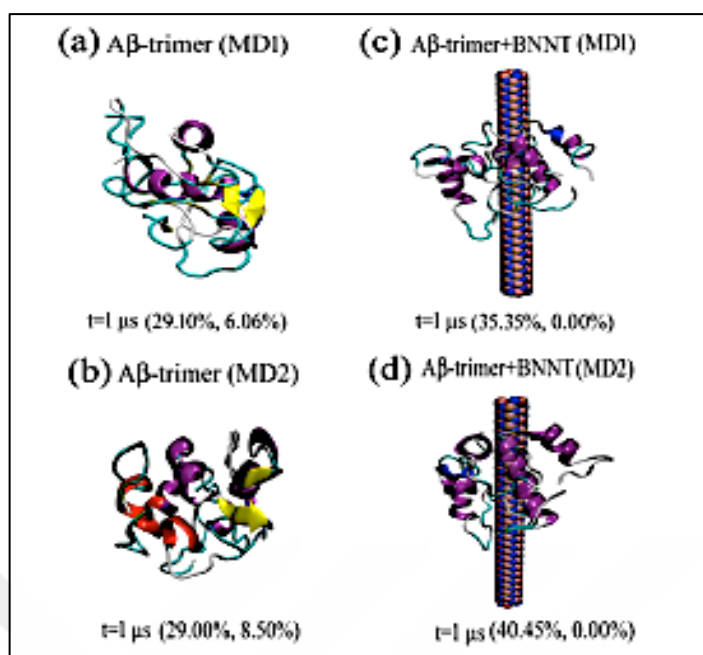


Figure 1.11. Representative image of two MD runs (MD1 and MD2) for both (a, b) A β -trimer and (c, d) A β -trimer with BNNT ($t=1 \mu s$). The percentage of helix and β -sheet are indicated in parentheses, respectively

In order to summarize, in the study, it was demonstrated that the absence of BNNTs provided a suitable environment for aggregate formation. On the other hand, the presence of BNNTs preserved the stability of the A β_{1-42} trimer by preventing the association of peptides and related aggregations [177].

1.5.2.4. Boron Treatment in Parkinson's Disease

A study using boron nitride nanoparticles as an alternative treatment has been a promising approach to Parkinson's disease (PD). In the study, the effect of hexagonal boron nitride nanoparticles (hBNs) against toxicity of 1-methyl-4phenylpyridinium (MPP⁺), a neurotoxin used in cellular models of Parkinson's Disease [192,193] was examined. When the experimental results were analysed, it was shown that hBN application increases cell viability against MPP⁺ toxicity observed in the PD model. As a result of Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) analysis, it was determined that the antioxidant capacity increased, and the oxidant level decreased as a result of hBNs application. In addition, according to the flow cytometry results, it was determined that MPP⁺ induced apoptosis was prevented by hBN application. In the light of these findings,

it has been demonstrated that hBNs can be used as a potential treatment method against MPP⁺ toxicity [194].

1.5.2.5. Boron Treatment in Amyotrophic Lateral Sclerosis (ALS)

One of the emerging factors associated with the occurrence of ALS disease is the excessive formation of reactive oxygen species (ROS), particularly in astrocytes [195]. In ALS, the up-regulation of ROS in CNS is primarily responsible for the motor neuron degeneration characteristic of the [196–198]. As mentioned several times before, one of the other main causes of ALS is hyperactivity of the superoxide dismutase (SOD1) enzyme, which catalyses the conversion of the toxic superoxide anion O₂⁻ to O₂ and H₂O₂, which constitute the main source of ROS [21].

A human ribonuclease, Angiogenin (ANG) is deficient in ALS patients. It has been shown to be a powerful neuroprotective and recovery agent in mouse models for ALS owing to its intracellular ribonucleolytic activity to provide neuroprotection [199]. Loss-of-function mutations in the secretory ribonuclease angiogenin are linked with the pathology of ALS [200]. Nevertheless, wild-type angiogenin treatment can also propagate undesired angiogenesis and tumour growth [201].

It has been known for years that hydrogen peroxide (H₂O₂) leads to oxidative cleavage of the boron-carbon bond and enables complete oxidation of the phenylboronic acid into phenol and boric acid (B(OH)₃) [202,203].

In the study conducted by Hoang and his colleagues based on this information, a boronic acid mask restraining the ribonucleic activity of ANG has been created [204]. They developed a phenylboronic acid (PBA) conjugate of ANG, which is inactive under normal conditions, however, activated in the existence of the most common ROS, H₂O₂. H₂O₂ leads to oxidative cleavage of the boron-carbon bond in PBA and releases the active ANG rather in cells only damaged through ROS-mediated toxicity [204,205]. This ROS-activatable phenylboronic acid conjugate of ANG was observed to increase catalytic activity when unmasked with H₂O₂. Endothelial cell proliferation is not enhanced by masked ANG; however, it guards astrocyte cells against oxidative stress. This strategy utilizing the two different biological functions of ANG could serve as a promising pharmacological approach in ALS treatment [204].

1.6. AIM OF STUDY

To understand the individual contribution of monocarboxylate transporters of CNS to the development of ALS disease, we aimed to analyse the changes in gene and protein expression levels of MCT1, MCT2, and MCT4 in the CNS of transgenic mice carrying ALS-causing mutant genes including SOD1 and C9ORF72 in a cell-type-specific manner by magnetic cell sorting in this study. We also designed *in vitro* experiments using NSC-34 motor neuron-like cells and investigated the use of lactate as an energy source, its importance for cell survival, and how its absence affects cell viability in a glucose-free environment. Next, we chemically blocked the function of MCTs in order to observe the extent of possible degeneration of neurons without MCT function and thus the consequences of loss of lactate trafficking.

In the second part of our study, inspired by the introduction of some boron derivatives and structures designed using boron as drug candidates for the treatment of neurodegenerative diseases, we aimed to test the effect of boron derivatives including boric acid and sodium pentaborate pentahydrate, antimicrobial, anti-inflammatory, and wound-healing properties of which have been proven in previous studies as stated in Section 1.5., however, has not yet been applied for the treatment of any neurodegenerative disease, and sodium perborate tetrahydrate (SPT; $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$), with no published studies of its use in any medical field, which is still used as a whitening agent and washing powder [209–211], on locomotor behaviours and molecular mechanisms including MCT expressions in animal models with ALS.

2. MATERIALS AND METHODS

2.1. ANALYSIS OF THE CELL-TYPE SPECIFIC GENE AND PROTEIN EXPRESSION OF MCTS IN CNS OF ADULT MICE CARRYING ALS-CAUSING MUTATIONS.

2.1.1. Ethical Approval

All animal procedures were conducted in accordance with the guidelines approved by the Institutional Animal Care and Welfare Committee of Yeditepe University (Turkey).

2.1.2. Mouse Model Handling and Genotyping

B6SJLTg (SOD1*G93A)1Gur/J and C57BL/6JTg (C9orf72_i2)112 Lutzky/J lines were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and six weeks old animals were bred in the live animal facilities of Yeditepe University using male transgenic and female wild-type C57BL6/J mice. They were fed ad libitum, with constant access to water under a 12:12-h light/dark cycle at a temperature of 18-23°C and 40-60 percent humidity [206].

DNA extracted from ear clippings of mice was used for genotyping material and genotyping was performed by standard PCR using specific primers as instructed by The Jackson Laboratory and Q5 Hot Start High-Fidelity 2X Master Mix (# M0270L, New England Biolabs, MA, USA) with 10 ng template DNA. The reaction was conducted in CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA). All primers and reagents that were used in the PCR procedure were given in Table 2.1. and Table 2.2.. PCR conditions were given in Table 2.3..

Table 2.1. Primers used in transgenic mice genotyping

Primer Name	Forward primer	Reverse primer	Expected Product Length
Internal Positive Control Primers for <i>SOD1</i> gene	5'CTA GGC CAC AGA ATT GAA AGA TCT'3	5'GTA GGT GGA AAT TCT AGC ATC ATC C '3	324 base pair
Transgene Primers for <i>SOD1</i> gene	5' CAT CAG CCC TAA TCC ATC TGA'3	5' CGC GAC TAA CAA TCA AAG TGA'3	236 base pair
Internal Positive Control Primers for <i>C9ORF72</i> gene	5' CAC GTG GGC TCC AGC ATT '3	5' TCA CCA GTC ATT TCT GCC TTT G '3	74 base pair
Transgene Primers for <i>C9ORF72</i> gene	5' AGG AGC TGG TGA GTT TCA AC '3	5' GCT CCC ACA GTG TAA TTC TCT '3	150 base pair

Table 2.2. The content of PCR mixture

Component	Volume	Final Concentration
Q5 Hot Start High-Fidelity 2X Master Mix	10 µl	1X
Forward Primer	1 µl	0,5 µM
Reverse Primer	1 µl	0,5 µM
Internal Positive Control Forward Primer	1 µl	0,5 µM
Template DNA (10 ng)	5 µl	
dH ₂ O	1 µl	
TOTAL volume	20 µl	

Table 2.3. Experimental protocol for standard PCR

Pre-denaturation	95°C	5 minutes
Denaturation	95°C	30 seconds
Annealing	60°C x 30	30 seconds
Extension	72°C	30 seconds
Last extension	72°C	2 minutes

The PCR products were loaded on 2.5 percent agarose gel prepared with TAE buffer. Samples were run at 110 volts for 35 minutes using an electrophoresis system, and the gel was visualized under ultraviolet with Bio-Rad ChemiDoc (Bio-Rad, Hercules, CA) via Image Lab Software.

2.1.3. Behavioural Characterization for SOD1-G93A Mice

2.1.3.1. Neurological Scoring System (NeuroScore)

In order to estimate the disease stages when early and late symptoms started to appear following the birth of animals, SOD1 transgenic animals' motor activity was monitored as it has been performed in a study with SOD1-G93A mutation (+) transgenic animals before [205]. As a result of this observation, two time points for the material collection were determined according to the duration of appearance of signs specific to early and late disease stages, and hereafter, it was planned to compare both gene expressions and protein levels collected from the samples of those two time points. Therefore, as indicated in the literature, the progression of the disease was graded based on a scoring system called NeuroScore (NS) [206].

As a protocol, which covers the daily evaluation of hindlimb function utilizing an easy neurological scoring system as elucidated below, the NeuroScore system is mainly focused on hindlimb dysfunction due to hindlimb deficiencies, which are the first symptom of the disease to occur in SOD1-G93A mouse according to the studies. The protocol enables a clear observation of the onset of paresis (mild or partial paralysis), progression, and

severity of the illness [206]. Each mouse was subjected to daily observation for the tests described below, and their performances were evaluated in order to determine their corresponding neuro scores.

1. Tail suspension test: The mouse was placed on top of the cage and suspended by the tail for 1-2 seconds. In the meantime, it was observed whether there was tremor and retraction in the hindlimbs. The test was repeated three times for consistency and the observation was recorded accordingly.

2. Gait analysis: The mouse was placed on a clean surface providing a walking distance of at least 25 cm and its gait was observed, allowing a total of 75 cm to walk.

3. Righting reflex test: In the “righting reflex” test, the mouse was placed on its left and right side, respectively, and the time it took to straighten itself without any assistance was recorded. This test was applied after the onset of paresis.

According to the findings obtained as a result of these tests, the symptom display levels of the animals were scored between 0-4 using the grading method mentioned below.

- **NS0 (Pre-symptomatic):** When the mouse is held by its tail and hung, it can normally fully extend its hindlimbs from the lateral midline to the side and remain in this position for at least two seconds or more. The mouse can walk normally when allowed (normal gait).
- **NS1 (Initial symptoms):** When the mouse is hung and held by its tail, its hindlimbs cannot be fully opened from the lateral midline, partially closed backward, or it is observed that its hindlimbs are trembling. His gait is normal or slightly slowed.
- **NS2 (Paresis):** As the mouse is held by its tail and hung, its hindlimbs are partially or completely closed or cannot be opened much, joint movements still continue. When the mouse is permitted to walk, it can use its hindlimbs for forward movements, but it is observed that its toes are bent backward shuffling at least twice during a 90 cm walk. When the mouse is tilted to the right or left, it can straighten itself within 10 seconds.
- **NS3 (Paralysis):** When the mouse is held by its tail and hung, its hindlimbs are clearly paralyzed or there is minimal joint movement. It is observed that when

allowed to walk, it can move forward but cannot use its hindlimbs. It is seen that the mouse can straighten itself within 10 seconds when tilted to the right or left.

- **NS4 (Human endpoint):** When the mouse is held by its tail and hung, its hindlimbs are completely paralyzed. When it is allowed to walk, no movement is observed, as the mouse is tilted to the right or left, it cannot straighten itself within 10 seconds, and the righting reflex disappears.

NS1 is characterized as a stage that covers the spectrum of early symptoms of the disease in SOD1-G93A mice. The duration may vary from mouse to mouse. Some mice have a tendency for their hindlimbs to collapse towards the midline while some have retraction tremor, clasp in their hindlimbs. Besides, in the initial stages of the disease, mice may display both NS 0 and NS1 characteristics [205]. The incidence of NS1 symptoms in other mice increases with time in SOD1-G93A mice, and these symptoms may have various phenotypical reflections. The NS1 phase in male and female SOD1-G93A mice generally lasts about 28-37 days.

One of the most distinguished features of the NS2 is that it is the first phase where paresis is observed. Symptoms specific to the NS1 phase can occur among wild-type animals, however, with the development of paresis, the onset of the pathology is quite pronounced in NS2. In the early NS2, the mouse is active, but serious deteriorations of moving ability occur later. Since severe paresis of the hindlimbs may not allow the mice to reach the food and water found in the upper part of the cage, food pellet should be positioned on the bottom of the cage or hydrated food should be given. In addition, water bottles can be supported with long sipper pipes so that they can reach the water more effectively. For SOD1-G93A mice, this phase usually takes 5 days.

NS3 is the easiest phase to identify with the development of complete hindlimb paralysis. Mice are quite ill during this period and need to be carefully and constantly observed. Symptoms are usually spastic, although they may display as flaccid or spastic. For SOD1-G93A mice, this stage usually takes 1-2 days.

In the NS4 phase, unlike other phases, the "righting reflex" which represents hindlimb capability completely disappears. Disease progression in mice may not always be symmetrical. For instance, the animal may use the righting reflex when placed in a certain direction, but not when placed in the opposite direction. This phase represents the

maximum duration time for reaching the humane endpoint for the termination of the experiments. For this reason, euthanasia should be performed if the reflex tests do not confirm that the animals right themselves on either side.

In this study, animals were sacrificed at the beginning of the NS1 phase (early-onset), on day 90 (postnatal day 90: PND 90) when symptoms first appear. Likewise, on day 60, which was accepted as part of the pre-symptomatic phase, a group of animals was sacrificed for comparative purpose and brain materials were collected, in order to use in magnetic cell sorting, qPCR, and western blot studies.

2.1.4. Behavioural Characterization of Tg(C9orf72_3) Line 112 Mice

As for C9ORF72 transgenic animals, it has been stated in the literature that no phenotypic symptoms have been observed until 16 months of age [207]. In the light of this information, we preferred to use 17 months old animals to collect CNS materials assuming molecular changes could probably be observed.

2.1.4.1. Rotarod Test

Since, unlike *sod1* animals, Tg(C9orf72_3) 112 line mice display no significant motor dysfunction or cognitive deterioration, a neuroscoring system has not been developed to rate the severity of the disease for this line. In order to understand whether excessive C9ORF72 expression results in MN degeneration or decline in motor function, C9ORF72 mice's locomotor activity was analysed via rotarod test. Mice of ages 7, 17, and 19 months old were trained for three consecutive days with three trials over on an accelerating rotarod (Ugo Basile, Varese, Italy) at 4 rpm with an acceleration of 0.2 rpm/s for 150 seconds and the latency to fall was recorded [207]. Data of last day's trials were collected as the real performance. Mice that rotated passively were evaluated as fallen. Behavioural tests were performed on ≥ 3 mice for each time point. Since there were not any 19 months old wild-type (wt) mice, 19 months old C9ORF72 mice were compared to 17 months old wild-type controls.

Table 2.4. Number of transgenic and wild-type mice used in the experiments (WO: weeks old)

Species	Strain	Sex	Age	Number of mice (n)		
				Pre-symptomatic	Early onset	Full brain
Mouse	C57/bl6 (wild-type)	Male	For breeding ≥ 8 WO; for experiments PND 60, PND 90 and 17 MO	3 (healthy controls for PND 60 SOD1-G93A)	3+3=6 (healthy controls for PND 90 SOD1-G93A and 17 MO C9ORF72 mice)	3+3+3=9 (normalization samples for PND 90 & PND 60 SOD1-G93A and 17 MO C9ORF72 mice)
Mouse	C57/bl6 (SOD1-G93A transgenic)	Male	For breeding ≥ 8 weeks old; for experiments; PND 60 and PND 90	3 (PND 60 SOD1-G93A)	3 (PND 90 SOD1-G93A mice)	-
Mouse	C57/bl6 (C9ORF72 transgenic)	Male	For breeding ≥ 8 WO; for experiments 17 MO	-	3 (17 MO C9ORF72 mice)	-
TOTAL				27		

2.1.5. Papain Disassociation

In order to determine cell-specific gene and protein expressions in mouse models, a cell disassociation and a magnetic cell separation method were optimized.

Mice were anesthetized with sevoflurane (Sevorane, #4456, Abbvie, İstanbul, Turkey) and decapitated. Papain disassociation was performed by optimizing the protocol suggested by Bekta et al.. [215]. Dissected full brain samples were placed on a plate kept at -20°C , then they were minced with a razor blade in serum-free Hibernate A-minus calcium medium (#HACA500, BrainBits, IL, USA) with 1 percent Glutamax (#35050061, Thermo-Fisher

Scientific, Gibco, Waltham, MA, USA), 1 percent Penicillin/ Streptomycin/ Amphotericin (PSA, Invitrogen, Gibco, UK), and 2 percent B27 (#17504044, Invitrogen, Gibco, UK), which was again kept at -20°C until it became semi frosted-semi gel in 35 mm dishes. Papain (#59H19406, BrainBits,) was dissolved in 3 ml, same Hibernate A medium, and DNase (2000U, 150 μ l) (#LK003172, Worthington Biochemical Corporation, NJ, USA) was added to prevent the mucoid layer that may form in the solution caused by the release of sticky DNA molecules from some damaged cells during the process and, AP5 (0.3 mM) (#ab120003, Abcam, Waltham, MA, USA) and CNQX (0.3 mM) (#ab120044, Abcam, Waltham, MA, USA) glutamate inhibitors were added aiming to minimize glutamate excitotoxicity and thereby neuronal cell death. The minced tissues were incubated in this Papain solution for 45 minutes at 37°C. After incubation, each minced brain tissue was triturated in a 15 ml falcon by passing them through a 1000 μ l pipette tip, a 200 μ l pipette tip, and finally a glass serological pipette, respectively, all three of which had their ends cut off and blunted by fire polishing. After trituration, the samples were incubated at room temperature for a minute to allow insoluble residues to sink to the bottom. The soluble part was taken into a new 15 ml falcon and Papain activity was stopped by adding one volume of Hibernate A medium with 10 percent fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK), and then centrifuged at 70 $\times$ g for 6 minutes. Meanwhile, the insoluble part was triturated for a while longer and added to the supernatant free sample after centrifugation. Again, the same volume of Hibernate with 10 percent FBS was added to the sample and centrifuged once more. After centrifugation, the pellet was diluted in serum-free Neurobasal A medium (#10888-022, Thermo-Fisher Scientific, Gibco, Waltham, MA, USA) with 1 percent Glutamax, 1 percent PSA, and 2 percent B27 and the process continued with magnetic cell sorting method. In order to measure the efficiency of the magnetic cell sorting experiment, whole-brain samples were collected from three wild-type mice for both C9ORF72 and SOD1-G93A lines to be used as normalization samples in qPCR trials. These materials were disassociated with papain but not subjected to magnetic cell sorting.

2.1.6. Magnetic Cell Sorting (MCS)

Symptomatic (PND 90) and pre-symptomatic SOD1 transgenic animals (PND 60), 17 months old C9ORF72 mice and their wild-type counterparts (n=3 per group) at the same

age as healthy controls were used to provide brain tissue material. The procedure is briefly described in Figure 2.1. [208].

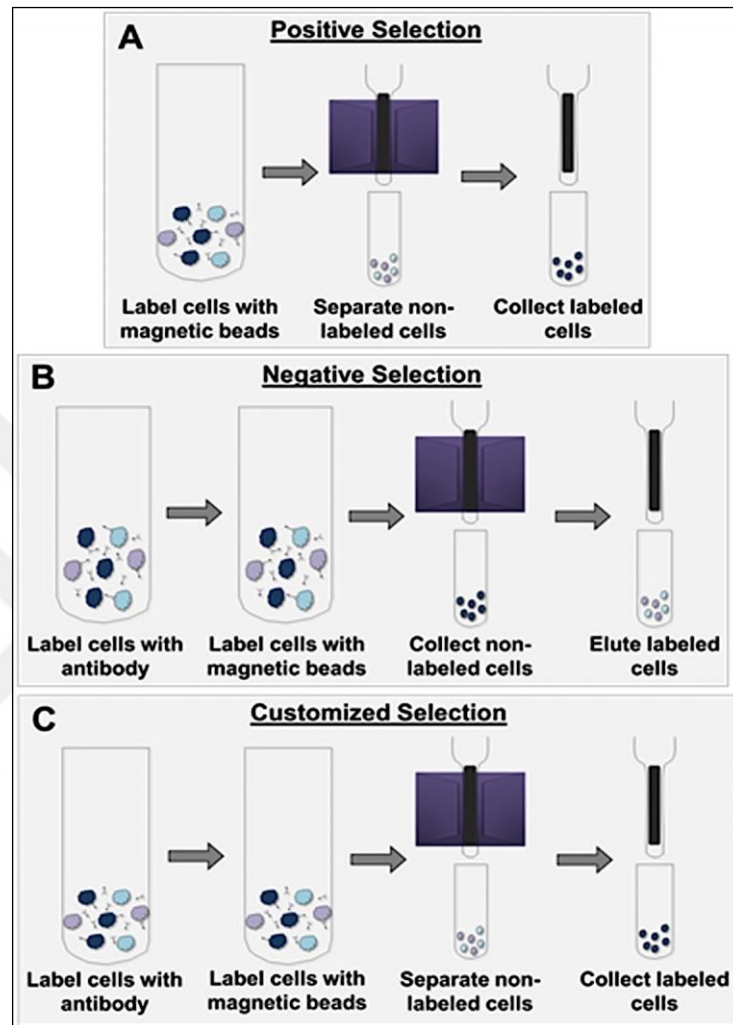


Figure 2.1. Schematic representation of cell separation techniques. A) Positive selection of astrocytes. B) Negative selection of neurons. C) Customizable positive selection of target cells [209]

2.1.6.1. Removal of Myelin and Separation of Oligodendrocytes

Before cell separation, myelin was collected to reach oligodendrocyte cell bodies before the extraction of astrocyte or neuronal cell populations. All procedures for magnetic separation of different cell types were conducted in the cold room (4°C) to prevent the death of relatively sensitive neuronal cells.

After dissociation, cells suspended in 0.5 percent Bovine serum albumin (BSA, # 37520, Thermo-Fisher, Waltham, MA, USA) /Phosphate buffered saline (PBS, # P3813, Sigma-Aldrich, Germany) buffer (1800 µl per adult whole-brain sample) are mixed with 200 µl of Myelin Removal Beads II (#130-096-731, Miltenyi Biotec, Germany) and incubated for 15 minutes at 4°C to reach the oligodendrocyte cell bodies and facilitate further isolation of astrocytes and neuronal cells.

After washing the cells with 0.5 percent BSA/PBS buffer, they were centrifuged at 300 xg for 10 minutes to clear unattached beads away from cells. The pellet, resuspended in the same buffer, was appended to an LS column prepared with 1000 µl of buffer placed in MACS MultiStand magnetic cell separator (#130-042-303, Miltenyi, Germany).

The column was washed twice with 1 ml of buffer to ensure the removal of unlabelled cells. For further isolation, flow-through was collected of astrocytes and neuronal cells. Cells in the column, mostly composed of oligodendrocytes, attached to the beads were eluted and stored at -80°C for protein and RNA extraction.

2.1.6.2. Separation of Astrocytes

The optimized protocol given by Miltenyi Biotec's Anti-ACSA-2 (astrocyte-specific cell surface antigen, #130097678, Miltenyi Biotec, Germany) was used for the positive selection of astrocytes. Cells obtained from the flow-through of the previous step were suspended in 80 µl of 0.5 percent BSA/PBS buffer. Then, they were incubated with 10 µl of FcR Blocking Buffer for 25 minutes at 4°C and with ACSA-2 microbeads for 25 minutes at 4°C, respectively. Following that, cells were again washed with 0.5 percent BSA/PBS buffer and centrifuged at 300 xg for 5 minutes to erase the remaining unbound beads. The pellet resuspended with the same buffer was added to a LS column, which was rinsed with 3 ml buffer and fitted in MACS MultiStand magnetic cell separator. The column was rinsed three times using 3 ml buffer to extract unlabelled cell cocktail for further neuronal isolation. Cells attached to the column, mostly containing astrocytes were eluted in the same buffer and stored at -80°C.

2.1.6.3. Separation of Neurons

Neuronal cell isolation was performed following the astrocyte separation step. Negative selection was performed as instructed by Miltenyi Biotec's Neuronal Isolation Kit

(#130098752, Miltenyi Biotec, Germany) protocol. Cells collected from flow-through of earlier step were resuspended in 0.5 percent BSA/PBS buffer, then incubated with 20 μ L of Non-Neuronal Cells Biotin-Antibody Cocktail for 10 minutes at 4°C. Cells rinsed with the buffer were centrifuged at 300 xg for 5 minutes to clear unattached antibody away from the cell pellet, then cells resuspended in 0.5 percent BSA/PBS buffer were incubated with 20 μ L of Anti-Biotin MicroBeads for 10 minutes at 4°C. The volume of the suspension was adjusted and applied to a LS column rinsed with 3ml buffer and fitted in MACS MultiStand magnetic cell separator. After two washes, neurons were depleted and the flow-through was extracted and stored at -80°C in order to be used as materials for following qPCR and Western Blotting experiments. Labelled and unwanted cells were eluted with the same buffer for later use.

Along with magnetically sorted CNS cells, spinal cord materials were obtained from some of the PND 90 SOD1-G93A mice and wild-type counterparts.

2.1.7. RNA Isolation and RT-PCR

Total RNA was extracted from magnetically sorted cells of transgenic PND 60 and PND 90 mice and their wild-type counterparts. Trizol reagent (#15596026, Thermo-Fisher Scientific, Ambion, Waltham, MA, USA) was used for RNA phase separation, and RNA isolation procedure was continued with RNeasy - Micro Total RNA Isolation Kit (#AM1931, Thermo-Fisher Scientific, Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Concentrations of isolated RNA were measured by using Nanodrop 2000 UV-Vis Spectrophotometer (#ND-2000, Thermo-Fisher Scientific, Waltham, MA, USA). Subsequently, 5 ng of RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription kit (#205311, Qiagen, Hilden, Germany).

2.1.8. qPCR for Relative mRNA Expression Analysis of Cell Markers and MCTs

Before proceeding to the observation of the target genes expressions including MCT1, MCT2, and MCT4, qPCR was performed to confirm the efficacy of the MCS procedure and to check if glial and neuronal cells were separated adequately. Even if, cell separation was not achieved with 100 percent efficiency, we expected a clear cell-type-specific

enrichment in the samples compared to its whole-brain counterparts as stated in Holt and Olsen's study [209]. The relative mRNA expressions were determined using real-time quantitative PCR by I-taq Universal Sybr Green Supermix (#1725124, Bio-rad, Hercules, CA). For oligodendrocyte characterization; MBP gene, for astrocyte characterization; GLAST gene, and for neuron characterization; RBFOX3 gene (NeuN) were chosen. ATP5b gene was used as housekeeping gene. Primers given in Table 2.4. were designed utilizing PrimerQuest tool of Integrated DNA Technologies. Relative mRNA expression levels were calculated via the delta-delta Ct method ($2^{-\Delta\Delta C_t}$), and normalized to their matched whole-brain sample values to evaluate the cell enrichment.

Table 2.5. Primers used in cell characterization qPCR

Primer Name	Forward primer	Reverse primer	Expected Product Length
<i>MBP</i>	5' CGA CAC CTC CTT ATC CCT CTA A 3'	5' ATC AAC CAT CAC CTG CCT TC 3'	117 base pair
<i>GLAST</i>	5' CAA GAT GTA GAC GGA CAG AGA AG 3'	5' CCT ATC AGA GTA GGG AGG AAA GA 3'	124 base pair
<i>GFAP</i>	5' GCA TTT CAG CCA CAC CTT TC 3'	5' CCT AAT TAC ACA GAG CCA GGA C 3'	122 base pair
<i>RBFOX3</i> (NeuN)	5' GGT CGT GTA TCA GGA TGG ATT T 3'	5' GCG GCA TAG ACT CTA CCA TAA C 3'	126 base pair
<i>MCT1</i>	5' GTG GCG TGA TCC TCA TCA TC 3'	5' CTT CTC CTC CGC TTT CTG TTC 3'	95 base pair
<i>MCT2</i>	5' CCA CGA ATC CAG TAC CTC TTC 3'	5' GTA GAC CAC CAA AGC TGA GTA G 3'	99 base pair
<i>MCT4</i>	5' CCT TCC CTC ATC ATG CTC AAT 3'	5' AGT GGA GAC AGT GCA CAA AG 3'	104 base pair
<i>COX10</i>	5' GAT CCT GTA CTC CTG GCA ATT C 3'	5' GAT GGG TGA CGG ACA TCA TAC 3'	101 base pair

Table 2.6. The content of PCR mixture

Component	Volume	Final Concentration
I-taq™ Universal Sybr® Green Supermix	6 µl	1x
Forward Primer	0,5 µl	0,5 µM
Reverse Primer	0,5 µl	0,5 µM
Template DNA (5 ng)	4 µl	-
dH ₂ O	1 µl	-
TOTAL volume	12 µl	

Table 2.7. Experimental protocol for qPCR

Cycle	Temperature	Duration	Repeats
Pre-denaturation	95°C	30 seconds	1
Denaturation	95°C	5 seconds	40
Annealing	58°C	30 seconds	
Melt Curve	65-95°C	0.5°C increment at 2-5 seconds/step	
Hold	4°C	∞	1

2.1.9. Western Blotting for Protein Expression Analysis of Cell Markers and MCTs

All reagents and chemicals used in western blot assays were purchased from Bio-rad Laboratories (Richmond, CA). Solutions prepared for western blot analysis are given in 2.7.. In order to determine the alteration in synthesized protein amount, total protein was isolated from cell pellets with RIPA buffer (1 percent sodium dodecyl sulfate (SDS), 100 mM Tris, pH 7.5), (#sc-24948, SantaCruz, TX, USA). The procedure is briefly indicated as follows: cell pellet, which was kept frozen at -80°C, was washed twice with ice-cold PBS. Then, RIPA buffer cocktail was prepared by adding PMSF (Phenylmethylsulfonyl

fluoride), protease inhibitor and sodium orthovanadate at a rate of 10 µl/ml to the RIPA solution. The RIPA cocktail was added to the cell pellets rinsed with PBS and mixed by pipetting about 40-50 times on ice. After approximately 30 minutes of incubation, the mixture was centrifuged for 15 minutes at 14,000 xg at 4°C. Following centrifugation, the protein-containing supernatant was taken and the pellet was discarded. Protein samples were stored at -80°C for further use. The BCA assay (Pierce BCA Protein Assay, #23225, ThermoFisher, Waltham, MA, USA) was performed to detect and normalize the protein concentrations before proceeding to western blot analysis. Proteins were heated to 95°C for 5 minutes with 6x Laemmli SDS sample buffer (375 mM Tris-HCl, 9 percent SDS (w/v), 50 percent Glycerol (v/v), 9 percent mercaptoethanol (v/v), 0.03 percent Bromophenol blue (w/v), #J61337, Thermo-Fisher Scientific, Alfa Aesar, Waltham, MA, USA).

Same amount of protein for each sample was loaded on a pre-casted SDS polyacrylamide gel (Mini-PROTEAN Precast Gel, #4561023, Bio-Rad, CA) The gel was first run at 110 V for about 15 minutes until the proteins migrated through separating gel, and subsequently at approximately 145 V for one hour, and finally transferred to a PVDF membrane (#1620177, Bio-Rad, CA) by wet transfer technique. Following transfer, the membrane was blocked for one hour with 1X Tris-Buffered Saline with 0.1 percent Tween (TBS-T) with 5 percent Blotting-Grade Blocker (#1706404, Bio-Rad, CA). For primary antibody incubation after blocking, MCT1 (dilution: 1:400, #ab93048, Abcam, Waltham, MA, USA) and GAPDH (dilution: 1:5000, # 10494-1-AP, Proteintech, UK) antibodies were diluted in the same blocking solution and incubated overnight at 4°C. After the removal of primary antibody by washing three times with TBS-T, the membrane incubation with anti-rabbit HRP-linked secondary antibody (dilution 1:10000, #ab6721, Abcam, Waltham, MA, USA) and anti-mouse HRP-linked secondary antibody (dilution 1:10000, #ab205719, Abcam, Waltham, MA, USA) for two hours at room temperature was performed. After washing the secondary antibody three times with TBS-T at the end of incubation, the membrane was treated with Immobilon ECL Ultra Western HRP Substrate (#WBULS0100, Merck Millipore, Burlington, MA, USA) for one minute to visualize proteins. Image J software was utilized for the protein bands' intensity analysis and proteins of interest were normalized to their respective GAPDH band intensities. Results were demonstrated as relative fold changes.

Table 2.8. Solutions used in western blotting

TBS-T	20 mM Tris-HCl 150 mM NaCl 0.1 percent Tween 20, pH 7.6
Running Buffer	25 mM Tris base 190 mM Glycine 0.1 percent SDS, pH 8.3
Transfer Buffer	25 mM Tris base 190 mM Glycine 20 percent Methanol
Blocking Buffer	5 percent Non-fat dry milk prepared in TBS-T

2.1.10. Protein Detection by Simple Western Analysis

The Simple Western™ system (Wes™, ProteinSimple, California, USA) utilizes capillary electrophoresis to characterize and quantify proteins of interest. The system consists of capillaries containing disposable stacking matrix and separation matrix prepared by the device each time during application; is based on the separation of proteins according to their size and charge. In about 3-5 hours, the proteins are separated, quantified according to the detection level, and the result is reported by the device by the end of the reaction [210]. Another advantage of this method is that it allows working in very low volumes of proteins (3 µl per sample). Since the total protein amount obtained per cell fraction after magnetic cell sorting was quite low, this method had provided us with convenience in this regard. The procedure was carried out as described previously [211]. For capillary electrophoresis analysis of the obtained proteins, the protein content was mixed directly with the ProteinSimple MasterMix (#PS-FL01-8, ProteinSimple, California, USA) containing DTT (ProteinSimple, California, USA) and ready-to-use Biontynylated Ladder (12-230 kDa, #PS-ST01EZ, ProteinSimple, California, USA) was used at a ratio of 1:5. The samples, which were incubated at 95°C for 5 minutes, were precipitated and kept on ice until the loading stage. Primary antibody, HRP-labeled secondary antibody, 2.5 percent BSA in PBS solution as blocking buffer, and Immobilon ECL Ultra Western HRP Substrate (#WBULS0100, Merck Millipore, Burlington, MA, USA) were added to the plates of the

Wes Simple Western system (13-Capillary Cartridge, #SM W001 or 25-Capillary Cartridge, #SM W003, ProteinSimple, California, USA) as specified in the protocol. The following primary and secondary antibodies were optimized, diluted in blocking buffer, and used for Wes: MCT4 (dilution: 1:50, #sc-376140, SantaCruz, TX, USA), MCT2 (dilution: 1:50, #ab81262, Abcam, Waltham, MA, USA), MBP (dilution: 1:100, #ab62631, Abcam, Waltham, MA, USA), EAAT2/GLT-1 (dilution: 1:50, #sc-365634, SantaCruz, TX, USA) RBFOX3/NeuN (dilution: 1:50, # NB300-322, Novus Biologicals, CO, USA), GAPDH (dilution: 1:100, #10494-1-AP, Proteintech, UK), Anti-rabbit IgG, HRP-linked Antibody (dilutions: 1:100, #7074, Cell Signalling Technology Leiden, The Netherlands), Anti-mouse IgG, and HRP-linked Antibody (dilutions: 1:100, #7074, Cell Signalling Technology Leiden, The Netherlands). Western Blot assay was performed for MCT1 protein in SOD1-G93A mice (PND 90) due to difficulties in antibody optimization in the first WES trials.

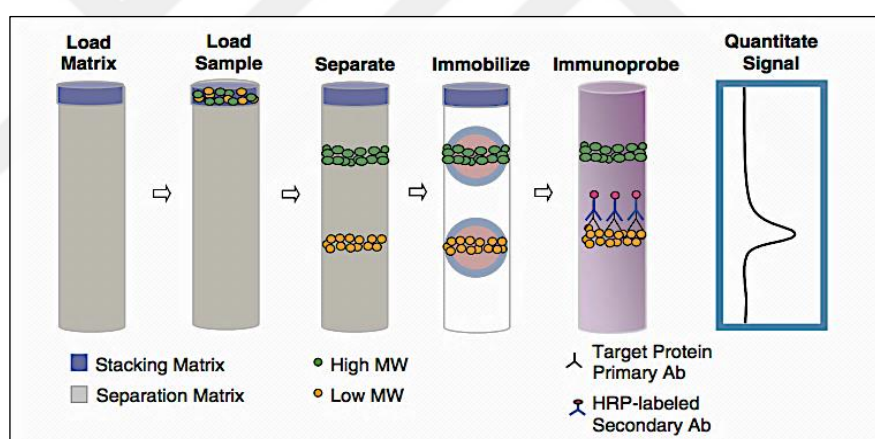


Figure 2.2. Simple Western automated procedure [209]

2.1.11. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 6.0e (GraphPad Software, Inc., San Diego, CA, USA) and results were indicated as mean $\pm$ standard error (SE). Statistical differences were analysed by two-way ANOVA and two-tailed Student's t-test. Results with P-values ≤ 0.05 were considered statistically significant.

2.2. INVESTIGATION OF THE INHIBITION OF MCT FUNCTION AND THE EFFECT OF LACTATE TRANSFER ON CELL SURVIVAL IN AN *IN-VITRO* CELL MODEL

2.2.1. NSC-34 Cell Line and Culture

The MN-like NSC-34 cell line (#CLU140, Cedarlane, CELLutions Biosystems, Burlington, CA) is a hybrid cell line produced by the fusion of mouse neuroblastoma and motor neuron-enriched embryonic mouse spinal cords. They display the characteristics of MNs morphologically and physiologically, such as choline acetyltransferase and acetylcholine storage and release, action potential forming, producing neurofilament triplet proteins, microtubule-associated protein 2 (MAP2) expression and neurite outgrowth. Since it is a promising cell model, especially for cholinergic degeneration, it is used mainly in research regarding ALS or Alzheimer's disease (AD) [212–214].

2.2.1.1. *Cell Thawing*

In order to re-culture the frozen NSC-34 cell line, the cryovials were removed from the liquid nitrogen tank and thawed by rapidly placing them in a water bath and heating to 37°C. Subsequently, 1 mL of cell suspension in cryovials was added dropwise to 2 mL of pre-warmed complete growth medium, and the mixture was centrifuged at 1300 rpm for 5 minutes.

The pellet was resuspended in complete growth medium and cells were transferred to the appropriate tissue culture flask. The culture was refreshed with fresh complete growth medium after 24 hours of incubation to eliminate possible dimethyl sulfoxide (DMSO) contamination. All *in-vitro* cell culture experiments were performed in a Laminar Flow Cabin (Heal Force Class II Biosafety Cabinet A2, China). And cells were incubated in the humidified incubator at 37°C, 95 percent (v/v) air and 5 percent (v/v) CO₂ conditions.

2.2.1.2. *Cell Lines and Culturing Conditions*

NSC-34 cells were cultured in proliferation medium consisting of Dulbecco's Modified Eagle's Medium/ high-glucose (DMEM/ high-glucose, #11965092, Thermo-Fisher Scientific, Gibco, Waltham, MA, USA) supplemented with 10 percent FBS, 1 percent PSA

and 1 percent 200 mM L-glutamine solution (#25030081, Thermo-Fisher Scientific, Cambridge, MA, USA) until they reach the experimental stage.

2.2.1.3. Cell Subculturing

NSC-34 cell line cultures should be split at ~80 percent confluency, at the ratio of 1:3-1:4. Cells were washed once with 1x PBS and then removed from the flasks by incubating with 0.05 percent (w/v) Trypsin-EDTA (0.25 percent, #25200056, Thermo-Fisher Scientific, Gibco, Waltham, MA, USA) for 5 minutes at 37°C. Complete medium was added to the cell suspension at a ratio of 1:1 to inhibit the trypsin activity. The cell suspension was then centrifuged at 1300 rpm for 5 minutes. The pellet was diluted in the suitable medium and cells were seeded in appropriate flasks to be used in future experiments.

2.2.1.4. Calculation of Cell Number

In order to reach the number of cells to be used in the following experiments, cells were counted. 10 µl of cell suspension was loaded into the hemocytometer and squares of the hemocytometer were counted using the 4x objective of an inverted light microscope.

$$\frac{\text{Cell number}}{\text{ml}} = \frac{\text{Total cells counted} \times \text{dilution factor} \times 10000 \frac{\text{cells}}{\text{ml}}}{\text{Number of squares counted}}$$

2.2.1.5. Cryopreservation of Cells

The freezing mix solution was prepared by mixing 10 percent (v/v) DMSO and 30 percent (v/v) FBS in DMEM/ high-glucose media according to the manufacturer's recommendations for the NSC-34 cell line. Cells were washed with 1x PBS, detached using Trypsin-EDTA, and counted as described in Section 2.3.1.4.. The cell pellet was dissolved in 1 ml of freezing mix with a cell density of 1 x 10⁶ cells/mL per cryovial. These cell suspensions were quickly placed in -80°C freezer and incubated there overnight, then for long-term storage, they were transferred to a liquid nitrogen tank.

2.2.2. Glucose Starvation

For the glucose-starvation experiment, in a previous study, DMEM/ no-glucose media was mixed with DMEM/ high-glucose media to prepare a medium containing 5 mM glucose

[214]. Similar to this study, DMEM/ low-glucose media (#12320032, Thermo-Fisher Scientific, Gibco, Waltham, MA, USA) consisting of 10 percent FBS, 1 percent PSA and 1 percent 200 mM L-glutamine solution with a glucose content of 5.5 mM were chosen and mixed with 80, 40, 20, and 10 mM Sodium L-lactate (#sc-220120A, SantaCruz, ChemCruz, TX, USA), respectively.

2.2.3. Cell Viability Assays

Cells (2000/well) were seeded in 96-well plates (#CLS6509, Corning Plasticware, Corning, NY) in high glucose complete medium, 24 hours before the experiment for 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay and nuclear staining assay. The media of the cells were changed the next day with media containing lactate at different concentrations as mentioned before. Cell survival was measured by MTS assay every other day for 12 days. Five wells were used for control groups and for each lactate concentration. For the MTS test, PBS/glucose solution (4.5 g/L glucose in PBS) containing 10 percent MTS reagent (#G3582, CellTiter 96 AQueous MTS Reagent Powder; Promega, Southampton, UK) was added to each well and after two hours of incubation, optical density was measured using the Elisa-Reader at 490 nm wavelength. The results obtained in the study, which was performed with three replicates, were expressed in proportion to the control group.

Hoechst/ Propidium iodide (PI) staining was performed to confirm cell survival results of the MTS assay. Cells were cultivated for 6-10 days in black 96-Well immuno plates (#437111, Thermo-Fisher Scientific, Gibco, Waltham, MA, USA) and cell survival was measured every other day during this period. Hoechst 33342 (#H3570, Thermo-Fisher Scientific, Waltham, MA, USA) and Propidium iodide (#P3566, Thermo-Fisher Scientific, Waltham, MA, USA) stock solutions were mixed and diluted in cell culture media. They were added at a final concentration of 1 µg/ml for each dye to each experimental well. After one hour of incubation, fluorescence signal intensity was measured using Varioskan LUX Multimode Microplate Reader (#N16045, Thermo-Fisher Scientific, Waltham, MA, USA). Images were captured with two software predetermined excitation/emission filter sets; 380/535 nm for Hoechst and 555/645 nm for PI. The results obtained in the study,

which was performed with three replicates, were expressed in proportion to the control group.

2.2.4. MCT Inhibition

AR-C155858; a cell-permeable thieno[2, 3 d]pyrimidinedione compound (#533436, Merck Millipore, Calbiochem, Burlington, MA, USA), which behaves as a highly effective inhibitor of MCT1 ($K_i=2.3$ nM for lactate uptake in rat erythrocytes) and has been proven to inhibit MCT2 function when associated with basigin ($IC_{50}=10$ nM) [215] was used assuming it would inhibit lactate entry into the cell. Although, the general distribution of MCT isoforms in CNS cells was determined via *in-vivo* and *in-vitro* studies, and NSC-34 cells were also designed to mimic mouse motor neurons, yet, undifferentiated NSC-34 cells might have been exhibiting a different MCT expression profile from adult neurons. Therefore, the expression profile of genes of interest including *MCT1*, *MCT2*, and *MCT4* were examined by qPCR in undifferentiated NSC-34 cells.

2.2.4.1. RNA Isolation and RT-PCR

Total RNA was isolated from undifferentiated NSC-34 cells. Trizol reagent was used for RNA phase separation and RNA isolation procedure was continued with PureLink RNA Mini Kit (#12183020, Thermo-Fisher Scientific, Invitrogene, Waltham, MA, USA) including a DNase treatment, according to the manufacturer's instructions. Concentrations of isolated RNA were measured by using Nanodrop 2000 UV-Vis Spectrophotometer. 1 μ g of RNA was reverse transcribed into cDNA using Qiagen's QuantiTect Reverse Transcription kit.

2.2.4.2. qPCR

The relative mRNA expressions were determined using real-time quantitative PCR by I-taq Universal Sybr Green Supermix (#1725124, Biorad, Hercules, CA). *MCT1*, *MCT2*, and *MCT4* gene primers and as housekeeping; *ATP5b* gene primers given in Table 2.4. were used. Assay conditions were arranged as indicated in Table 2.5. and Table 2.6.. After *MCT* genes expression profiles were determined, MCT inhibition studies were initiated.

2.2.4.3. Cell Culture

Cells (250,000/dish) were seeded in 35 mm dish (Falcon Easy-Grip Cell Culture Dish, # 353001, Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM/high glucose complete medium. After 48 hours, they were transferred 20mM sodium L-lactate-DMEM/no glucose medium mixture, in which the highest proliferation rate was seen after DMEM/high glucose medium earlier in the glucose starvation experiment. AR-C155858 stock solution was first diluted in DMSO and then added to each experimental dish at a final concentration of 200 nM in cell culture media as described before [217]. Media and cell pellet were collected at the end of various time points (0, 2, 4, 8, 16, and 32 minutes) in order to analyse lactate amount in both samples by HPLC analysis, in an effort to determine the inhibition level of lactate entry into the cell.

2.2.4.4. HPLC Method (LC-MS/MS)

Acquity H-Class UPLC device (Waters, Milford, MA, USA) was used for HPLC analysis. This device contains a quaternary mobile phase and is suitable for gradient and isocratic studies. The thermostat sample storage unit (20-90°C) has a capacity of 96 samples. The device contains polydiode array (PDA) $\epsilon\lambda$ detector, electrophoretic light scattering (ELS) detector, and fluorescent detector (FLR).

Gradient mobile phase elution system was used. This system contains as follows: A phase: 0.1 percent formic acid in water, B phase: 0.1 percent formic acid in acetonitrile. The phase ratios in the column change in a way that creates a gradient in the following time periods. 0 minutes= 35 percent A and 65 percent B, 1 minute= 35 percent A and 65 percent B, 3 minutes= 35 percent A and 65 percent B, 3.1 minutes=35 percent 10 and 90 percent B, 3.9 minutes=10 percent A and 90 percent B, 4 minutes=35 percent A and 65 percent B, 5 minutes=35 percent A and 65 percent B. Acquity UPLC BEH C18 1.7 μm , 2.1x50mm column was used. Waters Quattro Micro API MS (Waters, Milford, MA, USA) was used in mass spectrophotometer analyses. Lactic acid was analysed in Electrospray ionization (ESI) mode. RF lens was 0-0.1, ESI gas was N_2 , collision gas was Ar, capillary voltage was 3 kV, cone was 18-26 V, extractor was 3 V, source temperature was 120°C, and desolvation temperature was 300°C. Desolvation (gas flow rate) was 800 L/hr-900 L/hr. Media and cell pellet samples were dissolved in 500 μl of buffer prepared with water,

containing 1 percent formic acid and 60 percent acetonitrile. Masslynx 4.1 software was used to evaluate the data. Lactic acid was determined at a mass value of [M-H]-89.0 m/z.

2.2.4.5. Cell viability Assay

Cells were treated with AR-C155858 inhibitor for 12, 24, 48, and 72 hours in order to examine the effect of decreasing lactate flux on long-term cell survival. Cells were cultivated in black 96-Well immuno plates and cell survival was measured by Hoechst/PI nuclear staining. Stock solutions were mixed and diluted in cell culture media. Then, they were added at a final concentration of 1 µg/ml for each dye to each experimental well. After one hour of incubation, fluorescence signal intensity was measured via Varioskan LUX Multimode Microplate Reader (#N16045, Thermo-Fisher Scientific, Waltham, MA, USA). Images were captured with two software predetermined excitation/emission filter sets; 380/535 nm for Hoechst and 555/645 nm for PI. The results obtained in the study, which was performed with three replicates, were expressed in proportion to the control group.

2.2.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6.0e (GraphPad Software, Inc., San Diego, CA, USA) as described in Section 2.1.11..

2.3. INVESTIGATING THE EFFECT OF BORON DERIVATIVES ON MCTS AND EVALUATING IT AS AN ALTERNATIVE TREATMENT METHOD FOR ALS

2.3.1. Ethical Approval

All animal procedures were conducted in accordance with the guidelines approved by the Institutional Animal Care and Welfare Committee of Yeditepe University (Turkey).

2.3.2. Mouse Model Handling and Boron Application

B6SJLTg (SOD1*G93A)1Gur/J lines were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and six-week-old animals were bred at our own facility using transgenic male and wild-type female C57BL6/J mice. Female mice were preferred in order to conduct the study. They were fed ad libitum, with constant access to water under a 12:12-h light/dark cycle. All mice were genotyped as described earlier in Section 2.1.2..

In order to investigate the effect of boron derivatives on ALS animal models, pre-symptomatic 80 days old female mice and their wild-type counterparts were used. Boron derivatives dissolved in their drinking water were administered to mice by gavage at 200 mg/kg every other day for three weeks. Only water was given to the wild-type and transgenic control groups. Brain and spinal cord materials were collected from the animals after the treatment aiming to investigate the possible effects of applied boron derivatives on the onset and course of ALS symptoms. There were eight animals in each group, five of which are for histological studies in brain and spinal cord materials and three of which are for gene and protein expression analysis. The animal groups set up for the study and the applied boron derivatives are given in Table 2.9. and Table 2.10., respectively.

Table 2.9. Information about the laboratory animals used for boron application

Species	Strain	Gender	Age	Number
Mouse	C57BL/6 (wild-type)	Female	80 days old	32
Mouse	C57BL/6 (SOD1-G93A, transgenic)	Female	80 days old	32
				TOTAL: 64

Table 2.10. Information about the animal groups used for boron application

Groups (n=8)	Species of animal	Substance to be applied
Group I	SOD1-G93A, transgenic	Control (water)
Group II	SOD1-G93A, transgenic	Sodium pentaborate (NaB)
Group III	SOD1-G93A, transgenic	Boric acid (BA)
Group IV	SOD1-G93A, transgenic	Sodium perborate tetrahydrate (SPT)
Group V	Wild-type	Control (water)
Group VI	Wild-type	NaB (Sodium pentaborate)
Group VII	Wild-type	Boric acid (BA)
Group VIII	Wild-type	Sodium perborate tetrahydrate (SPT)

2.3.3. Motor Function Tests

In order to understand whether boron derivatives make a difference in the motor coordination of animals, especially transgenic mice, the animals were subjected to certain motor function tests before and after application.

- **Neurological scoring System (NS)** for each mouse was assessed along with the experiment as described in Section 2.1.3.1.. No significant effect of boron derivatives on the onset of pathology has been observed.
- **Rotarod test:** The motor functions were tested, the mice were trained three times with the rotarod tester and their performances were recorded at the end of the same day. Motor performances were measured three times for a maximum of 180 seconds at 0.2 rpm / s speed [217] on the accelerated rotarod and the longest residence times on the apparatus were recorded.

- **Hanging wire grip test:** The mice were attached to the metal cable apparatus, which was horizontally suspended at a height of 50 cm from the ground, with its front forepaws, and the length of their stay on the metal cable was recorded, including the hindlimbs. Each animal had three trials to stand for a maximum of 30 seconds and was evaluated as follows [219]: 0, falls off within 10 seconds; 1, hangs onto bar by two forepaws; 2, attempts to climb onto bar; 3, hangs onto bar by two forepaws and one or both hindlimbs; 4, hangs by all four paws, additionally tail wrapped around bar; 5, active escape to the end of bar. The longest latency was recorded and scored.

2.3.4. CNS material collection

Mice were anaesthetized with sevoflurane and decapitated [220]. Dissected full brain and spinal cord samples were immediately placed in liquid nitrogen for rapid freezing and stored at -80°C.

2.3.5. RNA Isolation

Total RNA was isolated from each group. Trizol reagent was used for RNA phase separation and RNA isolation procedure was continued with PureLink RNA Mini Kit (#12183020, Thermo-Fisher Scientific, Invitrogene, Waltham, MA, USA) including a DNase treatment, according to the manufacturer's instructions. Concentrations of isolated RNA were measured by using Nanodrop 2000 UV-Vis Spectrophotometer and the integrity of total RNA was determined by Bioanalyzer (AgilentTechnologies, The Netherlands). Samples with an RNA Integrity Number (RIN) greater than six were considered suitable for Microarray analysis.

2.3.6. Microarray Preparation and Data Pre-processing

A microarray is a laboratory technic, which allows detecting the expression of thousands of genes at the same time via a small DNA chip. Thousands of tiny spots in defined localization are printed in these small microarray chips, and each spot is marked with a known DNA sequence or gene. The DNA molecules printed on these small chips are also

called probes and utilizing hybridization technology, they detect gene expressions in transcriptome or mRNA sets [221,222].

Microarray Assay was conducted by SEM Laboratory. The prob array type, sample description, and required comments were entered by the application manager. In this study, the materials collected from the groups treated with NaB and SPT, which were found to cause a significant or a trend-level positive change in the motor activities of transgenic SOD1-G93A mice in the locomotor tests, were subjected to microarray analysis. Spinal cord and brain materials (n=3 per material type) were collected for the evaluation. Water groups for wild-type and transgenic mice were used as control groups (Table 2.11.).

Table 2.11. Information about the animal groups used for microarray analysis

Groups (n=6)	Species of animal	Collected materials	Substance to be applied
Group I	SOD1-G93A, transgenic	Brain material (n=3) Spinal cord material (n=3)	Control (water)
Group II	SOD1-G93A, transgenic	Brain material (n=3) Spinal cord material (n=3)	NaB (Sodium penta borate)
Group IV	SOD1-G93A, transgenic	Brain material (n=3) Spinal cord material (n=3)	Sodium perborate tetrahydrate (SPT)
Group V	Wild-type	Brain material (n=3) Spinal cord material (n=3)	Control (water)

The protocol was briefly performed as follows; samples were labelled with Cyanine 3 (Cy3) to measure gene expression in experimental and control samples, then hybridized, and washed according to the One-Color Microarray-Based Gene Expression Analysis protocol version 6.9.1 (Agilent Technologies, CA, USA, Figure 2.3.-2.4.). The Low Input Quick Amp Labeling Kit, One-Color (#p/n 5190-2305, Agilent Technologies, CA, USA) generates fluorescent cRNA (complementary RNA) with a sample input RNA range

between 10 ng and 200 ng of total RNA or a minimum of 5 ng of poly A⁺ RNA for one-color processing.

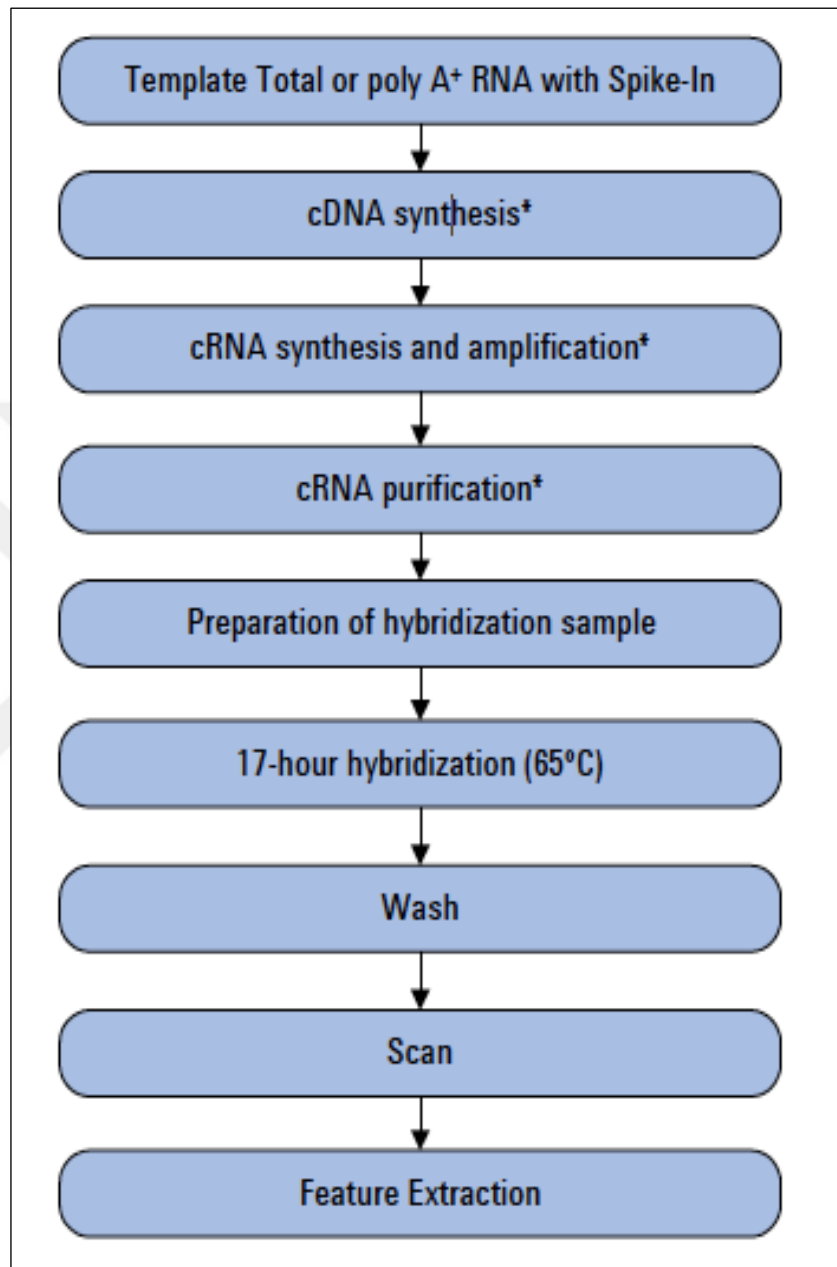


Figure 2.3. Workflow for sample preparation and array processing [222]

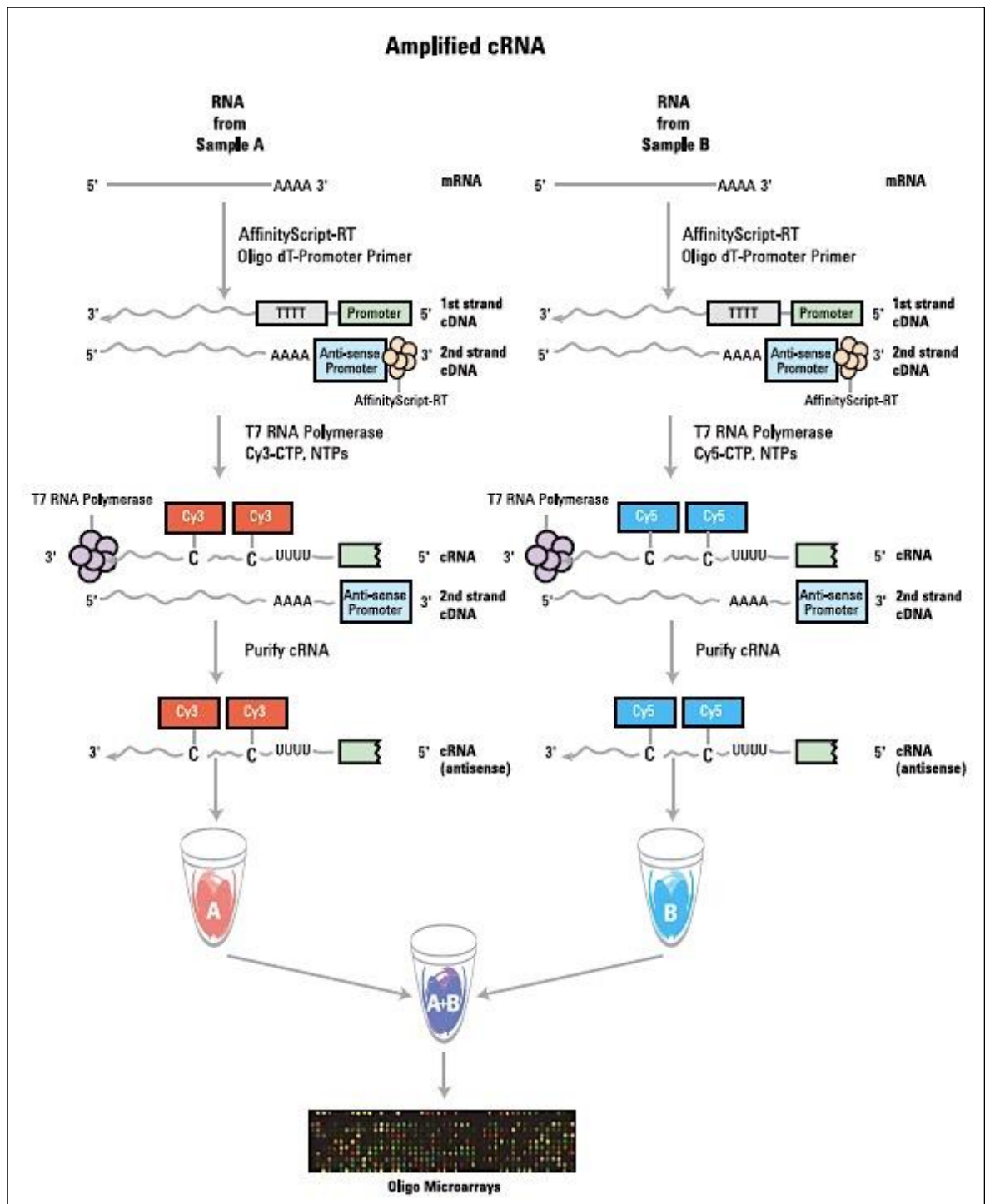


Figure 2.4. Schematic representation of amplified cRNA procedure. Generation of cRNA for a two-color microarray experiment is shown here [223]

Cy3-labeled cRNA was hybridized using Agilent's High-RPM Gene Expression Hybridization Kit (#p/n 5188-5242, Agilent Technologies, CA, USA). It was performed for 17 hours at 65 °C in a rotating hybridization oven at 10 rpm according to the

company's recommendations. After washing, samples were scanned using Agilent DNA Microarray Scanner (#p/n G4900DA, Agilent Technologies, CA, USA). The resulting raw data as TIFF images were extracted and processed by Agilent Feature extraction software (FES, version 10.7.3.1). Following that, data were transformed log₂ system and normalized using quantile normalization. Expression profiles of treated groups were compared with negative control groups.

2.3.7. Statistical Analysis

GeneSpring GX v14.9 software (GeneSpring Multi-Omic Analysis version 14.9–Build 11939 by Agilent Technologies) was used for the analysis of mRNA expressions from the spinal cord and brain materials. (n=12 per material type). mRNA raw data collected from both types of materials from the Agilent Technologies Microarray Scanner System G2505C were imported to GeneSpring GX v14.9 software. The results were expressed as the means $\pm$ standard error (SE) after quantile normalization. Statistical differences between the groups were compared using two-way ANOVA and two-tailed Student's t-test using GeneSpring GX v14.9 software. P-values ≤ 0.05 were considered to indicate statistically significant results.

2.3.8. PANTHER Gene Ontology (GO) Classification and Pathway System Analysis

As a result of the analysis performed with the GeneSpring GX v14.9 software, the genes expressions of which changed significantly after boron treatments were separated as up-regulated and down-regulated. All of the up-regulated and down-regulated genes were uploaded separately to PANTHER Gene Ontology (GO) Classification and Pathway System as described before [224]. The genes were separated based on their molecular function, biological process, and related pathways. Following that, genes involved in prominent pathways were selected and uploaded to the String database v11. Finally, their connections with each other were arranged as a diagram, which presents a pathway-based gene-gene interaction network between gene expression profiles. The selected genes, which were uploaded to the String database v11, were also imported into the GeneSpring GX v14.9 software and their expression profiles were extracted as heatmaps.

3. RESULTS

3.1. GENOTYPING OF TRANSGENIC SOD1-G93A AND C9ORF72 MICE

The transgenic SOD1-G93A mouse model, first reported in 1994, has since been the most widely used transgenic animal for modeling ALS disease [225]. This transgenic animal was genetically engineered to overexpress a mutant form of the human Cu/Zn superoxide dismutase 1 (SOD1) gene, which contains the glycine-alanine mutation at amino acid 93 (G93A) that has been discovered to involve in ALS development. This mutation is one of many other mutations in the SOD1 gene, which is responsible for about approximately 20 percent of familial ALS cases [22].

B6SJLTg (SOD1*G93A)1Gur/J and C57BL/6JTg (C9orf72_i2)112 Lutz/J lines were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and six weeks old animals were bred at our own facility using transgenic male and wild-type female C57BL6/J mice. They were fed ad libitum, with constant access to water under a 12:12-h light/dark cycle. Mice were weaned at 21-28 days old as described before [226,227]. All mice were genotyped using DNA extracted from ear clippings via standard PCR using specific primers as suggested by The Jackson Laboratory. The PCR products were loaded on agarose gel and it was visualized under ultraviolet with Bio-Rad ChemiDoc (Bio-Rad, Hercules, CA) via Image Lab Software. As stated in Figure 3.1., for all mouse colonies, the single band displayed on the gel represented wild-type mice, and the double band represented transgenic mice as one of the bands indicated the PCR product of the transgene.

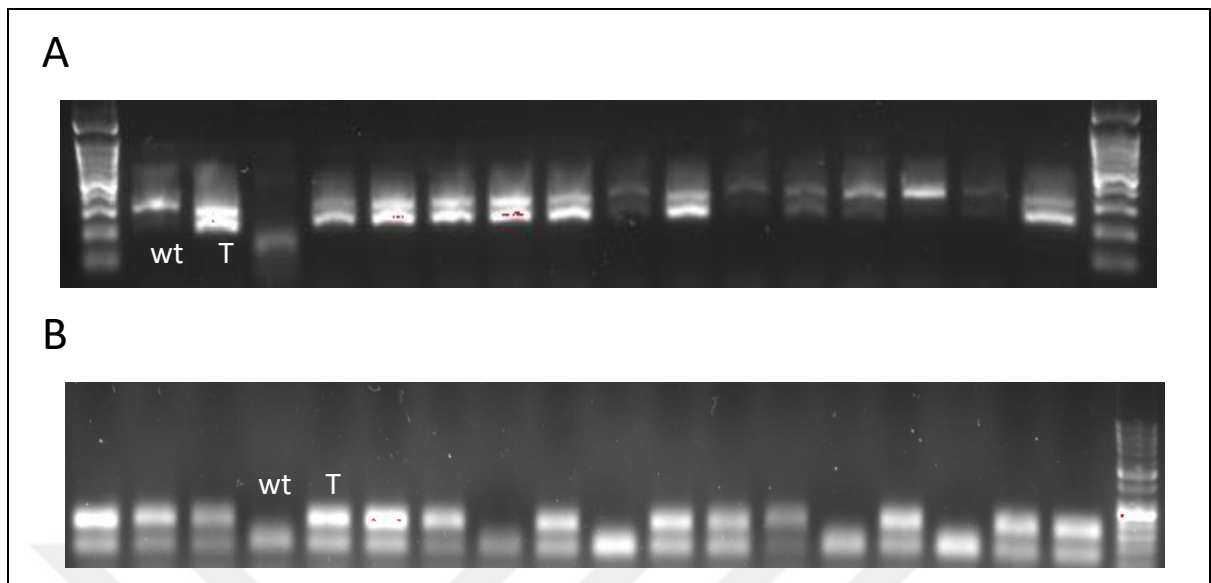


Figure 3.1. Gel images of mouse colony genotyping after maternal separation. (A) SOD1-G93A transgenic mouse line genotyping image. (B) C9ORF72 transgenic mouse line genotyping image. Wt: wild-type, T: transgenic

3.2. BEHAVIORAL TESTS

3.2.1. NeuroScore Evaluation Table of Transgenic SOD1-G93A Mice

The SOD1-G93A model has become the most preferred mouse model in ALS drug development research, as it carries many of the pathological features of ALS in humans, such as motor neuron loss, progressive muscle weakness, atrophy, and eventually paralysis and death [227]. Nevertheless, as a problem that is described in many transgenic animal models, SOD1-G93A mice also occasionally exhibit variation in the severity and timing of the onset of pathology [228]. Therefore, there was a need for an *in vivo* screening system that assesses the neuromuscular function of SOD1-G93A mice in a standardized manner. Thus, Hatzipetros et al developed a protocol in ALS Therapy Development Institute (ALS TDI) that allows a rapid and reliable assessment of the neuromuscular function of SOD1-G93A mice.

The protocol and related locomotor activity tests of this measurement system, called Neuroscore, was explained in detail in Section 2.1.3.1.. The tests performed for the

evaluation method can be summarized briefly as tail suspension test, gait analysis, and rightening reflex measurement, which are applied sequentially (Figure 3.2.-3.3., Table 3.1.)

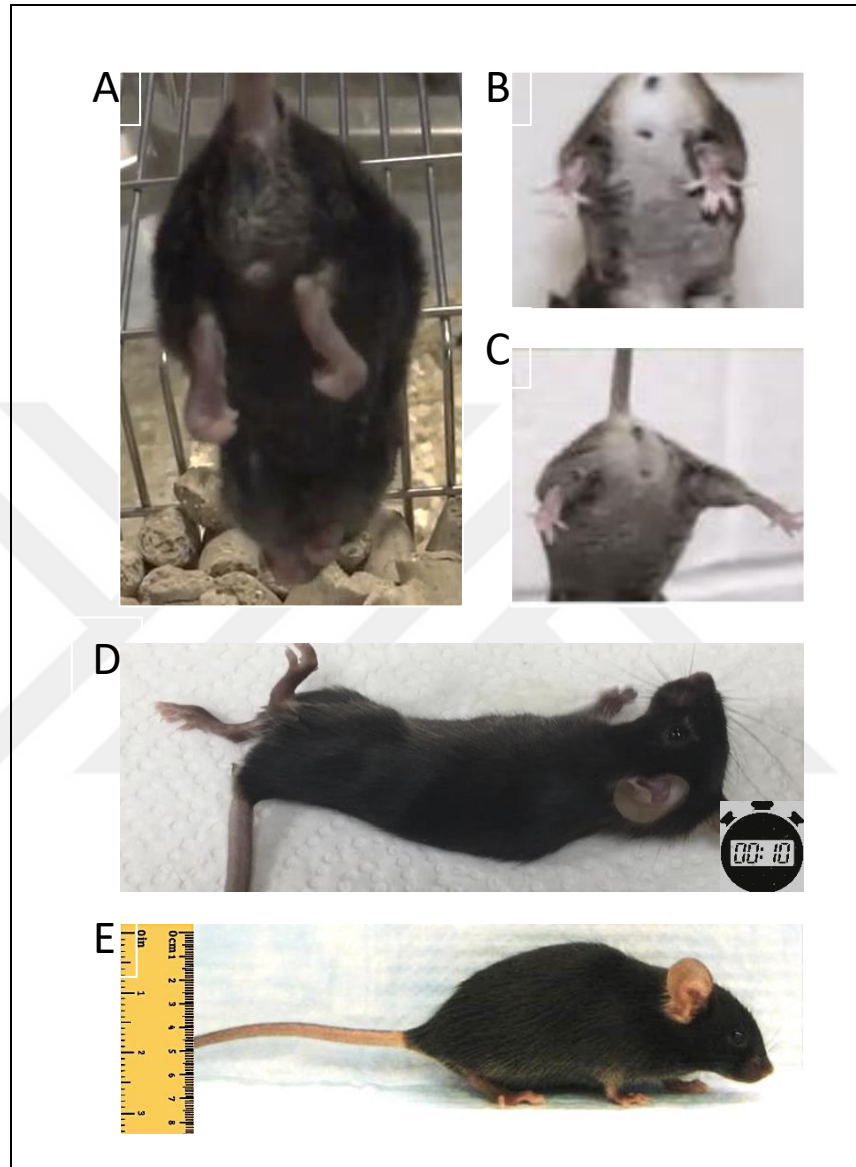


Figure 3.2. Locomotor activity tests applied for NeuroScore evaluation. (A), (B), and (C); tail suspension test, representing severe and mild hindlimb retraction in transgenic SOD1-G93A mouse, and no hindlimb retraction in wild-type counterpart, respectively. (D); rightening reflex measurement (duration: 10 seconds.) and (E) gait analysis; mice were allowed to walk across for 70 cm

Table 3.1. Neuroscore evaluation of one of the offspring colonies of SOD1-G93A mice line (DOB*: date of birth, DOE*: date of euthanasia)

Trans-gene	Strain	Animal No	Gender	Father	Mother	Geno-type	DOB	Cage no:	Notes	DOE*
SOD1* G93A	C57BL/ 6xSJL	108-02	F	129	2 wt	T	11/26/2017	1	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait NS2_Day 123_03/29/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/05/18: Paralysis in the hindlimb. Hindlimb is not used for forward motion: Tail suspension test: successful. NS4_Day 133_04/08/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	04/12/18 Day 137
SOD1* G93A	C57BL/ 6xSJL	108-07	F	129	2 wt	T	11/26/2017	1	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait NS2_Day 125_03/31/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/05/18: Severe paralysis in the hindlimb. Hindlimb is not used for forward motion: Tail suspension test: successful. NS4_Day 133_04/08/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test:	04/12/18 Day 137
SOD1* G93A	C57BL/ 6xSJL	108-03	F	129	2 wt	T	11/26/2017	1	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, normal gait. NS2_Day 126_04/02/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 132_04/07/18: Paralysis in the hindlimb. Hindlimb is not used for forward motion: Tail suspension test: successful. NS4_Day 135_04/10/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test:	04/21/18 Day 146

Trans-gene	Strain	Animal No	Gender	Father	Mother	Geno-type	DOB*	Cage no:	Notes	DOE*
SOD1* G93A	C57BL /6xSJL	108-06	F	129	2 wt	T	11/26/2017	1	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 123_03/29/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/05/18: Paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 133_04/08/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	04/12/18 Day 137
SOD1* G93A	C57BL /6xSJL	108-05	F	129	2 wt	T	11/26/2017	1	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 124_03/30/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 131_04/05/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 134_04/09/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	04/12/18 Day: 137
SOD1* G93A	C57BL /6xSJL	113-03	F	129	2 wt	T	11/26/2017	2	NS1_Day 92_02/24/18: Hindlimb is retracted, trembles during tail suspension test, normal gait. NS2_Day 126_04/01/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/05/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 132_04/07/18: Rigid paralysis in the hindlimb. No forward motion.	04/21/18 day: 146
SOD1* G93A	C57BL /6xSJL	113-01	F	126	2 wt	T	11/26/2017	2	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 123_03/29/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/05/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 133_04/08/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	ex: 04/09/18 Day: 134

Trans-gene	Strain	Animal No	Gender	Father	Mother	Geno-type	DOB*	Cage no:	Notes	DOE*
SOD1 *G93A	C57BL/ 6xSJL	106-01	M	129	2 wt	T	11/26/2017	2	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 123_03/29/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/05/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 133_04/08/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	04/12/18 Day: 137
SOD1 *G93A	C57BL/ 6xSJL	106-04	M	129	2 wt	T	12/11/2017	3	NS1_Day 91_03/13/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 123_04/14/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 129_04/20/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 131_04/22/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	04/28/18 Day: 137
SOD1 *G93A	C57BL/ 6xSJL	106-05	M	129	2 wt	T	12/11/2017	3	NS1_Day 90_03/12/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 128_04/19/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 133_04/24/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 135_04/26/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	04/28/18 Day: 137
SOD1 *G93A	C57BL/ 6xSJL	112-02	M	126	2 wt	T	12/11/2017	3	NS1_Day 92_02/24/18: Hindlimb is retracted, trembles during tail suspension test, normal gait. NS2_Day 126_04/17/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/21/18: Paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 132_04/23/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: unsuccessful.	05/05/18 Day: 144

Trans gene	Strain	Animal No	Gender	Father	Mother	Geno-type	DOB*	Cage no:	Notes	DOE*
SOD1 *G93 A	C57BL /6xSJL	112-04	M	126	2 wt	T	12/11/2017	3	NS1_Day 90_02/24/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 121_04/12/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 125_04/16/18: Paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 127_04/18/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: successful.	04/28/18 Day: 137
SOD1 *G93 A	C57BL /6xSJL	106-04	M	129	2 wt	T	12/11/2017	3	NS1_Day 91_03/13/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 123_04/14/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 129_04/20/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 131_04/22/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: successful.	04/28/18 Day: 137
SOD1 *G93 A	C57BL /6xSJL	106-05	M	129	2 wt	T	12/11/2017	3	NS1_Day 90_03/12/18: Hindlimb is retracted, trembles during tail suspension test, slower gait. NS2_Day 128_04/19/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 133_04/24/18: Severe/partial paralysis in the hindlimb. Hindlimb is not used for forward motion. Tail suspension test: successful. NS4_Day 135_04/26/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: successful.	04/28/18 Day: 137
SOD1 *G93 A	C57BL /6xSJL	112-02	M	126	2 wt	T	12/11/2017	3	NS1_Day 92_02/24/18: Hindlimb is retracted, trembles during tail suspension test, normal gait. NS2_Day 126_04/17/18: Hindlimb mostly collapsed, toes curl downwards at least twice during walking test. Tail suspension test: successful. NS3_Day 130_04/21/18: Paralysis in the hindlimb. Hindlimb is not used for forward motion: Tail suspension test: successful. NS4_Day 132_04/23/18: Rigid paralysis in the hindlimb. No forward motion. Tail suspension test: successful.	05/05/18 Day: 144

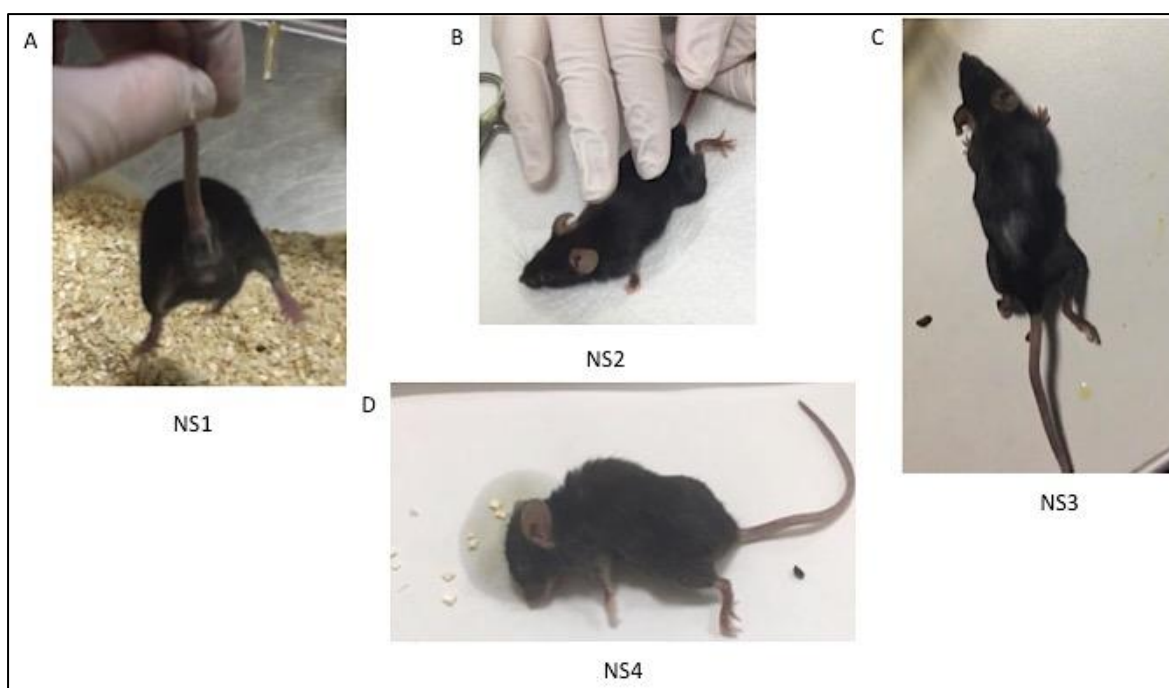


Figure 3.3. Phenotypal aspects of NeuroScore stages. (A) NS1: Slight hindlimb retraction, (B), (C) NS2 and NS3: ataxia and tremor during walking, toes are bent backward in NS3, (D) NS4: complete paralysis

3.2.2. Behavioural Characterization for Transgenic C9ORF72 Mice – Rotarod Test

Since, unlike SOD1-G93A animals, Tg(C9orf72_3) 112 line mice display no significant motor dysfunction or cognitive deterioration, a neuroscoring system has not been developed to rate the severity of the disease for this line. In order to understand if excessive C9ORF72 expression leads to motor neuron degeneration or abnormal motor function, we analysed transgenic C9ORF72 mice's locomotor activity via rotarod test. Mice of ages 7, 17, and 19 months old mice (MO) were used for performance comparison, however since there were not any 19 months old wild-type (wt) mice, 19 months old C9ORF72 mice were compared to 17 months old wild-type controls. Statistical analysis was performed by two-way ANOVA using GraphPad Prism Version 6.0e.

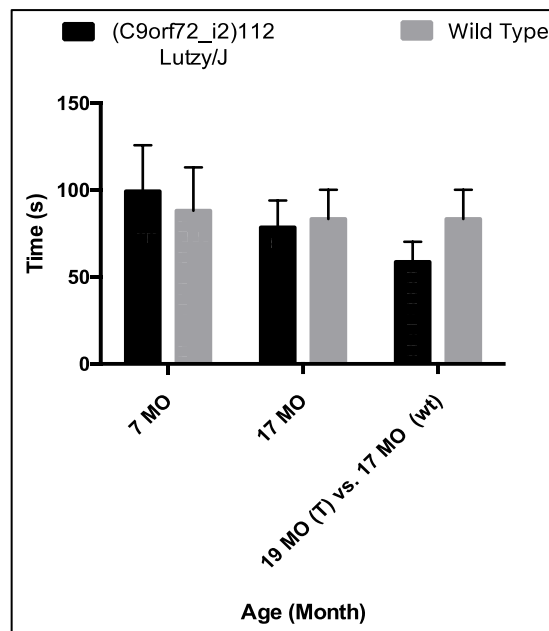


Figure 3.4. Rotarod test results of 7, 17, and 19 months old C9ORF72 mice and wild-type counterparts, MO: month old. Data represents the longest residence time of three test trials for each mouse (n=5 per group) $\pm$ SEM (P-value for interaction factor, row factor, and column factor are 0,1856, 0,0787, and 0,435, respectively)

As shown in Figure 3.4., no significant difference was found in the rotarod performances at the three selected time points. In the 19th month, however, C9ORF72 mice showed a trend-level decrease in performance compared to wild-type 17 MO controls. Consequently, this time point was chosen as the symptomatic stage for CNS material collection. Nevertheless, the pre-symptomatic stage could not be determined due to some emerging difficulties in the maintenance of the transgenic line.

3.3. MAGNETIC CELL SORTING (MCS) CONFIRMATION

3.3.1. qPCR Analysis

Before the examination of target genes expressions including *MCT1*, *MCT2*, and *MCT4*, qPCR was performed in order to determine whether MCS was successfully carried out for brain samples collected from wild-type mice (PND 90, n=3) and cells were separated as oligodendrocyte, astrocyte and neuron. Even if cell separation did not occur 100 percent

successfully, we expected a clear cell-type-specific enrichment in the samples compared to its whole-brain counterparts (collected from wild-type PND 90 mice as well, $n=3$) as stated in Holts and Olsen's study [208].

For oligodendrocyte characterization, *MBP* gene, for astrocyte characterization, *SLC1A3* (GLAST) gene, and for neuron characterization, *RBFOX3* (NeuN) gene were used as stated in Section 2.1.8. before. *ATP5b* gene was chosen as housekeeping gene.

According to the qPCR results, *SLC1A3* (GLAST) gene was expressed at a higher level in sorted astrocytes compared to other marker genes. A very low level of *MBP* gene expression indicates that oligodendrocytes in sorted astrocytes were almost completely eliminated. The fact that the *RBFOX3* gene was expressed, albeit slightly, means that there was a neuronal contamination, but in general, a significant enrichment of astrocytes could be observed as indicated in Figure 3.5. (B) ($P \leq 0.0001$). Another cell population in which magnetic separation was successful was oligodendrocytes. *MBP* gene expression was quite high compared to other marker genes, and the *SLC1A3* (GLAST) gene was almost not expressed, indicating that the oligodendrocytes were almost completely separated from astrocytes. However, as in astrocytes, there was a certain degree of neuronal population in sorted oligodendrocytes indicating neurons could not be efficiently separated from oligodendrocytes (Figure 3.5. (A), $P \leq 0.05$). Unlike other cell populations, in sorted neurons, the fact that the astrocytic marker *SLC1A3* (GLAST) gene was expressed at a higher level than the neural marker *RBFOX3* gene in neurons showed that magnetic cell sorting could not make a complete neural separation and could not enrich neural cells at a sufficient rate.

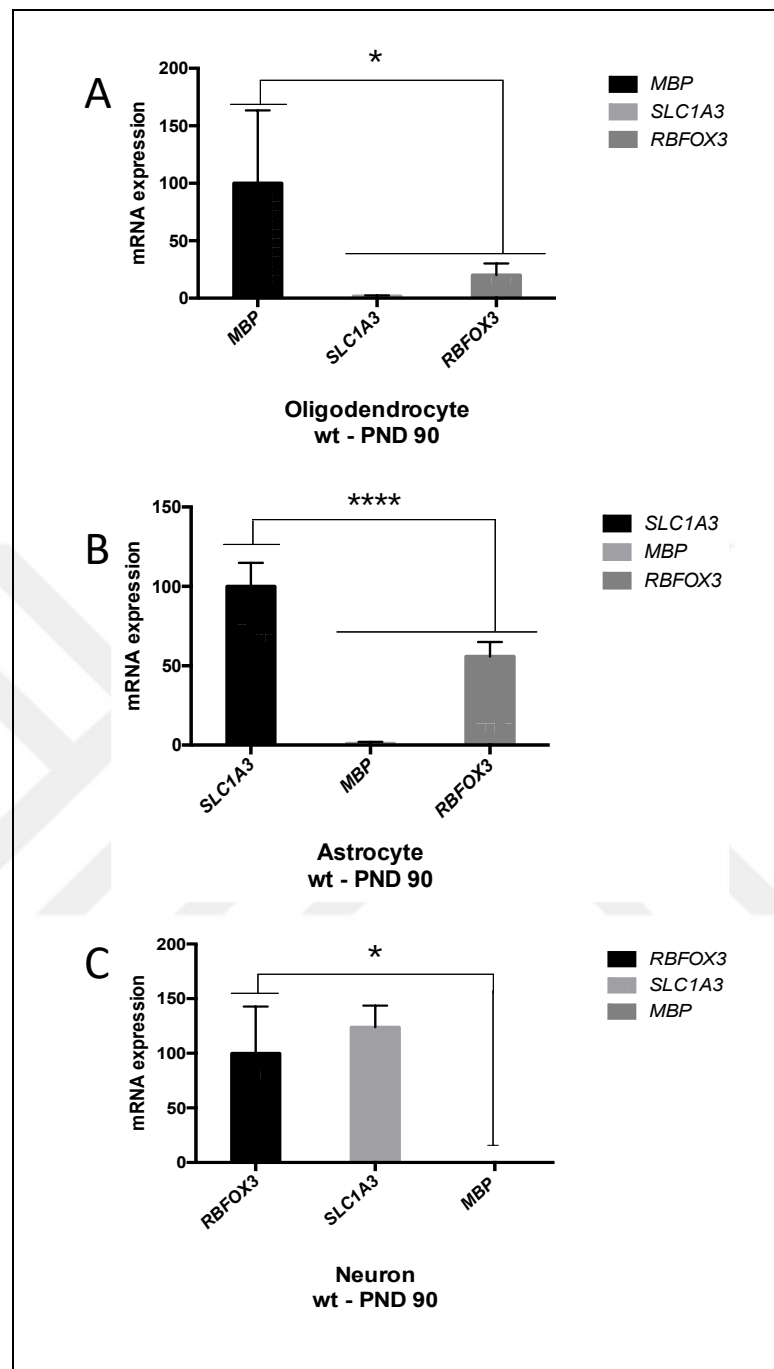


Figure 3.5. Relative mRNA levels of marker genes in magnetically sorted (A) oligodendrocytes, (B) astrocytes, and (C) neurons in PND 90 wild-type mice. Data represents average mRNA expression of three biological replicates (n=3) for each cell sorting experiment $\pm$ SEM (* $P \leq 0.05$, **** $P \leq 0.0001$)

The expression of marker genes in sorted cell populations and their expression in whole-brain samples were also compared, and even in populations in which separation did not 100 percent successfully occur, cell enrichment was observed very clearly (Figure 3.6.).

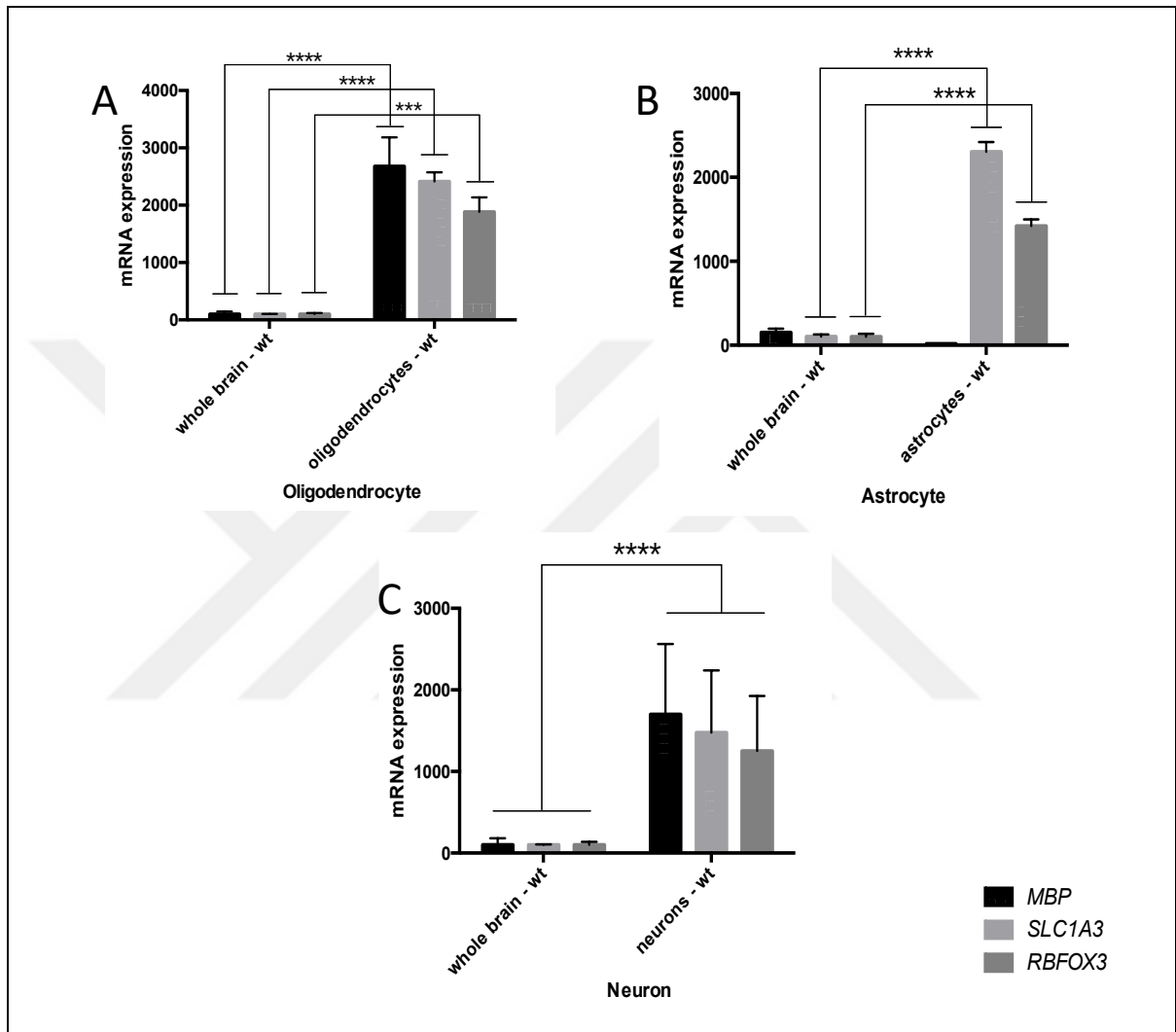


Figure 3.6. Relative mRNA levels of marker genes in magnetically sorted (A) oligodendrocytes, (B) astrocytes, and (C) neurons comparing whole-brain samples in PND 90 wild-type mice. Data represents average mRNA expression of three biological replicates (n=3) for each cell sorting experiment $\pm$ SEM (*** $P \leq 0.001$, **** $P \leq 0.0001$)

qPCR for the genes of interest; *MCT1* and *MCT4*, expressed in magnetically sorted cell fractions isolated from brain materials of SOD1-G93A PND 90 mice was performed. *MCT1* expression was surprisingly higher in oligodendrocytes of SOD1-G93A animals (3,62 fold), contrary to the study of Lee et al. [14], which we referred to, while *MCT4* expression in sorted astrocytes was lower (2,7 fold) in transgenic animals than in their wild-type healthy counterparts (Figure 3.7.).

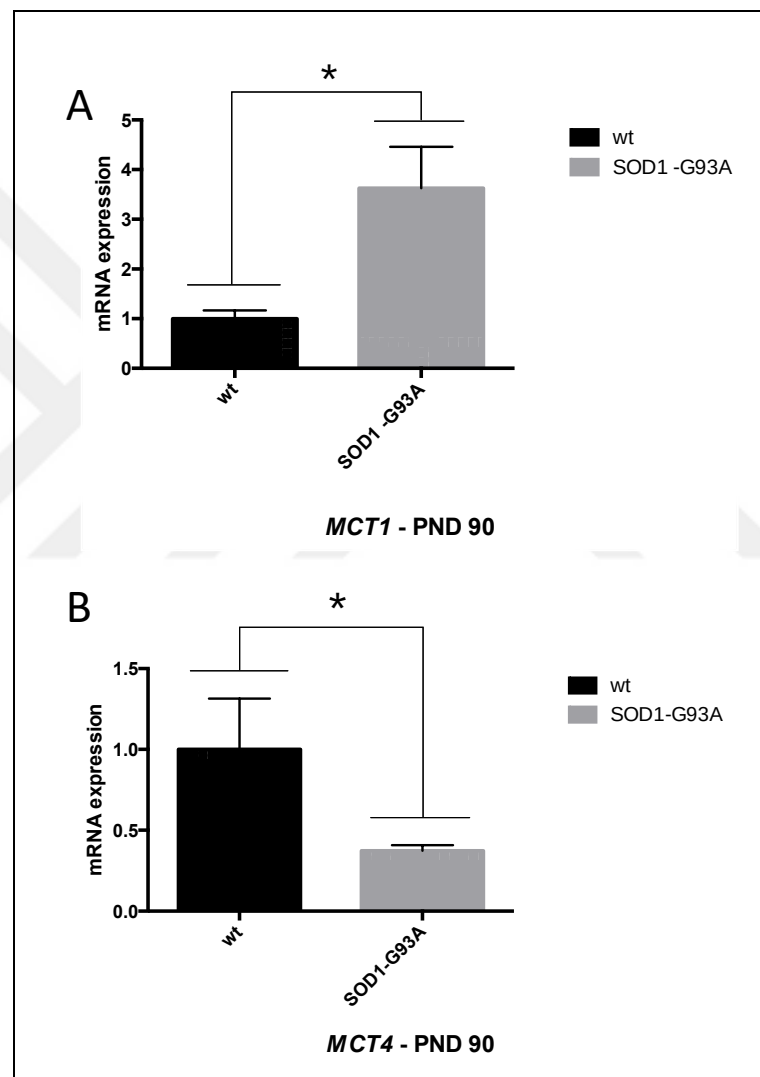


Figure 3.7. Gene expressions of *MCT1* in sorted oligodendrocytes (A) and *MCT4* in sorted astrocytes (B) in PND 90 SOD1-G93A mice. Data represents average mRNA expression of three biological replicates (n=3) for each group $\pm$ SEM (* $P \leq 0.05$)

In addition to the cells isolated from brain materials and then subjected to magnetic cell sorting, MCT expressions were also examined in whole-spinal cord materials collected from the same PND 90 transgenic and wild-type animals. While all MCT isoforms functioning in the spinal cord were expressed higher in SOD1-G93A mice than in wild-type controls (MCT1; 3,87 fold, MCT2; 2,68 fold, and MCT4; 11,99 fold), the expressions of *MCT1* and *MCT2* genes were not statistically significant (Figure 3.8., P-values are 0,0888 and 0,1856, respectively).

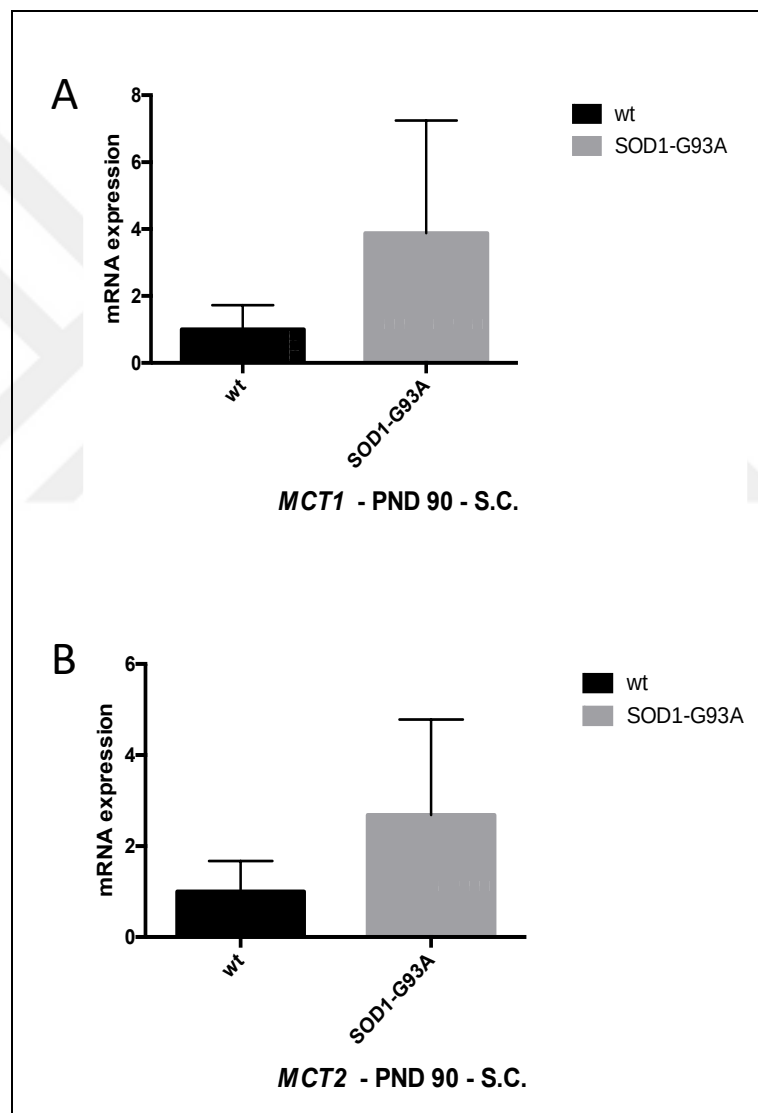


Figure 3.8. *MCT1* (A) and *MCT2* (B) gene expressions in whole-spinal cord (s.c.) material of PND 90 SOD1-G93A mice. Data represents average mRNA expression of three biological replicates (n=3) for each group $\pm$ SEM (P-values are 0,0888 and 0,1856, respectively)

The *MCT4* gene, on the other hand, was significantly higher expressed in SOD1-G93A mice than in their wild-type counterparts (11,99 fold), unlike the other isoforms as indicated in Figure 3.9., (A) ($P \leq 0.01$). The expression of the *COX10* gene which encodes a mitochondrial enzyme catalysing the electron transfer from reduced cytochrome c to oxygen [230] was also tested in whole-spinal cord materials in order to understand whether a mitochondrial dysfunction might occur along with ALS pathogenesis due to a possible lactate metabolism disruption. The *COX10* gene was relatively less expressed (3,13 fold) in SOD1-G93A mice, confirming this suspicion (Figure 3.9., (B), $P \leq 0.05$).

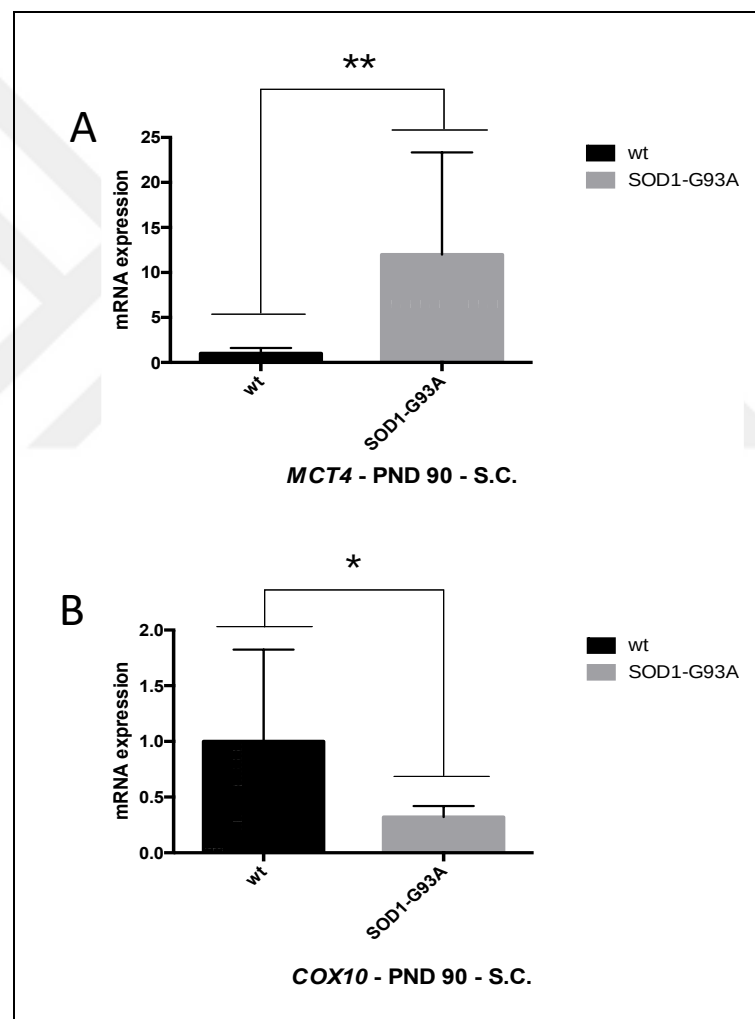


Figure 3.9. *MCT4* (A) and *COX10* (B) gene expressions in whole-spinal cord (s.c.) material of PND 90 SOD1-G93A mice. Data represents average mRNA expression of three biological replicates ($n=3$) for each group $\pm$ SEM (* $P \leq 0.05$, ** $P \leq 0.01$)

MCT1 and *MCT4* genes were also examined in magnetically sorted cells isolated from pre-symptomatic PND 60 SOD1-G93A mice in order to understand whether MCT expression differences still existed before the onset of pathology. Although not statistically significant; both *MCT1* expressions in oligodendrocytes and *MCT4* expressions in astrocytes were slightly similar to that of PND 90 animals at a trend-level as represented in Figure 3.9. (P-values are 0,8588 and 0,9047, respectively), suggesting that pathology might begin at the molecular level before it was displayed phenotypically.

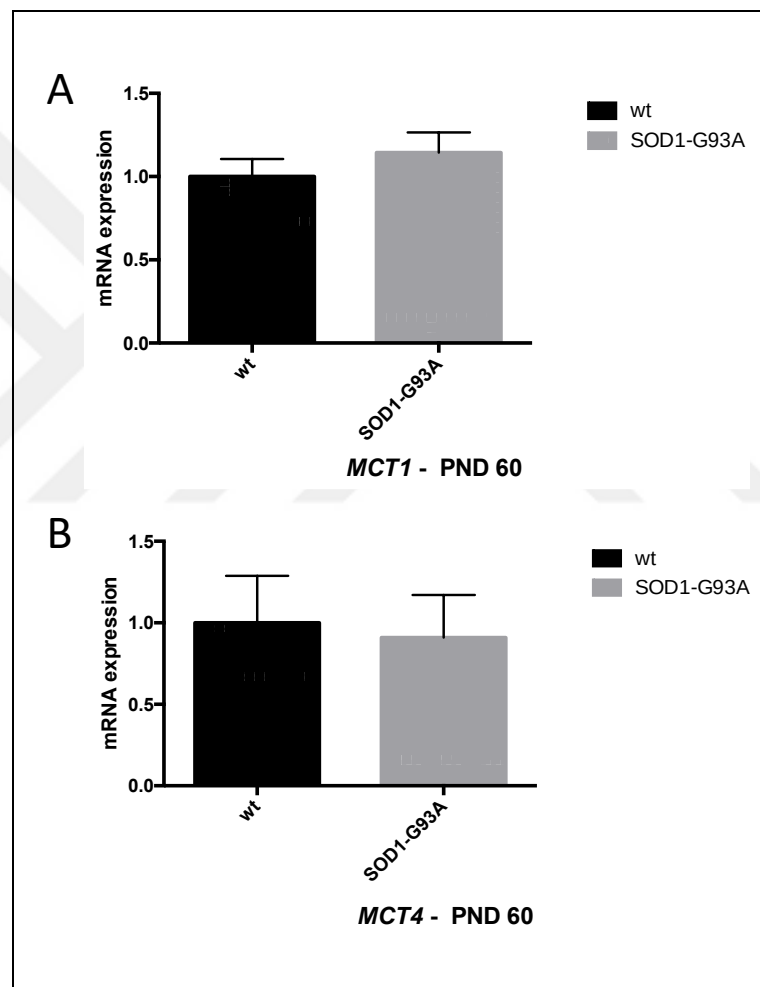


Figure 3.10. Gene expressions of *MCT1* in sorted oligodendrocytes (A) and *MCT4* in sorted astrocytes (B) in PND 60 SOD1-G93A mice. Data represents average mRNA expression of three biological replicates (n=3) for each group $\pm$ SEM (P-values are 0,8588 and 0,9047, respectively)

qPCR for *MCT1* and *MCT4* genes expressed in magnetically sorted cell fractions isolated from 17 months old C9ORF72 transgenic mice and their wild-type counterparts was also

performed. Despite the fact that *MCT1* expression difference was not significant in sorted oligodendrocytes, just as in PND 60 SOD1-G93A transgenic animals, its expression was higher at a trend-level in C9ORF72 transgenic mice than in wild-type controls at the same age (Figure 3.11., (A), p-value is 0,1935). As for the *MCT4* gene in sorted astrocytes, its expression was quite higher in C9ORF72 transgenic mice than in their wild-type counterparts (28,95 fold), as in spinal cord materials of PND 90 SOD1-G93A mice (Figure 3.10., (B), $P \leq 0.0001$).

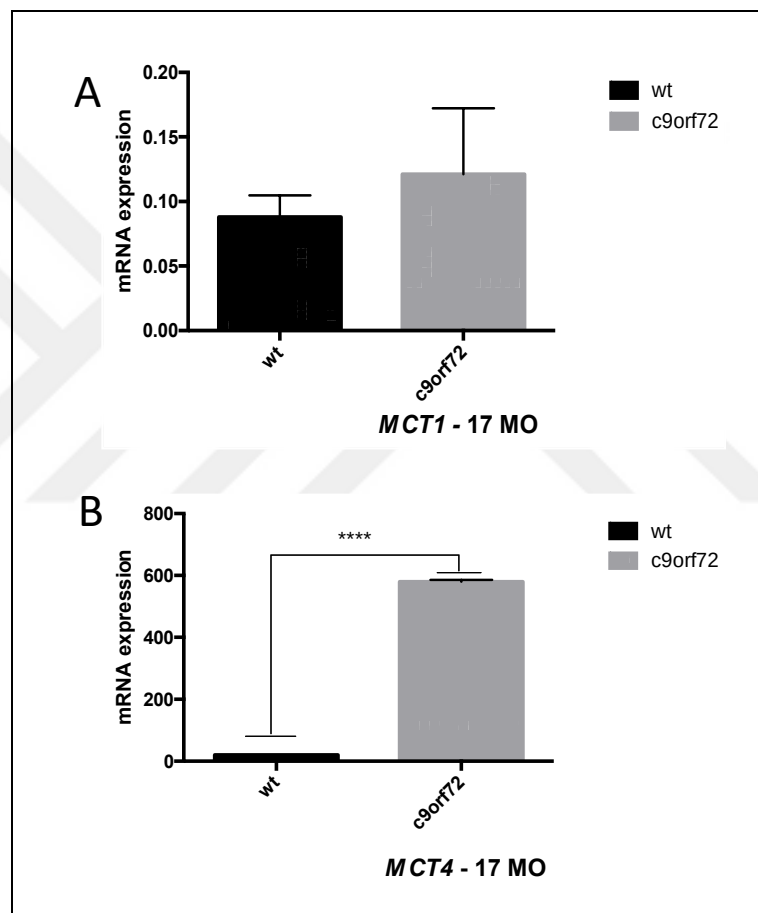


Figure 3.11. Gene expressions of *MCT1* in sorted oligodendrocytes (A) and *MCT4* in sorted astrocytes (B) in 17 MO C9ORF72 mice. Data represents average mRNA expression of three biological replicates (n=3) for each group $\pm$ SEM (P-values are 0,1935 and **** ≤ 0.0001 , respectively)

3.3.2. Western Blot and Simple Western (WES) Analysis

Prior to the examination of target proteins expressions including MCT1, MCT2, and MCT4, protein expression analysis was performed for marker proteins as well as qPCR analyses of marker genes in order to verify whether the MCS process occurred successfully. All protein analyses were carried out for brain samples collected from wild-type mice (PND 90, n=3) assuming sorted cells were separated as neuronal and glial cells. As pointed out in qPCR trials, even though cell separation did not occur 100 percent successfully, we expected a confirmation for a clear cell-type-specific enrichment in the samples compared to its whole-brain counterparts (collected from wild-type PND 90 mice as well, n=3) in protein expression analysis as well.

For oligodendrocyte characterization, MBP antibody; for astrocyte characterization, EAAT2/GLT-1 antibody, and for neuron characterization, RBFOX3 (NeuN) antibody were used as stated in Section 2.1.9. and 2.1.10. before.

While the expression of MCT1 protein in PND 90 SOD1-G93A mice was evaluated with the traditional western blot, other protein expressions for both remaining MCTs and marker proteins were examined by Simple Western (WES) method, which is more advantageous in many respects as mentioned in Section 2.1.10.. GAPDH antibody was used as a loading control for both western blot and Wes applications. Results were analysed by two-way ANOVA and Student's t-test using GraphPad Prism Version 6.0e.

3.3.2.1. *Protein Expression Analysis of Neuronal and Glial Markers*

According to the Wes results, MBP protein expression was quite high compared to other marker proteins as represented in Figure 3.12. (*** $P \leq 0.001$, **** $P \leq 0.0001$). The presence of low rates of NeuN and Glt-1 expressions indicated a low amount of neuronal and astroglial cell contamination in the oligodendrocyte fraction, but overall, low expression of both whole-brain controls and mentioned marker proteins revealed a significant oligodendroglial cell enrichment in the sorted samples confirming qPCR experiments.

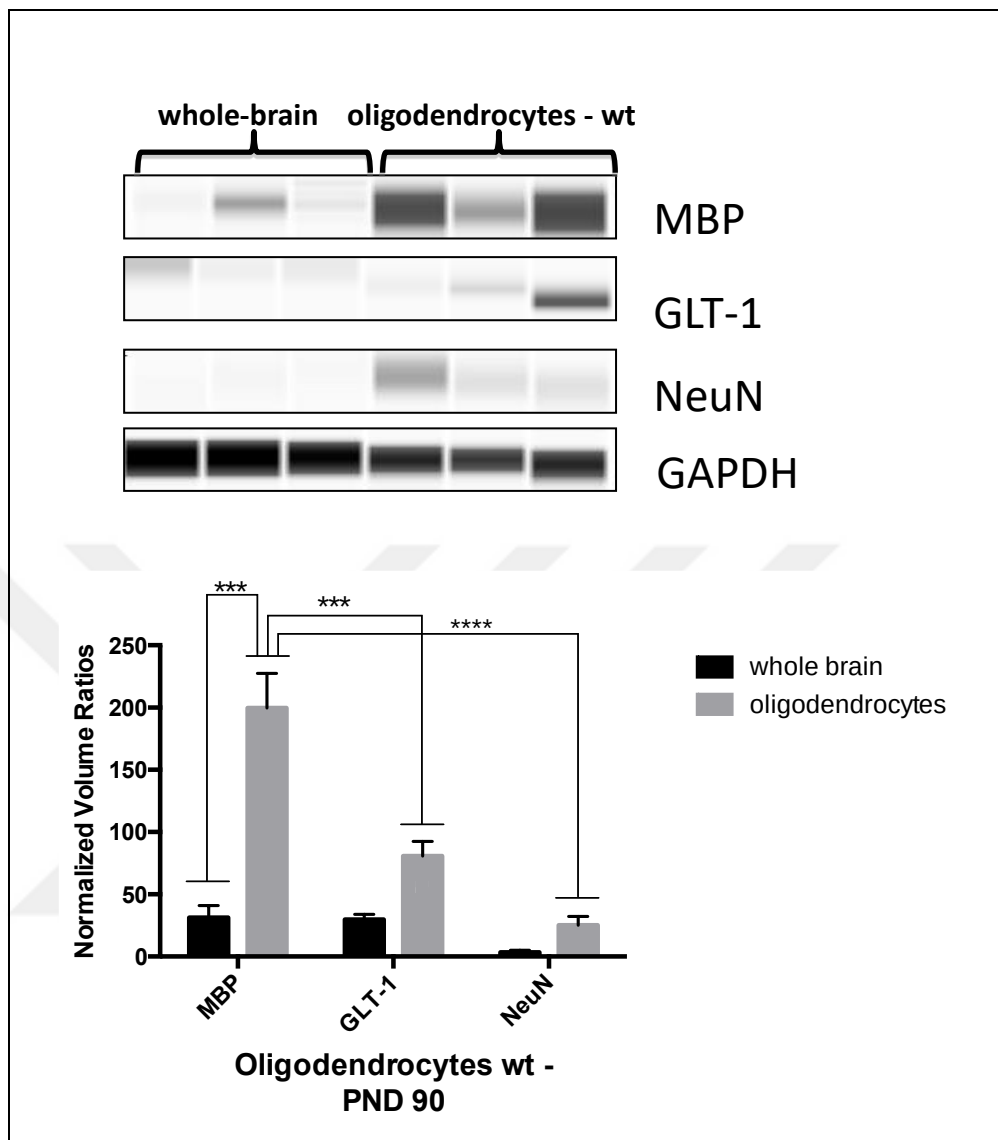


Figure 3.12. Protein expressions of MBP, GLT-1, and NeuN markers in sorted oligodendrocytes in PND 90 SOD1-G93A mice. Data represents average normalized volume ratios of three biological replicates ($n=3$) for each group $\pm$ SEM (*** $P \leq 0.001$, **** $P \leq 0.0001$)

Although it does not seem as successful as oligodendroglial cell separation, it was observed that there was certain cell enrichment for magnetically sorted astrocyte cells as stated in Figure 3.12. (*** $P \leq 0.001$, * $P \leq 0.05$). The results showed that there might be a certain amount of oligodendrocyte contamination in the astrocyte cell fraction, as the expression level of MBP protein was not very low as displayed in Figure 3.13.. However, the low expression of the MBP gene in qPCR experiments indicates that this MBP protein

contamination may be due to the incomplete removal of the remnants of the oligodendroglial myelin sheath rather than the cell bodies of the oligodendrocytes.

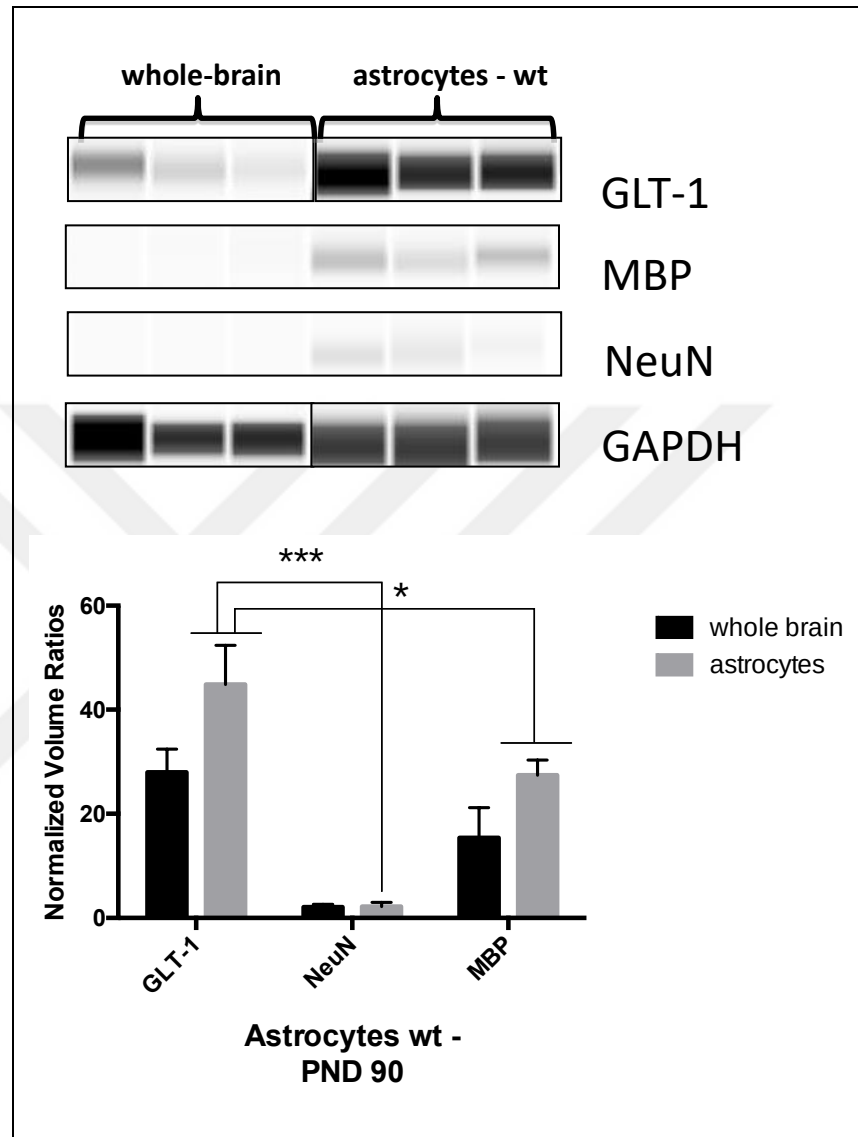


Figure 3.13. Protein expressions of GLT-1, NeuN, and MBP markers in sorted astrocytes in PND 90 SOD1-G93A mice. Data represents average normalized volume ratios of three biological replicates (n=3) for each group $\pm$ SEM (** $P \leq 0.001$, * $P \leq 0.05$)

Just as in the qPCR experiments, protein expression analysis show that the astroglial marker GLT-1 was overexpressed, approximately two fold more than the neuronal marker NeuN in neuronal cell fraction as indicated in Figure 3.14.. Again, although not as intense as GLT-1 expression, MBP expression was also found to be higher than NeuN expression. In addition, the extensive standard deviation in the expression values was also not allowed

to conduct a clear assessment for significant positive or negative results. In conclusion, both qPCR and Wes studies determined that glial markers were still intensely expressed in the neuronal cell fraction after cell sorting, suggesting that MCS might be an inefficient method for neuronal cell extraction.

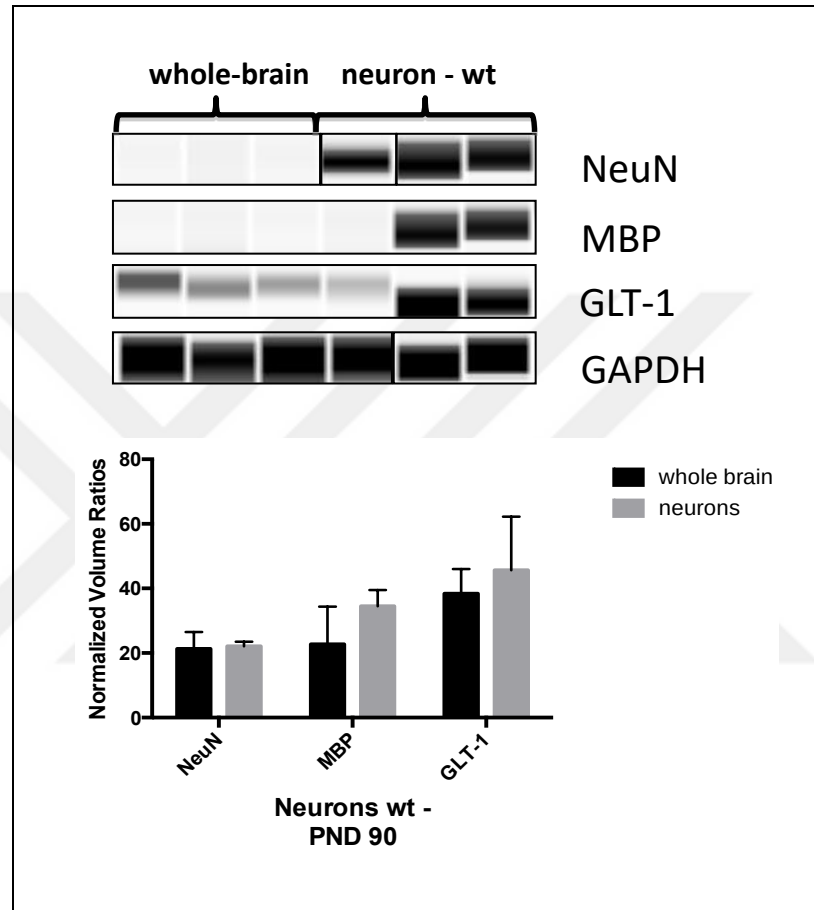


Figure 3.14. Protein expressions of NeuN, MBP, and GLT-1 markers in sorted neurons in PND 90 SOD1-G93A mice. Data represents average normalized volume ratios of three biological replicates (n=3) for each group $\pm$ SEM (P-values for interaction, row factor and column factor are 0,8404, 0,1288, and 0,4047, respectively)

3.3.2.2. Protein Expression Analysis of MCTs

Interesting results were obtained for MCT expressions by qPCR experiments. Protein expression analyses were performed to investigate whether these results were reflected in MCTs' protein translations. As mentioned before, while MCT1 protein expression was examined by the classical western blot method, the expressions of the remaining isoforms were evaluated by novel the Simple Wes application.

As indicated in Figure 3.15. ($***P \leq 0.001$), MCT1 expressions in sorted oligodendrocytes were higher in SOD1-G93A PND 90 mice (4.5 fold) than in wild-type counterparts, confirming qPCR experiments, in contrast to Lee's study [14].

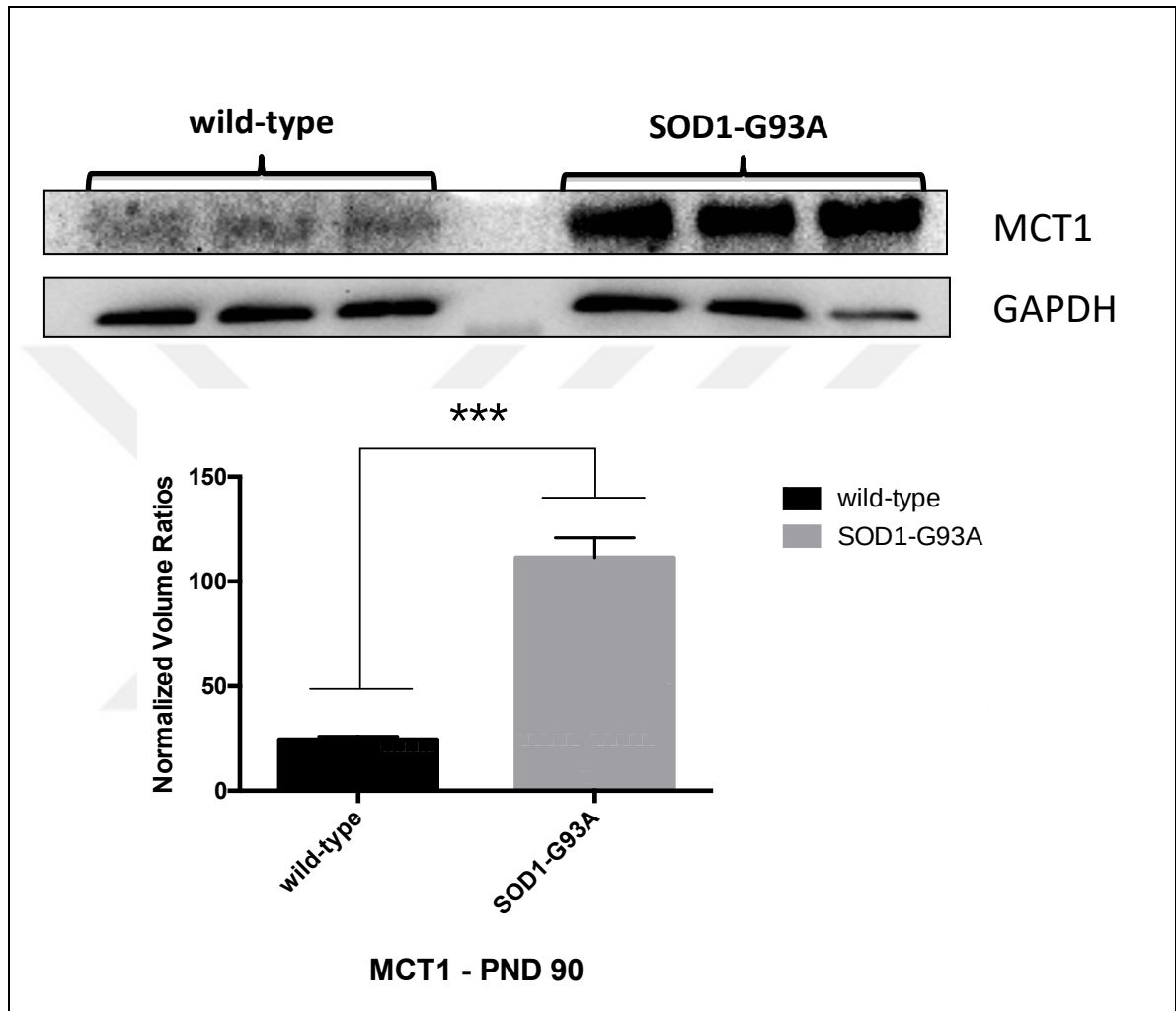


Figure 3.15. Protein expressions of MCT1 in magnetically sorted oligodendrocytes in PND 90 SOD1-G93A mice. Data represents average normalized volume ratios of three biological replicates ($n=3$) for each group $\pm$ SEM ($***P \leq 0.001$)

Unlike qPCR experiments, via Wes method, MCT2 expressions were also examined and its expressions were found to be slightly higher in SOD1-G93A PND 90 mice comparing wild-type mice of the same age. Nevertheless, these results were not significant and remained at a trend-level (Figure 3.16., P-value: 0,2780).

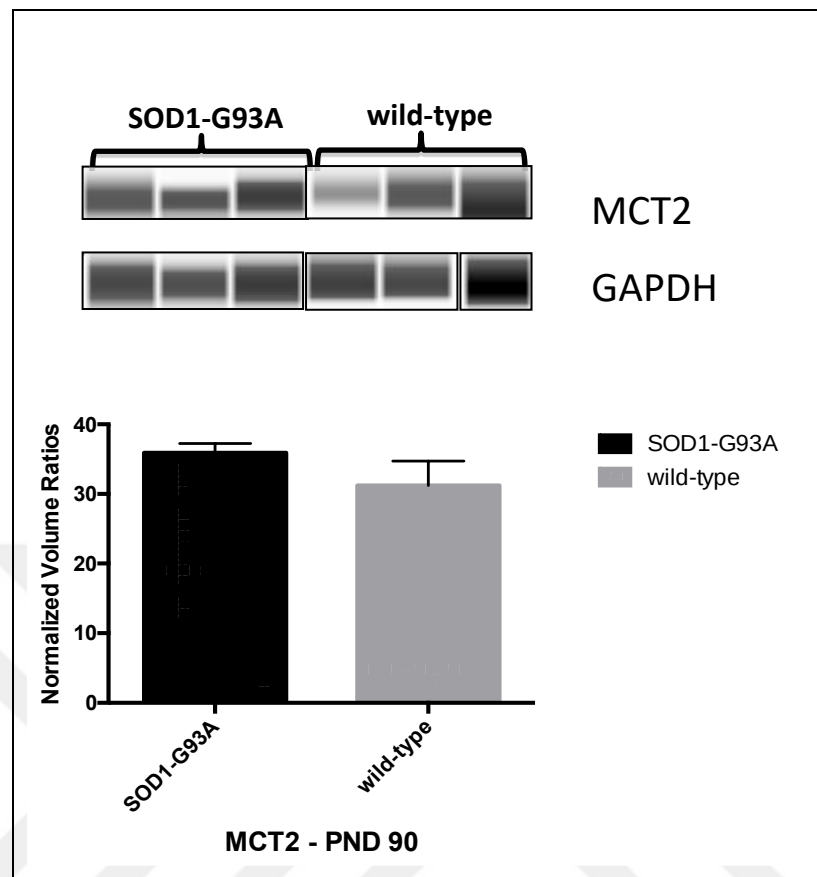


Figure 3.16. Protein expressions of MCT2 in magnetically sorted neurons in PND 90 SOD1-G93A mice. Data represents average normalized volume ratios of three biological replicates (n=3) for each group $\pm$ SEM (P-value: 0,2780)

As for the MCT4 protein, its expressions were significantly lower (1,52 fold) in SOD1-G93A PND 90 mice comparing wild-type healthy controls, supporting Lee's study and our qPCR results, pointing out that low MCT4 expression may contribute directly or indirectly to the onset of ALS pathology (Figure 3.17.).

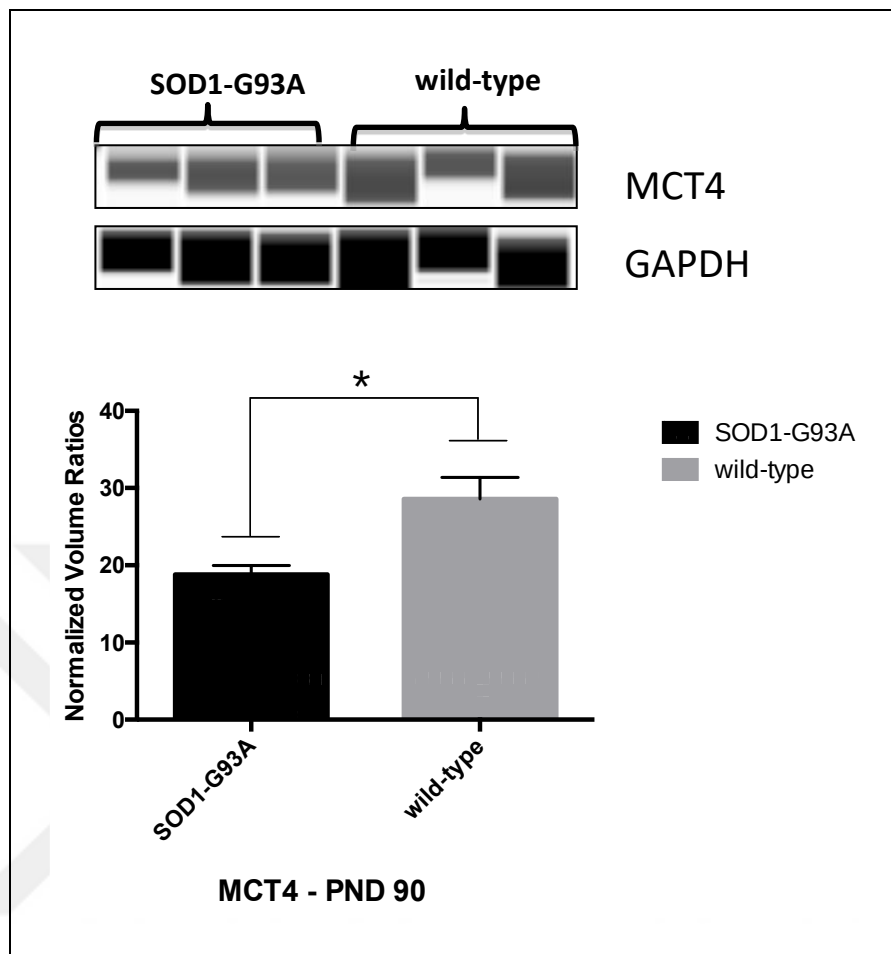


Figure 3.17. Protein expressions of MCT4 in magnetically sorted astrocytes in PND 90 SOD1-G93A mice. Data represents average normalized volume ratios of three biological replicates (n=3) for each group $\pm$ SEM (* $P \leq 0.05$)

Protein expression profiles of MCT1, MCT2, and MCT4 were also investigated in magnetically sorted cells isolated from pre-symptomatic PND 60 SOD1-G93A mice in order to understand whether MCT expression differences still existed before the onset of pathology. Although not statistically significant; protein expressions of MCT1 in sorted oligodendrocytes, MCT2 in sorted neurons, and MCT4 in sorted astrocytes were similar to the expression levels in PND 90 mice at a trend-level as indicated in Figure 3.18. (P-values are 0,4532, 0,4929, and 0,3630, respectively), confirming qPCR results and again, suggesting the pathogenesis might begin at the molecular level before phenotypical onset.

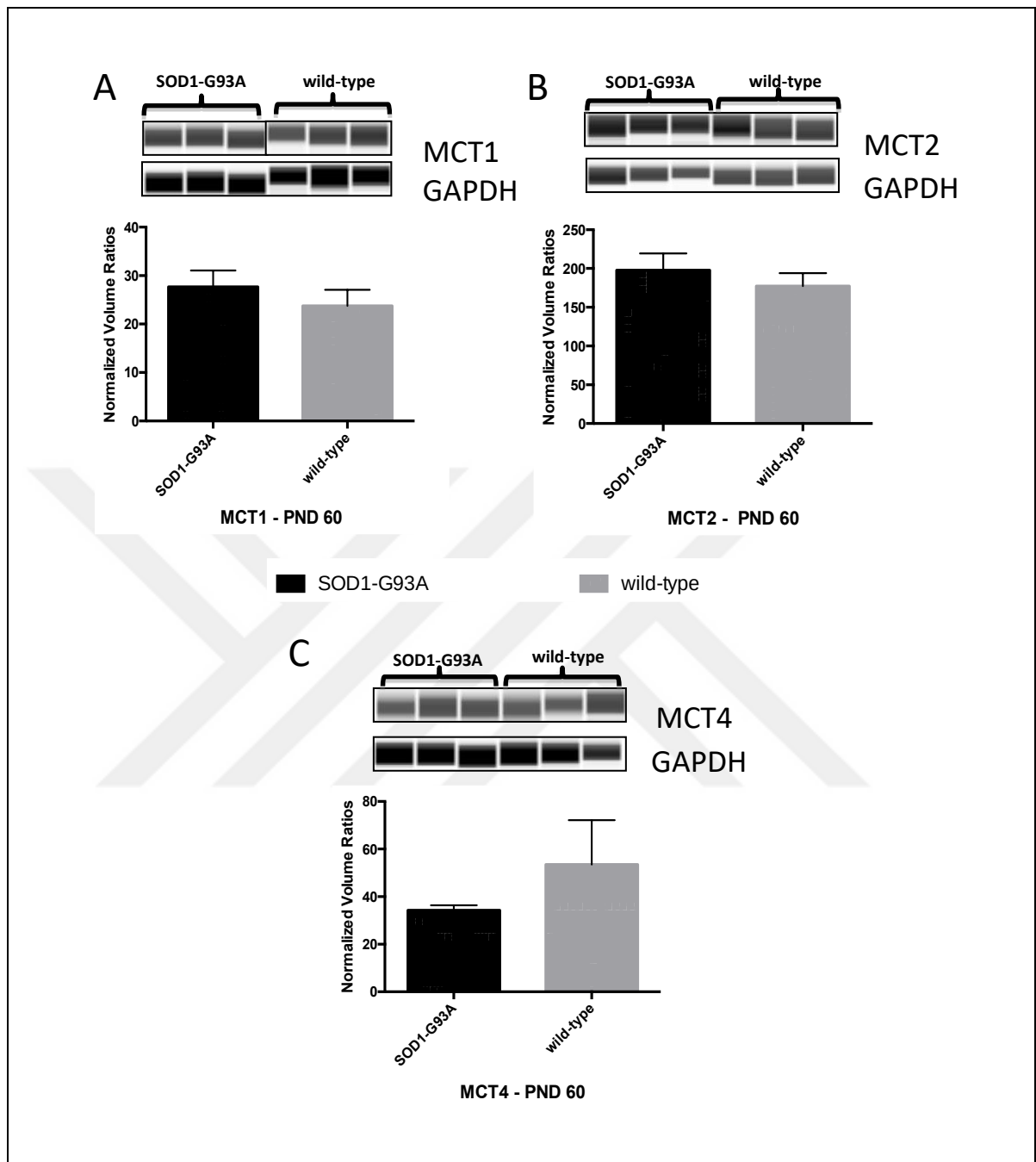


Figure 3.18. Protein expressions of (A) MCT1 in magnetically sorted oligodendrocytes, (B) MCT2 in magnetically sorted neurons, and (C) MCT4 in magnetically sorted astrocytes in PND 60 SOD1-G93A mice comparing wild-type controls. Data represents normalized volume ratios of three biological replicates ($n=3$) for each group $\pm$ SEM (P-values are 0,4532, 0,4929, and 0,3630, respectively)

3.4. INVESTIGATION OF THE INHIBITION OF MCT FUNCTION AND THE EFFECT OF LACTATE TRANSFER ON CELL SURVIVAL IN AN *IN-VITRO* CELL MODEL

3.4.1. Cell Viability Assays

NSC-34 is a hybrid cell line generated by the fusion of embryonic mouse spinal cord cells, engineered to contain a high number of motor neuron cells with mouse neuroblastoma. These cells, as mentioned in Section 2.3.1., are suitable for use in *in-vitro* studies of neurodegenerative diseases as it recapitulates many characteristics of motor neurons, including synthesis storage and release of acetyltransferase, acetylcholine, and possessing neurofilament triplet proteins [230].

The role and crucial importance of lactate existence in CNS cells have been a long-debated topic since Pellerin and Magistretti's pioneering study [74], which also serves as a basis for our study. In the light of these findings, here, we investigated whether lactate is a preferable energy source for NSC-34 cells in the absence of glucose as well, and glucose starvation assay was applied on NSC-34 cell culture. For the experiment, DMEM/low glucose media with a glucose content of 5.5 mM were used and mixed with 80, 40, 20, and 10 mM Sodium L-lactate, respectively to estimate the optimal lactate concentration.

Cells (2000/well) were seeded in 96-well plates in high glucose complete medium, 24 hours before the experiment for MTS and nuclear staining (Hoechst/ PI) assays. The media of the cells were changed the next day with media containing lactate at various concentrations as mentioned above. Five wells were used for the positive control group treated with DMEM high-glucose media, negative control group treated with DMEM low-glucose media, and other remaining groups treated with various lactate concentrations.

As an initial step to measure cell survival, MTS assay was performed every other day for 12 days for long-term observation of cell growth in lactate-contained media. Following that, Hoechst/PI staining was performed to compare the cell survival results of MTS assay. This time, cells were cultivated for 6-10 days to reach the appropriate cell number considering their growth rate in MTS experiment, and cell survival was measured every other day. The results obtained in both assays, which were performed with three replicates,

were expressed in proportion to the control groups. They were analysed by two-way ANOVA and Tukey's test using GraphPad Prism Version 6.0e.

In MTS assay, cell survival of the low-glucose and low-glucose + lactate treated groups was evaluated relatively to the high glucose treated control group, where cell survival was considered maximum. According to the results, the group treated with 20 mM L-lactate + low-glucose media gave the closest average cell viability (~35 percent decrease) to the group treated with classic high glucose complete media between day six and day 10, when cell proliferation peaked as represented in Figure 3.19., (A) (** $P \leq 0.001$, **** $P \leq 0.001$). In addition to that, the group treated with 40 mM L-lactate + low-glucose media showed ~42 percent, and the group treated with 10 mM L-lactate + low-glucose media showed ~40 percent average decrease in cell viability comparing positive control groups between the same time points. The cell viability in the negative control group treated with only DMEM low-glucose media surprisingly reduced by only 39 percent. Due to the fact that 80 mM Na L-lactate was most probably toxic for the cells, the group treated with 80 mM lactate + low-glucose showed the lowest cell viability (~81 percent decrease).

As for Hoechst/ PI staining, quite interestingly; the high glucose treated group exhibited quite a low cell viability rates (average decrease of 40 percent) throughout the experiment in contrast with its MTS assay results. Nevertheless, except for this inconsistency, cell viability of the group treated with 20 mM L-lactate + low-glucose media bore a close resemblance to its MTS measurement and possess the highest average cell survival rates (~10 percent decrease) among lactate treated groups as demonstrated in figure 3.19., (B) between day six and day 10. It is followed by 40 and 10 mM Na L-lactate treated groups with an average decrease of ~12 and ~22 in cell viability, respectively. Data were expressed as percentage of cell viability relative to the lowest rate of cell death (Hoechst/ PI) obtained for each day.

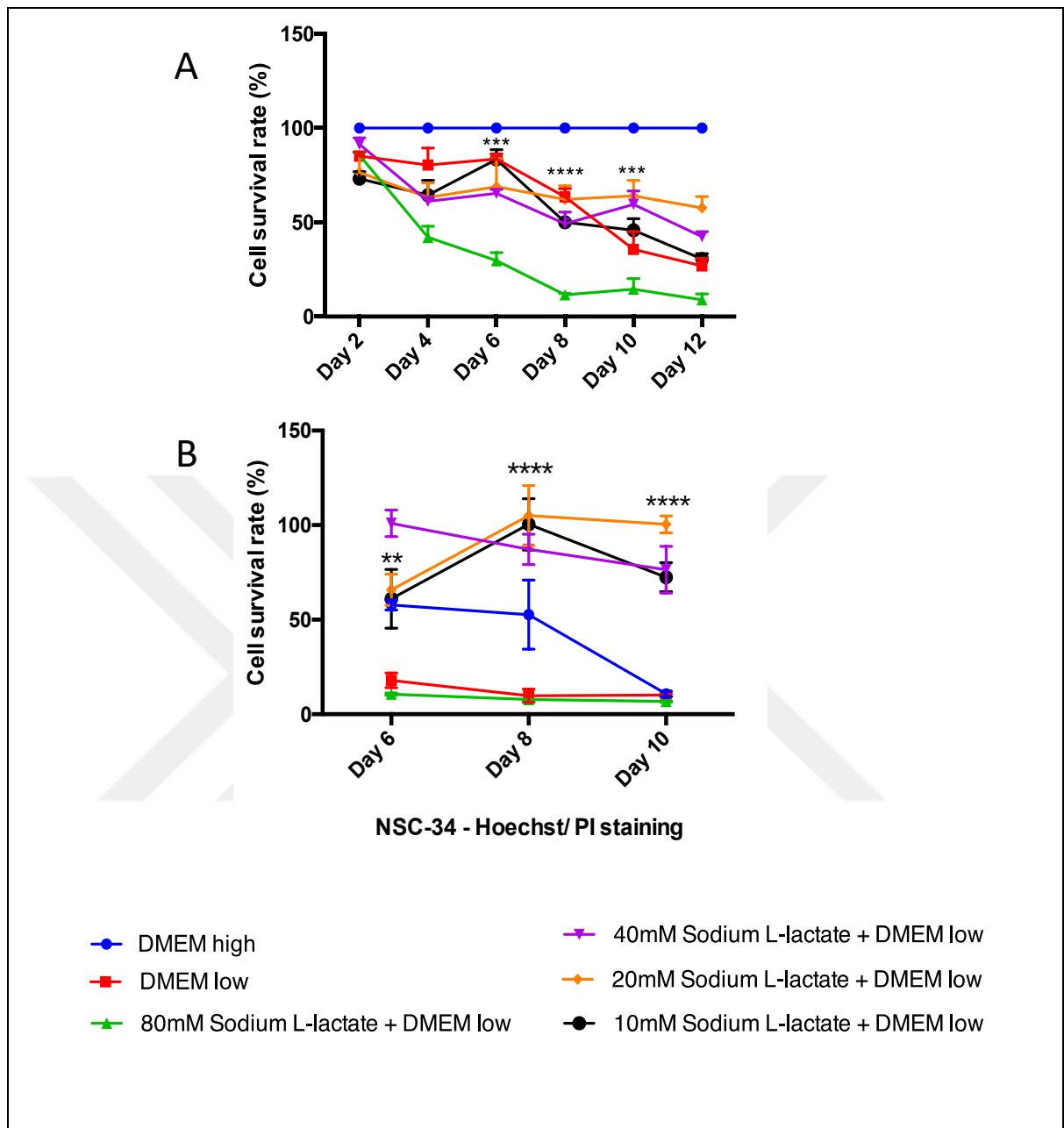


Figure 3.19. The effect of lactate treatment on NSC-34 cell line's survival. The line graph (A) shows the effect of selected Na L-Lactate doses on cell viability by percentage, measured by MTS assay. The line graph (B) represents cell viability comparison of the same line and same lactate treatment with nuclear (Hoechst/ PI) staining. Data represent average cell survival ratios of three independent experiments (n=3) for each group $\pm$ SEM (** $P \leq 0.001$, **** $P \leq 0.001$)

3.4.2. MCTs Expressions in NSC-34 Cell Lines and MCTs Inhibitions

3.4.2.1. qPCR Analysis

As mentioned in Section 2.2.4., AR-C155858; a highly effective inhibitor of MCT1 ($K_i=2.3$ nM for lactate uptake in rat erythrocytes) and proven to inhibit MCT2 function when associated with basigin [215] was utilized, suggesting it would restrain lactate flux into the cell. However, prior to MCT inhibition experiments; in order to fully comprehend undifferentiated NSC-34 cell line characteristics, qPCR was performed for CNS marker genes including *MBP*, *SLC1A3* (*GLAST*), and *RBFOX3* (*NeuN*). *ATP5b* gene was used as housekeeping and mRNA isolated from whole-brain samples was used as a normalization sample (not included in the graphics). Following that, the general distribution of MCT isoforms in NSC-34 cells was also examined via qPCR by measuring gene expressions of *MCT1*, *MCT2*, and *MCT4*. As represented in Figure 3.20., *MBP* was the highest expressed marker gene comparing *GLAST* and *NeuN* (* $P \leq 0.05$).

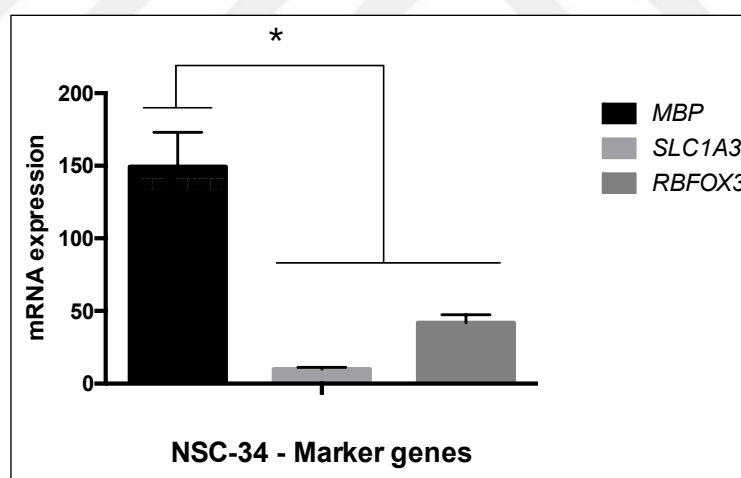


Figure 3.20. Relative mRNA levels of marker genes in NSC-34 cells. Data represents average mRNA expression of three independent experiments, with three technical replicates ($n=3$) $\pm$ SEM (* $P \leq 0.05$)

In addition to that, as indicated in Figure 3.21., the *MCT1* gene appeared to be the most abundant isoform expressed among others, implying that undifferentiated NSC-34 cells have glial cell characteristics rather than neuronal, and more specifically, they resemble oligodendrocyte cells with high *MBP* and *MCT1* gene expressions. Results were analysed

by two-way ANOVA using GraphPad Prism Version 6.0e.

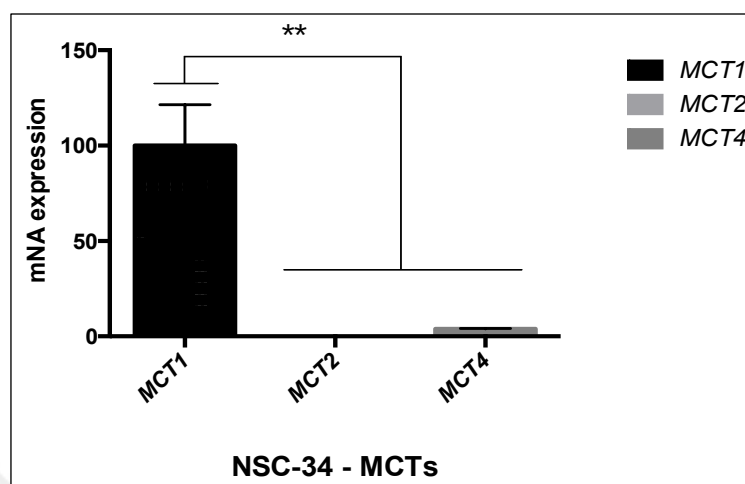


Figure 3.21. Relative mRNA levels of *MCT1*, *MCT2*, and *MCT4* genes in NSC-34 cells. Data represents average mRNA expression of three independent experiments, with three technical replicates ($n=3$) $\pm$ SEM (** $P \leq 0.01$)

3.4.2.2. *MCT Inhibition by AR-C155858 and HPLC (LC-MS/MS) Analysis*

As mentioned in Section 1.4.3. and 2.3.4. before, cells were incubated in DMEM-high complete media for two days, then transferred to 20mM sodium L-lactate-DMEM/no glucose medium mixture, in which the highest proliferation rate was seen after DMEM/high glucose medium earlier in glucose starvation experiment. Cells were then treated with 200 nM AR-C155858 inhibitor and media and cell pellets were collected at certain time points (0, 2, 4, 8, 16, and 32 minutes). The amount of L-lactate in the collected samples was measured by the HPLC (LC-MS/MS) method. Measurement results obtained from HPLC were converted to area and intensity graphs via Waters's Masslynx Version 4.1. Calculations were performed using area graphs of standards and samples indicated in Figure 3.22., 3.23., and 3.24. and statistical analysis was performed by two-way ANOVA using GraphPad Prism Version 6.0e. P-values ≤ 0.05 were considered to indicate statistically significant results. Since an efficient L-lactate measurement could not be achieved from the pellets by HPLC, the time-dependent alteration in lactate uptake was interpreted indirectly by determining the amount of lactate in the media.

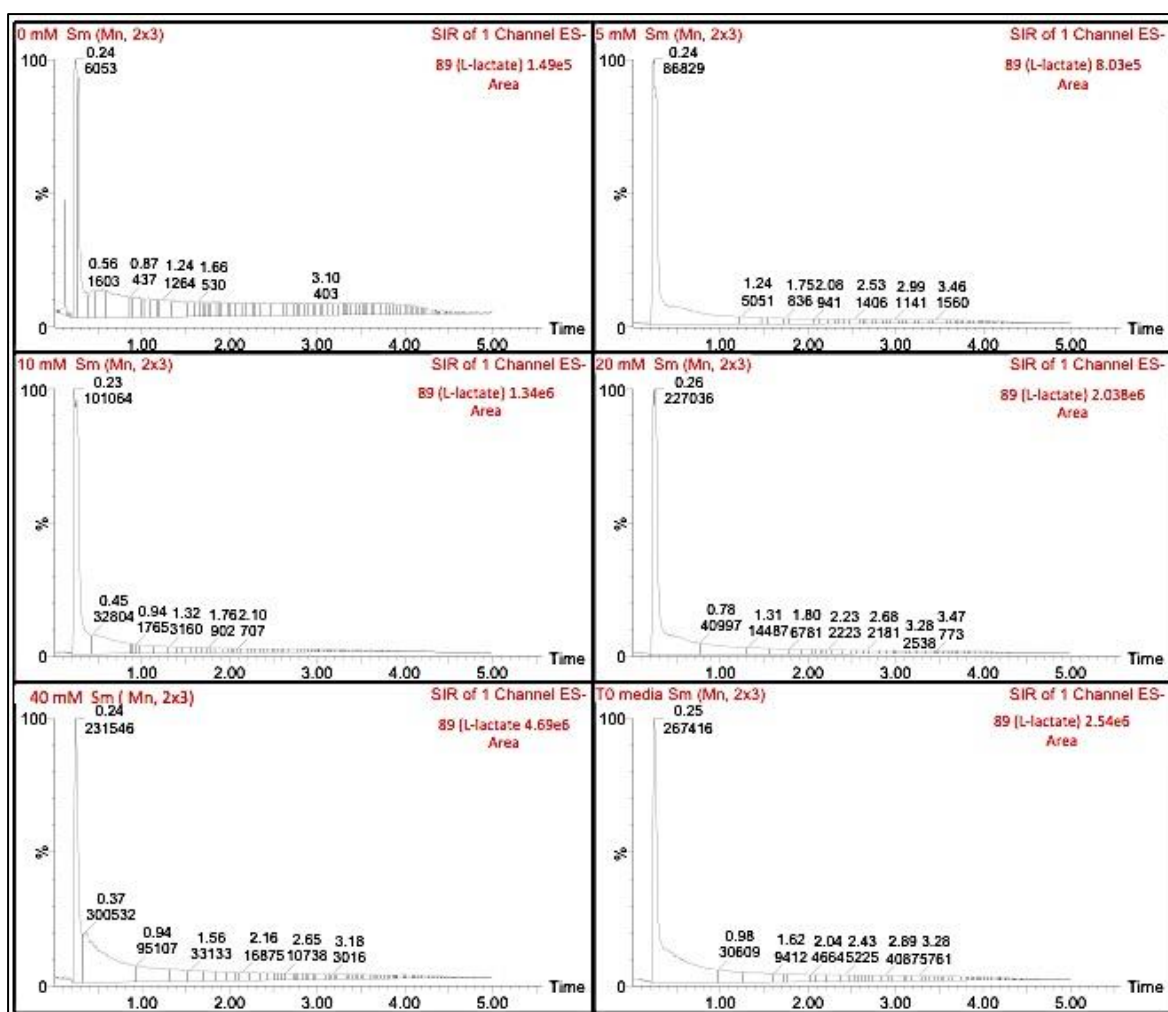


Figure 3.22. Area graphics of 0-40 mM L-lactate standards and L-lactate representation collected from media at t=0 min, which show the amount of lactate obtained by processing the data of HPLC (LC-MS/MS) assay via Masslynx Version 4.1

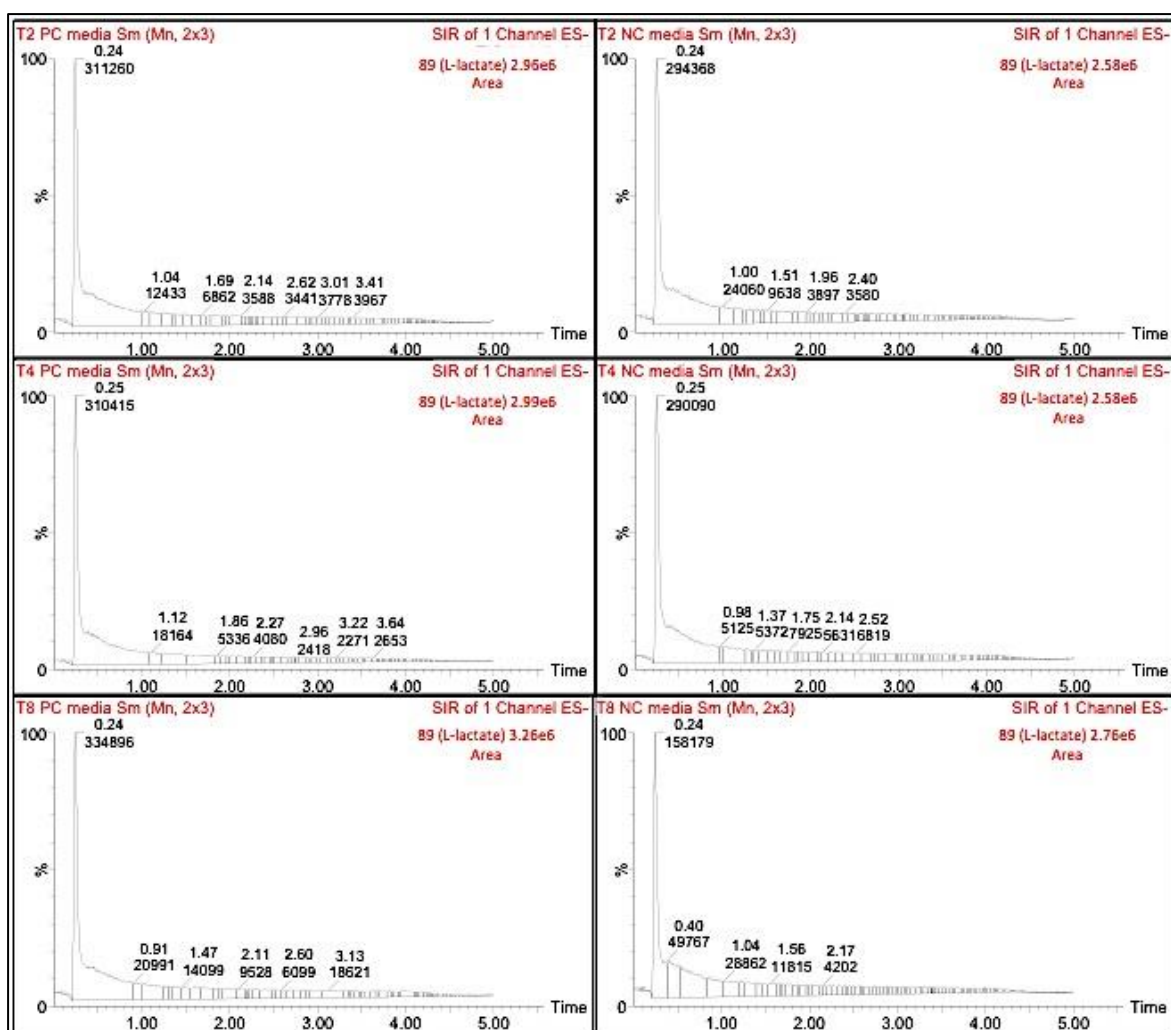


Figure 3.23. Area graphics of negative and positive controls of t=2, 4, and 8 min, which show the amount of L-lactate in media obtained by processing the data of HPLC (LC-MS/MS) assay via Masslynx Version 4.1

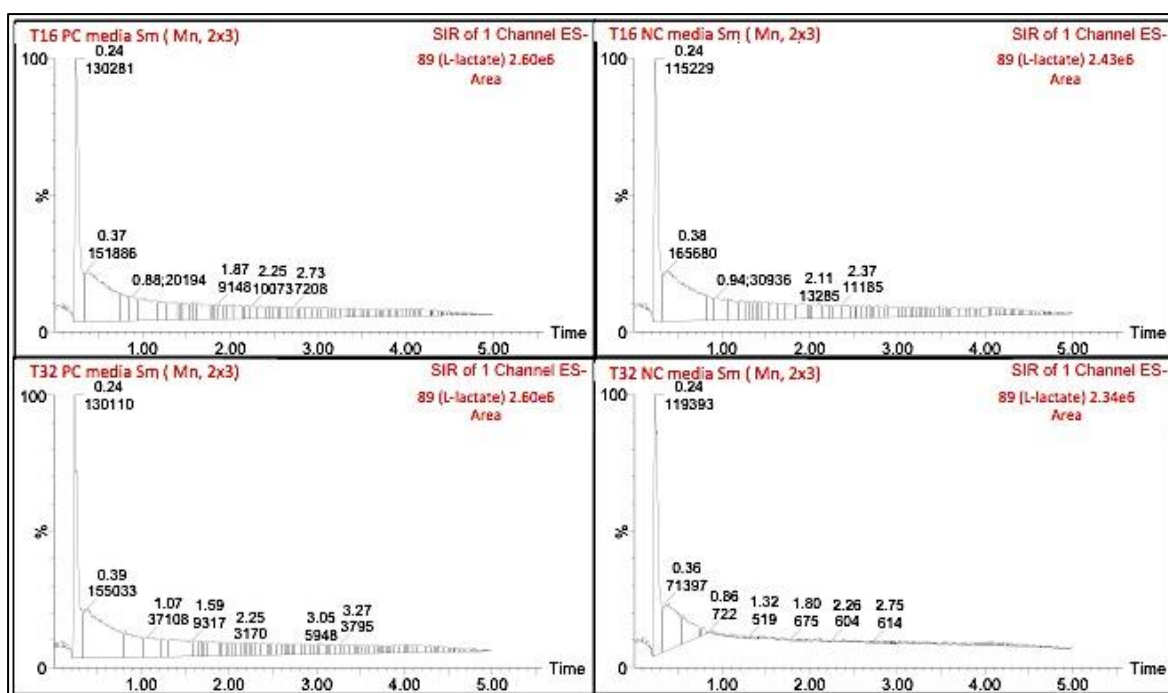


Figure 3.24. Area graphics of negative and positive controls of t=2, 4, and 8 min, which show the amount of L-lactate in media obtained by processing the data of HPLC (LC-MS/MS) assay via Masslynx Version 4.1

Concentration calculations were performed by creating a standard curve from the area graphs extracted from Masslynx software. As indicated in Figure 3.25. below, the amount of L-lactate exhibited a decrease in the media depending on time, suggesting the fact that lactate has been used at certain levels, regardless of the presence of AR-C155858 inhibitor. Despite this, when the positive controls and the negative controls (without inhibitor) belonging to the same time points were examined, it was clearly seen that the amount of lactate in the media was higher than in their negative controls, meaning the inhibitor prevents the lactate entry to the cell to a certain extent. Specifically, this difference was even more pronounced between the media of negative and positive controls collected at two and eight minutes (* $P \leq 0.01$).

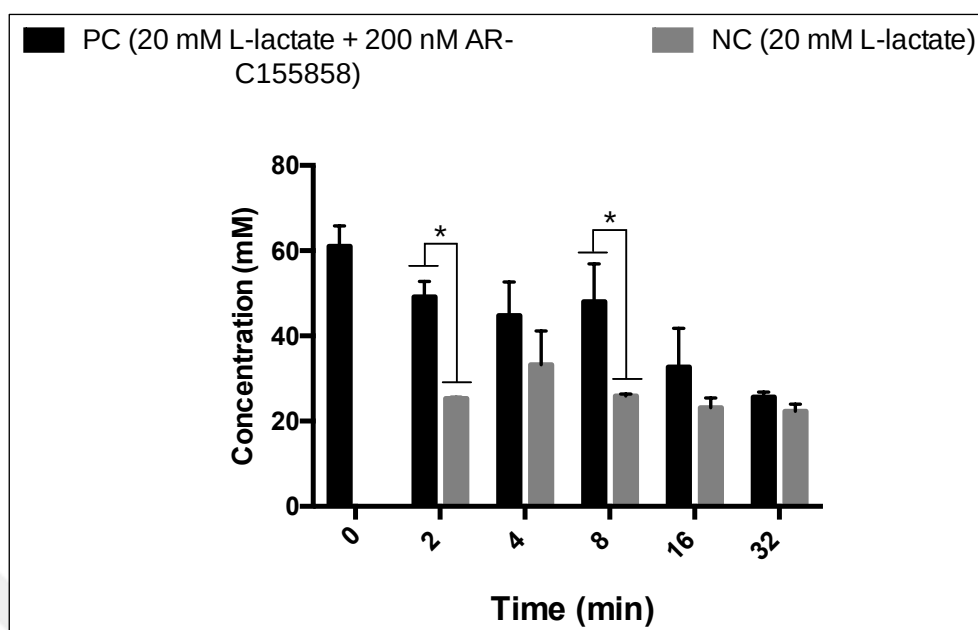


Figure 3.25. The time-dependent inhibition of L-lactate uptake by AR-C155858. Data represents average concentration obtained by three independent measurements from three different experiments ($n=3$) $\pm$ SEM (* $P \leq 0.01$, * $P \leq 0.01$)

3.4.2.3. *Effect of MCT Inhibition on Cell Survival*

Once it was revealed that AR-C155858 inhibitor prevented lactate entry into the cell to a certain level, in an attempt to investigate the effect of the long-term presence of the inhibitor on lactate consumption and its reflection on cell survival, cells were incubated with AR-C155858 for 12, 24, 48, and 72 hours and their cell viability was measured by nuclear staining method as explained in Section 2.3.3.. Statistical analysis was performed by two-way ANOVA using GraphPad Prism Version 6.0e. As demonstrated in Figure 3.26., cell viability decreased in all groups depending on time, however, the rate of viability in the control group; DMEM/high media treated cells decreased to 81 percent at the end of 72 hours, while this rate abated to 49 percent in AR-C155858 + 20 mM L-lactate treated cells. DMEM/no glucose media treated cells showed the lowest survival rate as anticipated with 39 percent. On the other hand, the cell viability of cells treated with DMEM/no glucose media + 20 mM L-lactate was 60 percent, closest to the control group. As can be seen from the results; L-lactate was a preferable energy source after high glucose for undifferentiated NSC-34 cells and AR-C155858 inhibitor clearly blocked the entry of lactate into the cell, which significantly reduced cell survival comparing the

control group (**** $P \leq 0.0001$).

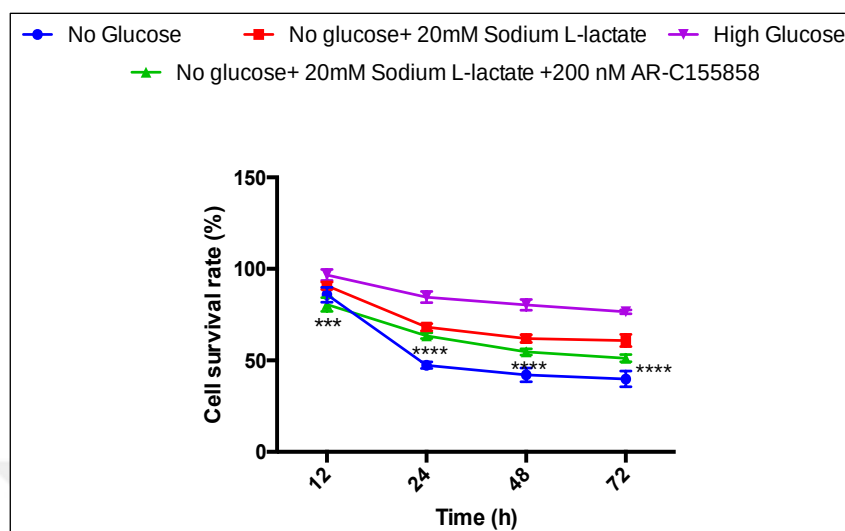


Figure 3.26. The effect of inhibition of L-lactate uptake by AR-C155858 for various time points on cell viability. Data represent average cell survival rates obtained by three independent measurements from three different experiments ($n=3$) $\pm$ SEM (**** $P \leq 0.0001$)

3.5. INVESTIGATING THE EFFECT OF BORON DERIVATIVES ON MCTS AND EVALUATING IT AS AN ALTERNATIVE TREATMENT METHOD FOR ALS

In an attempt to explore the effect of boron derivatives on ALS animal models, pre-symptomatic 80 days old female mice and their wild-type counterparts were used. Boron derivatives dissolved in their drinking water were administered to mice by gavage at 200 mg/kg every other day for 3 weeks. Only water was given to the wild-type and transgenic control groups. Brain and spinal cord materials were collected from the animals after the treatment aiming to investigate the possible effects of applied boron derivatives on the onset and course of ALS symptoms. There were eight animals in each group, five of which are for histological studies in brain and spinal cord materials and three of which are for gene and protein expression analysis (histological studies was postponed to a later date, as the facility where histological studies will be conducted was closed due to pandemic conditions). The animal groups set up for the study and the applied boron derivatives are given in Table 2.8. and Table 2.9., respectively.

3.5.1. Motor Activity Tests

In order to understand whether boron derivatives cause an alteration in the motor coordination of animals, especially transgenic mice, the animals were subjected to certain motor function tests before and after boron applications.

3.5.1.1. Neurological Scoring System (NS)

Neurological scoring for each mouse was assessed along with the experiment as described in Section 2.1.3.1.. No significant effect of boron derivatives on the onset of pathology has been observed. At the end of the experiment, the neuroscores of the mice, neuroscore levels of which were measured regularly were again measured as NS1. Nonetheless, mice in the NaB and SPT treated groups were observed to exhibit significantly milder NS1 symptoms as the experiment progressed and at the end of the experiment, as indicated in detail in Table 3.1..

Table 3.1. Detailed comparison of the phenotypic characteristics of the NS1 stage in the experimental groups (wt: wild-type, tg.: transgenic, s: symptom, s1: hindlimb retraction, s2: tremor, s3: ataxia)

	Day 80 (t=0)			Day 90-NS1 (symptomatic onset of the disease)			Day 110-NS1 (end of the experiment)		
	s1	s2	s3	s1	s2	s3	s1	s2	s3
wt	-	-	-	-	-	-	-	-	-
tg. su	-	-	almost none	moderate	moderate	moderate	severe	severe	severe
tg. SPT	-	-	almost none	almost none	almost none	almost none	mild	mild	mild
tg. NaB	-	-	almost none	mild	mild	mild	moderate	moderate	moderate

3.5.1.2. *Rotarod Test*

Mice were trained three times with the rotarod tester and their performances were recorded at the end of the same day. Motor performances were measured three times for a maximum of 180 seconds at 0.2 rpm / s speed [218] on the accelerated rotarod (Figure 3.27., (A)) and the longest residence times on the apparatus were recorded. Statistical analysis was performed by two-way ANOVA using GraphPad Prism Version 6.0e.

As a result of the comparison of the motor functions of these transgenic animals; it was observed that SPT increased the performance of the rotarod test by 88.4 percent in the SOD1-G93A model compared to the transgenic water control group ($P \leq 0.01$) (Figure 3.27., (B)). In the NaB applied group, the increase (62,45 percent) in the rotarod performance was determined as the trend-level compared to the transgenic water group, but no statistically significant result could be obtained. Although a motor activity improvement was also observed at the trend-level in the BA treated group (26,6 percent), it was even lower than NaB provided. In the transgenic control group, it was observed that the symptoms intensified over time and the rotarod performance decreased.

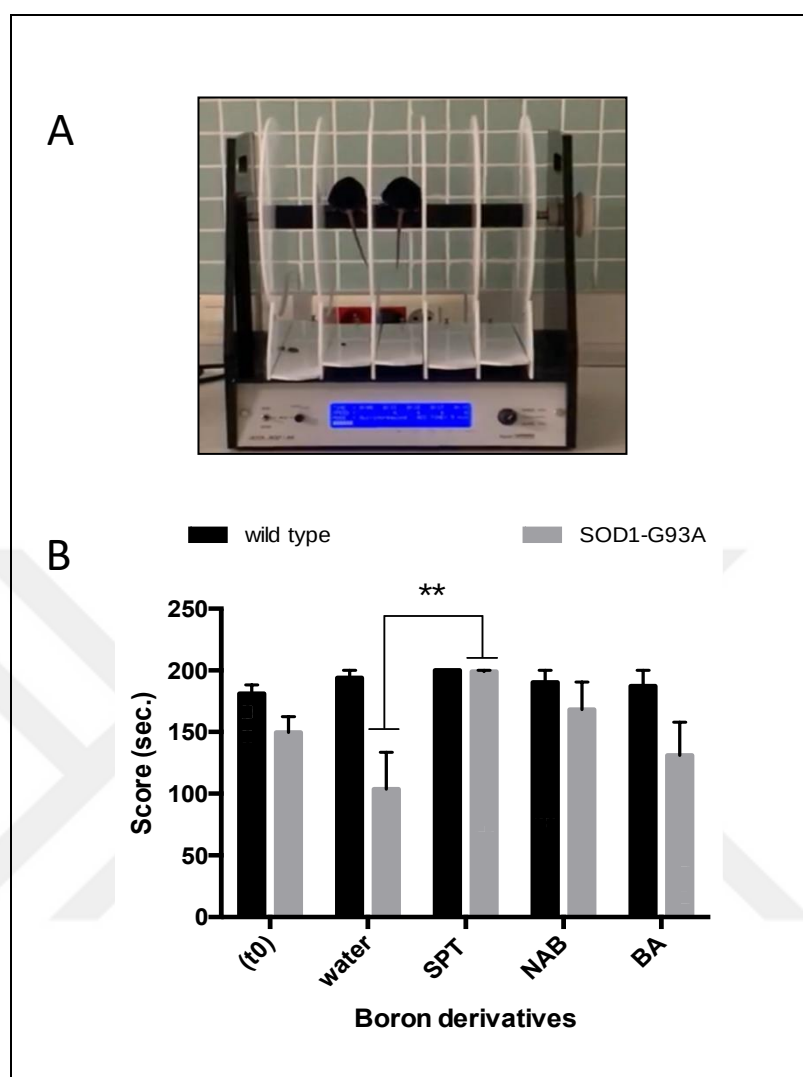


Figure 3.27. Rotarod test results of mice before and after SPT, NaB and BA applications. (A) Rotarod test apparatus, (B) Bar graphic of the motor performances including before and after boron applications. Data represents the longest residence time of three test trials for each mouse (n=8 per group) $\pm$ SEM (** $P \leq 0.01$)

3.5.1.3. Hanging Wire Grip Test

The mice were attached to the metal cable apparatus, which was horizontally suspended at a height of 50 cm from the ground, with its front forepaws, and the length of their stay on the metal cable was recorded, including the hindlimbs. The animals had three attempts to stand for a maximum of 30 seconds and scored as described in Section 2.3.3.. Statistical analysis was performed by two-way ANOVA using GraphPad Prism Version 6.0e.

No significant difference was observed in the motor activities of the wild-type and

transgenic mice via the hanging wire grip test before and after boron administration. (Figure 3.28., P values for interaction, row factor and column factor are 0,2868, 0,9525 and 0,5714, respectively).

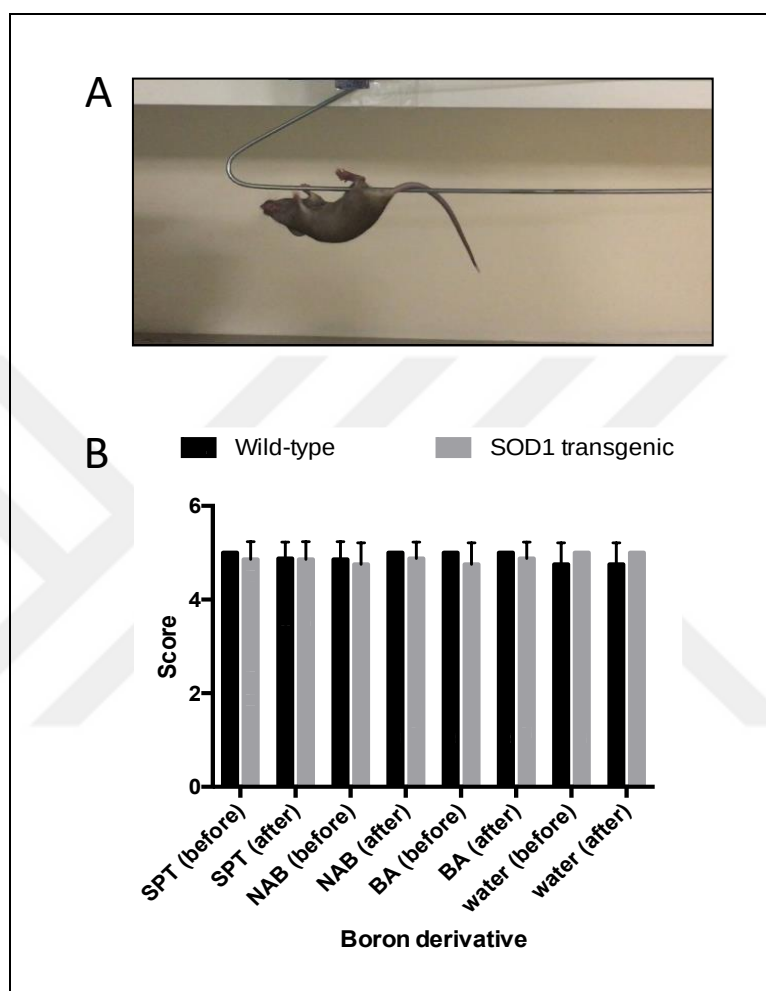


Figure 3.28. Hanging wire grip test results of mice before and after SPT, NaB, and BA applications. (A) Hanging wire test apparatus (B) Bar graphic of the hanging performance of mice including before and after boron applications, data represents the longest residence time of three trials for each mouse (n=8 per group) $\pm$ SEM

3.5.2. Microarray Analysis

As described in Section 2.2.6., in this study, spinal cord and brain materials were collected from the transgenic groups in which NaB and SPT derivatives were applied, which were found to cause a significant or a trend-level positive change in the motor activities of transgenic SOD1-G93A mice in the locomotor activity tests, were subjected to microarray

analysis. In addition, the transgenic water group was used as the untreated ALS model control, and the wild-type water group was used as the untreated healthy control group (Table 2.10.). BA was excluded from the study because it did not show a positive, significant, or trend-level effect, especially in the rotarod test. Microarray analysis results were evaluated by examining the spinal cord and brain materials' data separately.

3.5.2.1. Spinal Cord Materials

Raw micororray data obtained from spinal cord materials was first uploaded into the GeneSpring GX v14.9 software and then evaluated with Volcano Plot analysis (two-way ANOVA was performed for all groups comparison and two-tailed Student's t-test was performed for dual comparisons). NaB and SPT treated transgenic groups and water treated wild-type group were compared to the transgenic water group. Fold change ± 2 and more in gene expressions and P-values ≤ 0.05 were accepted as significant.

Initially, data obtained from the overall examination of the groups were extracted as a heatmap. As shown in Figure 3.29., when the transgenic water group was compared with the wild-type water group, it was observed that there was a significant up-regulation in the general gene expression profile of the transgenic water group. However, the general expression profiles of the transgenic mouse groups treated with NaB and SPT showed a serious tendency towards down-regulation and surprisingly exhibited a clear resemblance to the expression profile of the healthy wild-type water group.

Afterward, scatter plot analysis was performed to compare the gene expression profiles of the transgenic NaB and SPT treated groups with that of the transgenic water group again (Figure 3.30.). In the groups treated with boron derivatives, there was a significant change in the expression levels of a large number of genes. According to the scatter plot results, 7882 genes in the NaB treated group and 7382 genes in the SPT treated group were up or down-regulated more than 2 folds (P-values ≤ 0.05). Likewise, the wild-type water group was also compared with the transgenic water group and 7332 genes in the wild-type water group were up or down-regulated more than 2 folds comparing the transgenic water group (P-values ≤ 0.05).

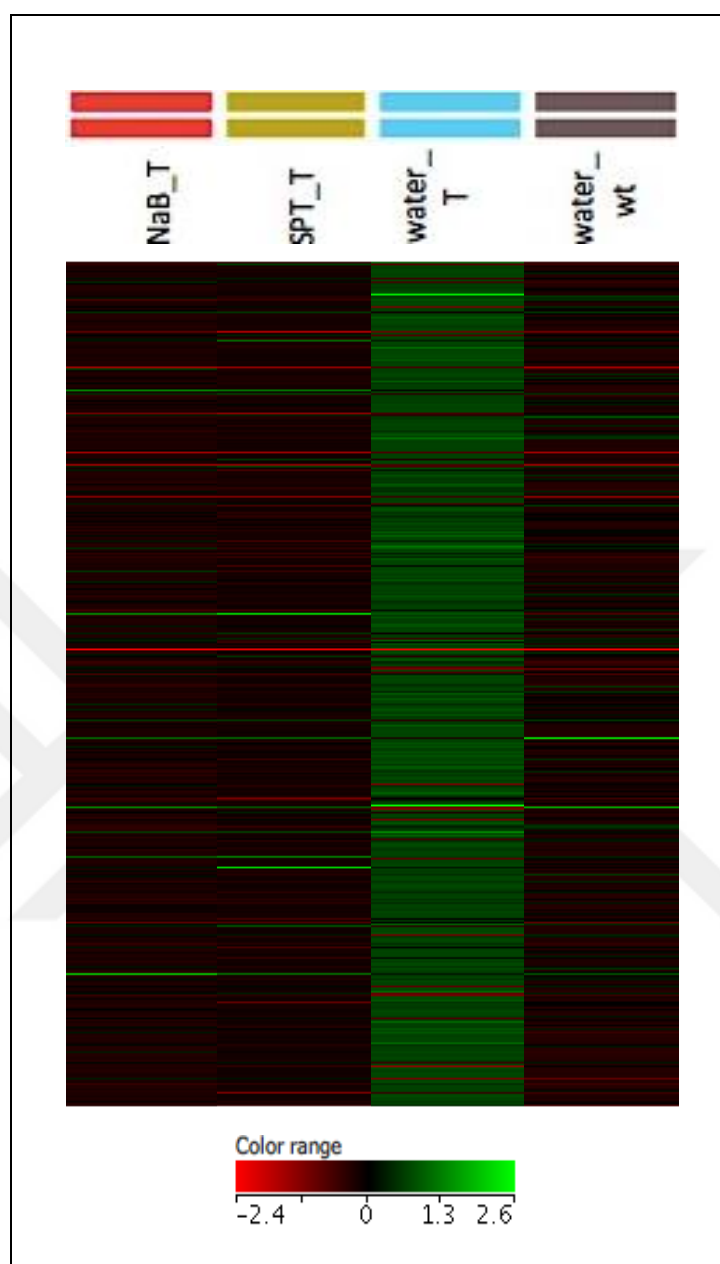


Figure 3.29. Heatmap image of microarray data obtained from spinal cord materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups (n=3). Statistical differences between the groups were compared using Volcano plot analysis (two-way ANOVA; P-values ≤ 0.05 and fold change ± 2 and more were considered statistically significant)

After the scatter plot analysis, the genes for each group were separated as up-regulated and down-regulated, and then uploaded to the PANTHER Gene Ontology (GO) Classification and Pathway System, and related molecular functions, biological process, and pathway analyses were performed. Comparative expressions of some of the prominent genes after boron administration in the spinal cord materials collected from groups are listed in Table 3.2.. Statistical differences between the groups were again compared using Volcano plot analysis (two-way ANOVA was performed; P-values ≤ 0.05 and fold change ± 2 and more were considered statistically significant).

Among these affected genes, there was also *Slc16a3* gene, (*MCT4*), which is mostly expressed in astrocytes in the CNS and has a quite important place in our studies, and more importantly; its expression increased in the untreated transgenic water group, whereas it decreased in the transgenic NaB (-2,35 fold) and SPT (-2,19 fold) treated groups, just as in the wild-type water group mice (-2,43 fold), confirming previous qPCR results performed using spinal cord materials.

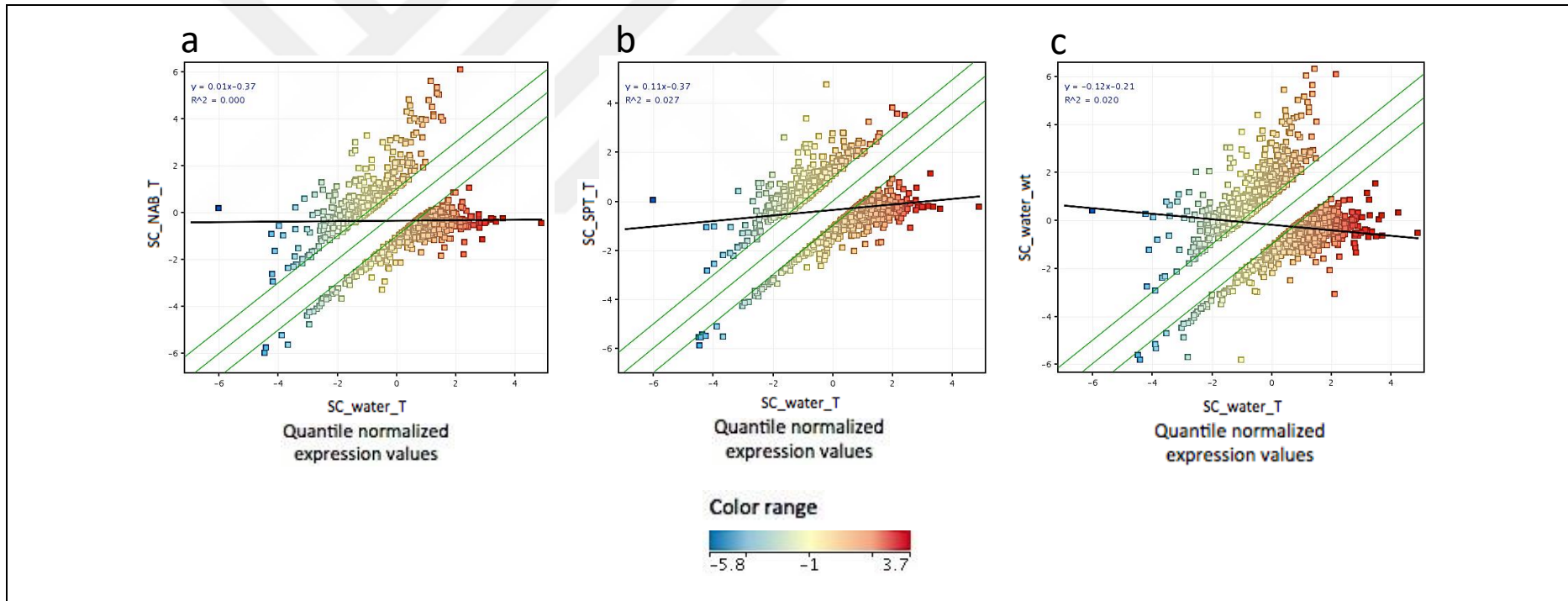


Figure 3.30. Scatter plot demonstration of the up-regulated and down-regulated genes in the (a) transgenic NaB, (b) SPT group, and (c) water treated wild-type group compared to the transgenic water group. The colour range represents $\pm$ fold change is indicated. SC: Spinal cord material. Data represents quantile normalized gene expression values (two-way ANOVA, P-values ≤ 0.05 , fold change ± 2 and more, $n=3$)

Table 3.2. Regulation of genes in the treatment groups belonging to certain important pathways in samples collected from spinal cord materials (P-values ≤ 0.05 and fold change ± 2); Tg: transgenic, wt: wild-type

Pathways	Gene Symbol	Regulation			
		wt-water	tg-NaB	tg-SPT	tg-Water
Adrenaline and noradrenaline biosynthesis (P00001)	<i>Nnmt</i>	Down	Down	Down	Up
- Alzheimer disease-amyloid secretase pathway (P00003) - Angiogenesis (P00005) - Apoptosis signalling pathway (P00006) - B cell activation (P00010) PDGF signalling pathway (P00047) - Oxidative stress response (P00046) Parkinson's disease (P00049) - Ras Pathway (P04393) - T cell activation (P00053) TGF-beta signalling pathway (P00052)	<i>Mapk8</i>	Down	Down	Down	Up
Alzheimer disease-amyloid secretase pathway (P00003)	<i>Cacna1f</i>	Down	Down	Down	Up
- Alzheimer disease-amyloid secretase pathway (P00004) - Alzheimer disease-presenilin pathway (P00003)	<i>Pcsk6</i>	Down	Down	Down	Up
- Alzheimer disease-presenilin pathway (P00004) - Wnt signalling pathway (P00057)	<i>Mmp7</i>	Down	Down	Down	Up
	<i>Wnt3a</i>	Down	Down	Down	Up
	<i>Spink6</i>	Down	Down	Down	Up
- Alzheimer disease-presenilin pathway (P00004) - Angiogenesis (P00005) - Wnt signalling pathway (P00057)	<i>Wnt4</i>	Down	Up	Up	Up
	<i>Wnt10b</i>	Down	Down	Down	Up
- Alzheimer disease-presenilin pathway (P00004) - Wnt signalling pathway (P00057)	<i>Lef1</i>	Down	Down	Down	Up
Alzheimer disease-presenilin pathway (P00004)	<i>Mmp1a</i>	Down	Down	Down	Up
	<i>Mmp20</i>	Down	Down	Down	Up
	<i>Ern1</i>	Down	Up	Down	Up
	<i>Mmp27</i>	Down	Down	Down	Up
- Alzheimer disease-presenilin pathway (P00004) - Huntington disease (P00029) - Inflammation mediated by chemokine and cytokine signalling pathway (P00031) - Wnt signalling pathway (P00057)	<i>Actb12</i>	Down	Down	Down	Up

• Angiogenesis (P00005) • Axon guidance mediated by netrin (P00009) • Inflammation mediated by chemokine and cytokine signalling pathway (P00031) • T cell activation (P00053) • VEGF signalling pathway (P00056) • p53 pathway feedback loops 2 (P04398) • p53 pathway (P00059)	<i>Pik3c2g</i>	Down	Down	Down	Up
• Angiogenesis (P00005)	<i>Fgf2</i>	Down	Down	Down	Up
	<i>Nr1i2</i>	Down	Down	Down	Up
• Angiogenesis (P00005) • PDGF signalling pathway (P00047) • PDGF signalling pathway (P00047) • Ras Pathway (P04393) • VEGF signalling pathway (P00056) • p38 MAPK pathway (P05918)	<i>Rps6kc1</i>	Down	Down	Down	Up
• Apoptosis signalling pathway (P00006) • B cell activation (P00010) • Parkinson's disease (P00049)	<i>Blk</i>	Down	Down	Down	Up
• Apoptosis signalling pathway (P00006) • Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032) • Oxidative stress response (P00046) • Ras Pathway (P04393) p38 MAPK pathway (P05918)	<i>Map2k3</i>	Down	Down	Down	Up
• Apoptosis signalling pathway (P00006)	<i>Bcl2l10</i>	Down	Down	Down	Up
	<i>Rax</i>	Down	Down	Down	Up
• Axon guidance mediated by Slit/Robo (P00008)	<i>Slit2</i>	Down	Down	Down	Up
• Axon guidance mediated by Slit/Robo (P00008) Axon guidance mediated by netrin (P00009)	<i>Ntn2</i>	Up	Down	Down	Down
• Axon guidance mediated by Slit/Robo (P00008) Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Cxcr4</i>	Down	Down	Down	Up
• Axon guidance mediated by Slit/Robo (P00008) Huntington disease (P00029)	<i>Rhoj</i>	Down	Down	Down	Up
• Axon guidance mediated by netrin (P00009) • PDGF signalling pathway (P00047) • p53 pathway feedback loops 2 (P04398) • p53 pathway (P00059)	<i>Pik3r5</i>	Down	Down	Down	Up
• B cell activation (P00010) • T cell activation (P00053)	<i>Ptpnc</i>	Down	Down	Down	Up
• B cell activation (P00010)	<i>Cd19</i>	Down	Down	Down	Up
	<i>Syk</i>	Down	Down	Down	Up
	<i>Cd22</i>	Down	Down	Down	Up
• Cell cycle (P00013) • p53 pathway (P00059)	<i>Ccnb1</i>	Down	Down	Down	Up

• DNA replication (P00017)	<i>Hist1h3a</i>	Down	Down	Down	Up
• GABA-B receptor II signalling (P05731) • Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway (P00027) • Inflammation mediated by chemokine and cytokine signalling pathway (P00031) • Metabotropic glutamate receptor group III pathway (P00039) • Wnt signalling pathway (P00057)	<i>Gng5</i>	Down	Down	Down	Up
• Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway (P00027)	<i>Rgs12</i>	Down	Down	Down	Up
	<i>Gnrhr</i>	Down	Down	Down	Up
• Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway (P00027) • Metabotropic glutamate receptor group III pathway (P00039)	<i>Grm6</i>	Down	Down	Down	Up
• Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway (P00027) • Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Rgs13</i>	Down	Down	Down	Up
• Heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway (P00027)	<i>Adora1</i>	Down	Down	Down	Up
• Huntington disease (P00029)	<i>Arl4c</i>	Down	Down	Down	Up
	<i>Dnah12</i>	Down	Up	Up	Up
	<i>Dnah3</i>	Down	Down	Down	Up
	<i>Dnal4</i>	Down	Down	Down	Up
	<i>Capn8</i>	Down	Down	Down	Up
	<i>Capn6</i>	Down	Down	Down	Up
	<i>Dnah6</i>	Down	Down	Down	Up
• Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Ccr1</i>	Down	Down	Down	Up
	<i>Ccr11</i>	Down	Down	Down	Up
	<i>Mylk3</i>	Down	Down	Down	Up
	<i>Myh10</i>	Down	Down	Down	Up
	<i>Fpr-rs4</i>	Down	Down	Down	Up
	<i>Ccr4</i>	Down	Down	Down	Up
	<i>Plce1</i>	Down	Down	Down	Up
	<i>Myo3a</i>	Down	Down	Down	Up

Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Plce1</i>	Down	Down	Down	Up
	<i>Myo3a</i>	Down	Down	Down	Up
	<i>Ccr9</i>	Down	Down	Down	Up
	<i>Cxcl10</i>	Down	Down	Down	Up
	<i>Bcl3</i>	Down	Down	Down	Up
	<i>Ccl8</i>	Down	Up	Up	Up
	<i>Plcz1</i>	Down	Down	Down	Up
	<i>Arr3</i>	Down	Down	Down	Up
	<i>Alox12e</i>	Down	Down	Down	Up
	<i>Ccl26</i>	Down	Down	Down	Up
	<i>Alox12</i>	Down	Down	Down	Up
- Ionotropic glutamate receptor pathway (P00037) - Metabotropic glutamate receptor group III pathway (P00039)	<i>Ccl26</i>	Down	Down	Down	Up
Ionotropic glutamate receptor pathway (P00037)	<i>Stx11</i>	Down	Down	Down	Up
Metabotropic glutamate receptor group III pathway (P00039)	<i>Adcy10</i>	Down	Down	Down	Up
Oxidative stress response (P00046)	<i>Dusp21</i>	Down	Down	Down	Up
PDGF signalling pathway (P00047)	<i>Ehf</i>	Down	Down	Down	Up
	<i>Arhgap26</i>	Down	Down	Down	Up
	<i>Rerg</i>	Down	Down	Down	Up
	<i>Elf3</i>	Down	Down	Down	Up
Parkinson's disease (P00049)	<i>Fgr</i>	Down	Down	Down	Up
- Parkinson's disease (P00049) - Adrenaline and noradrenaline biosynthesis (P00001)	<i>Stx7</i>	Down	Down	Down	Up
Pentose phosphate pathway (P02762)	<i>Hk1</i>	Down	Down	Down	Up
	<i>Hkdc1</i>	Down	Down	Down	Up
Ras Pathway (P04393)	<i>Exoc2</i>	Down	Down	Down	Up
	<i>Rgl2</i>	Down	Down	Down	Up
T cell activation (P00053)	<i>Cd28</i>	Down	Down	Down	Up
	<i>H2-DMb1</i>	Down	Down	Down	Up

	<i>Cd247</i>	Down	Down	Down	Up
	<i>Pdha2</i>	Down	Down	Down	Up
• TGF-beta signalling pathway (P00052)	<i>Fndc7</i>	Down	Down	Down	Up
	<i>Bmp5</i>	Down	Down	Down	Up
• Wnt signalling pathway (P00057)	<i>Cdh16</i>	Down	Down	Down	Up
	<i>Tbl1xr1</i>	Down	Down	Down	Up
	<i>Mycs</i>	Down	Down	Down	Up
	<i>Ankrd6</i>	Down	Down	Down	Up
	<i>Cdh23</i>	Down	Down	Down	Up
	<i>Cer1</i>	Down	Down	Down	Up
	<i>Pcdh12</i>	Down	Down	Down	Up
	<i>En1</i>	Down	Down	Down	Up
• p38 MAPK pathway (P05918)	<i>Eif4e1b</i>	Down	Down	Down	Up
No hit for PANTHER pathway	<i>Slc16a3</i>	Down	Down	Down	Up
• p53 pathway (P00059)	<i>Kat6b</i>	Down	Down	Down	Up
	<i>Sumo1</i>	Down	Down	Down	Up

The raw data of the selected prominent genes were processed, imported back into the String database v11, and the heatmap related to their regulations were extracted as demonstrated in Figure 3.31. and 3.32.. Statistical differences between the groups were compared using Volcano plot analysis (two-way ANOVA; P-values ≤ 0.05 and fold change ± 2 and more were considered statistically significant).

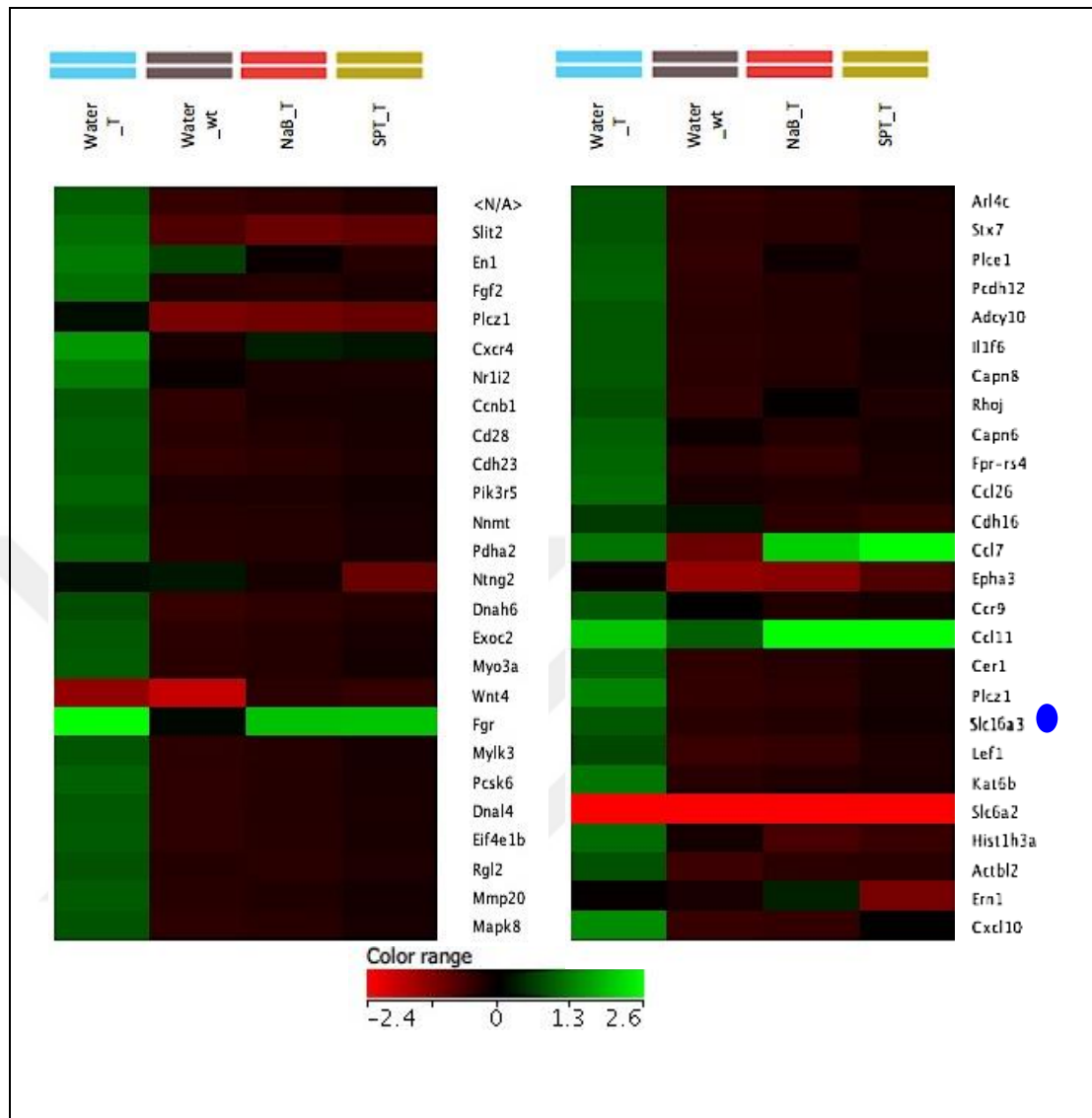


Figure 3.31. The first part of heatmap representation of microarray data of selected genes belonging to spinal cord materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups (n=3). *Slc16a3* (MCT4) gene expression is marked with blue

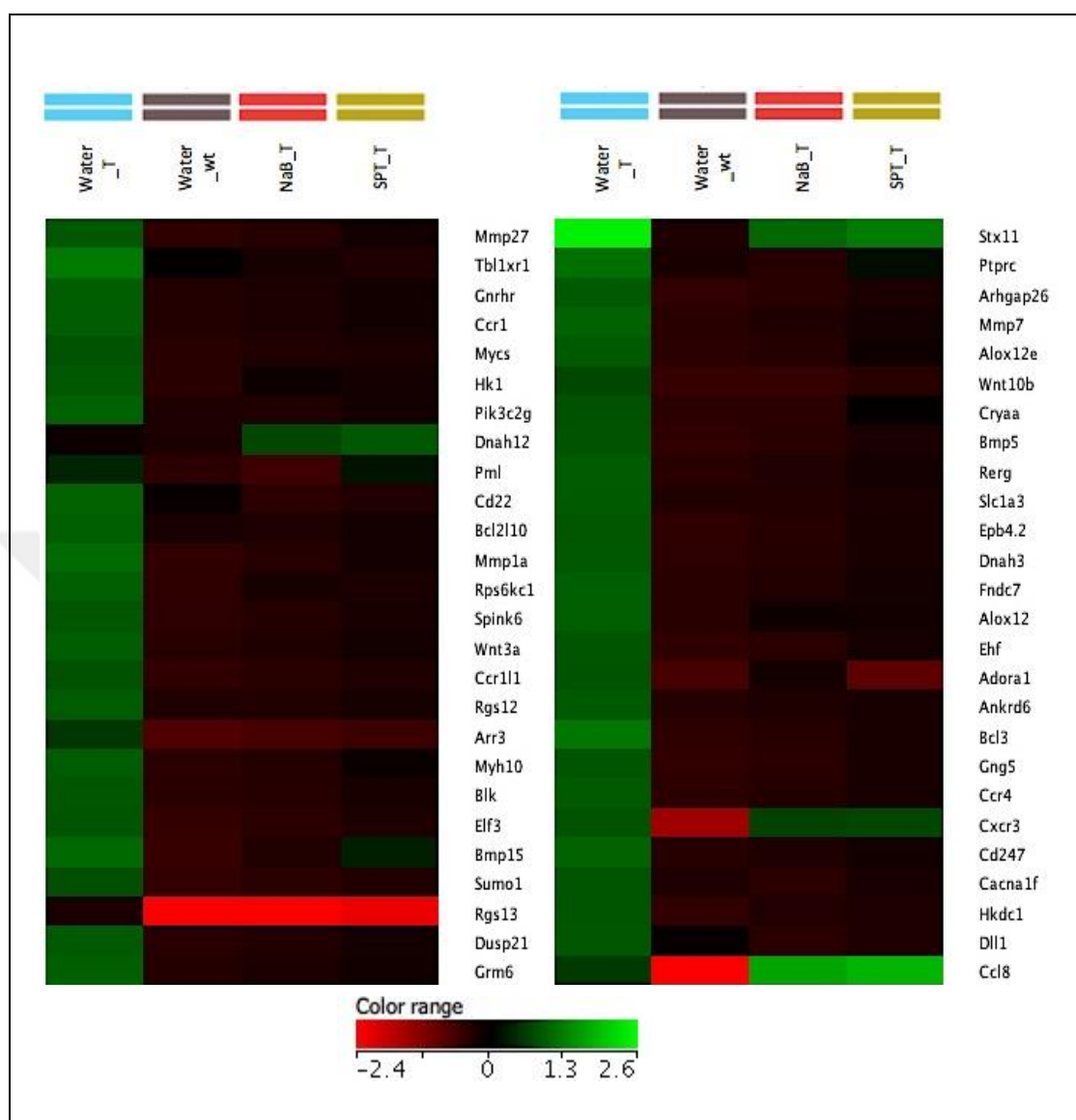


Figure 3.32. The second part of heatmap representation of microarray data of selected genes belonging to spinal cord materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups (n=3)

Afterward, certain molecular functions, biological processes, and various pathways affected by boron derivatives were represented as pie chart diagrams by uploading the selected genes from GeneSpring GX v14.9 software to PANTHER Gene Ontology (GO) Classification and Pathway System (Figure 3.33.-3.38.).

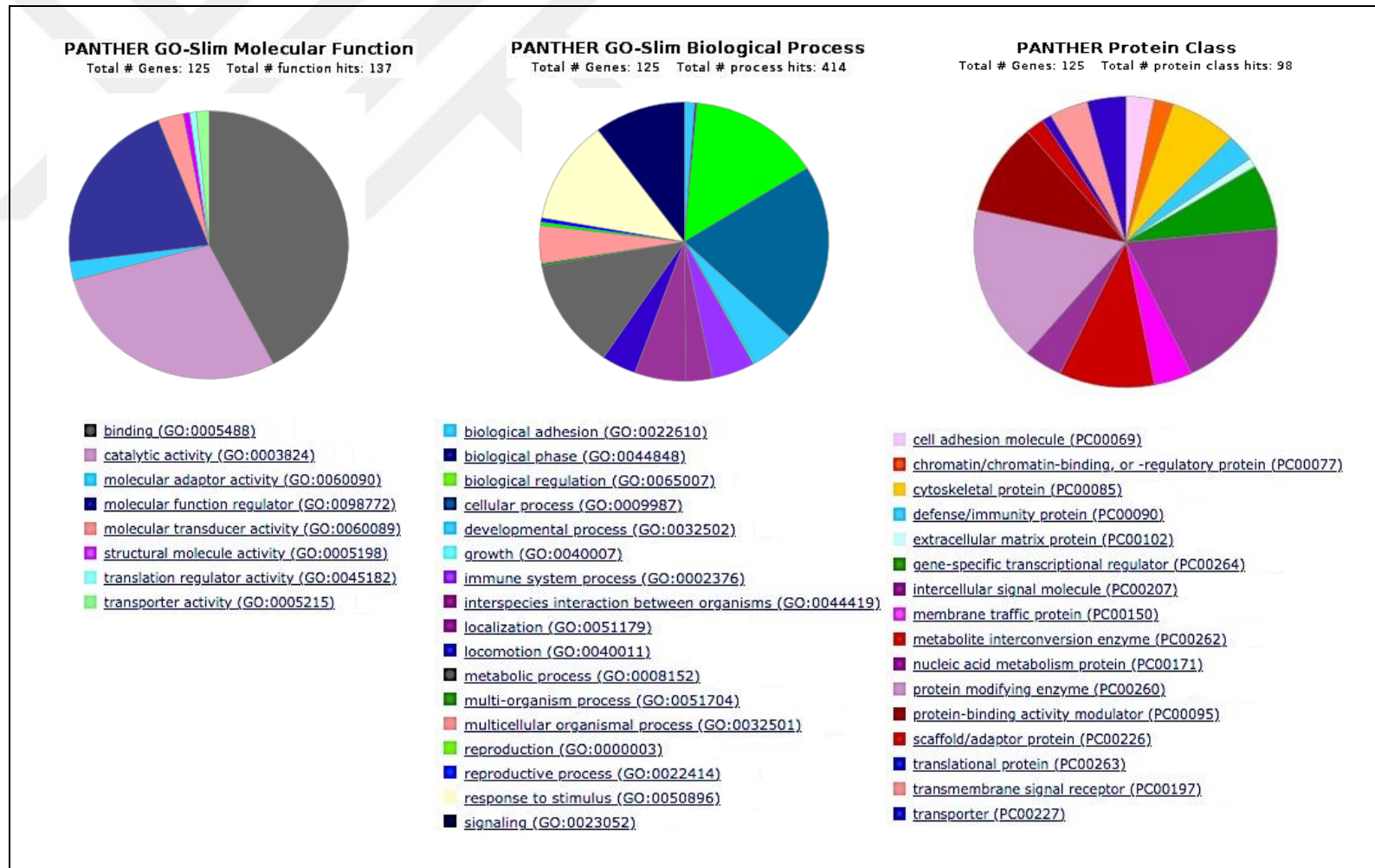


Figure 3.33. Molecular function, biological processes, and protein classes of the genes, which are up and down-regulated as a result of NaB treatment in the spinal cord

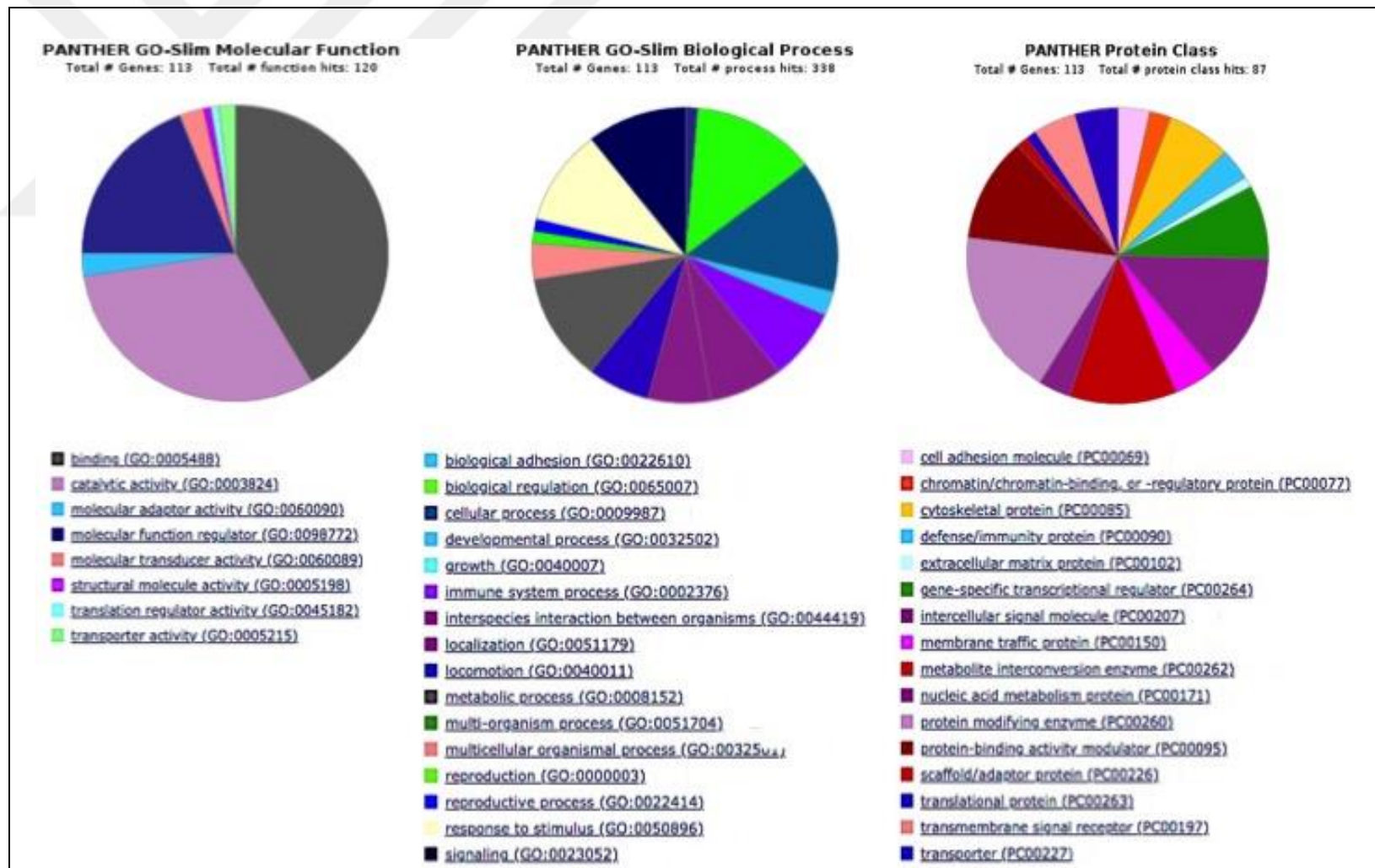


Figure 3.34. Molecular function, biological processes, and protein classes of the genes, which are up and down-regulated as a result of SPT treatment in the spinal cord

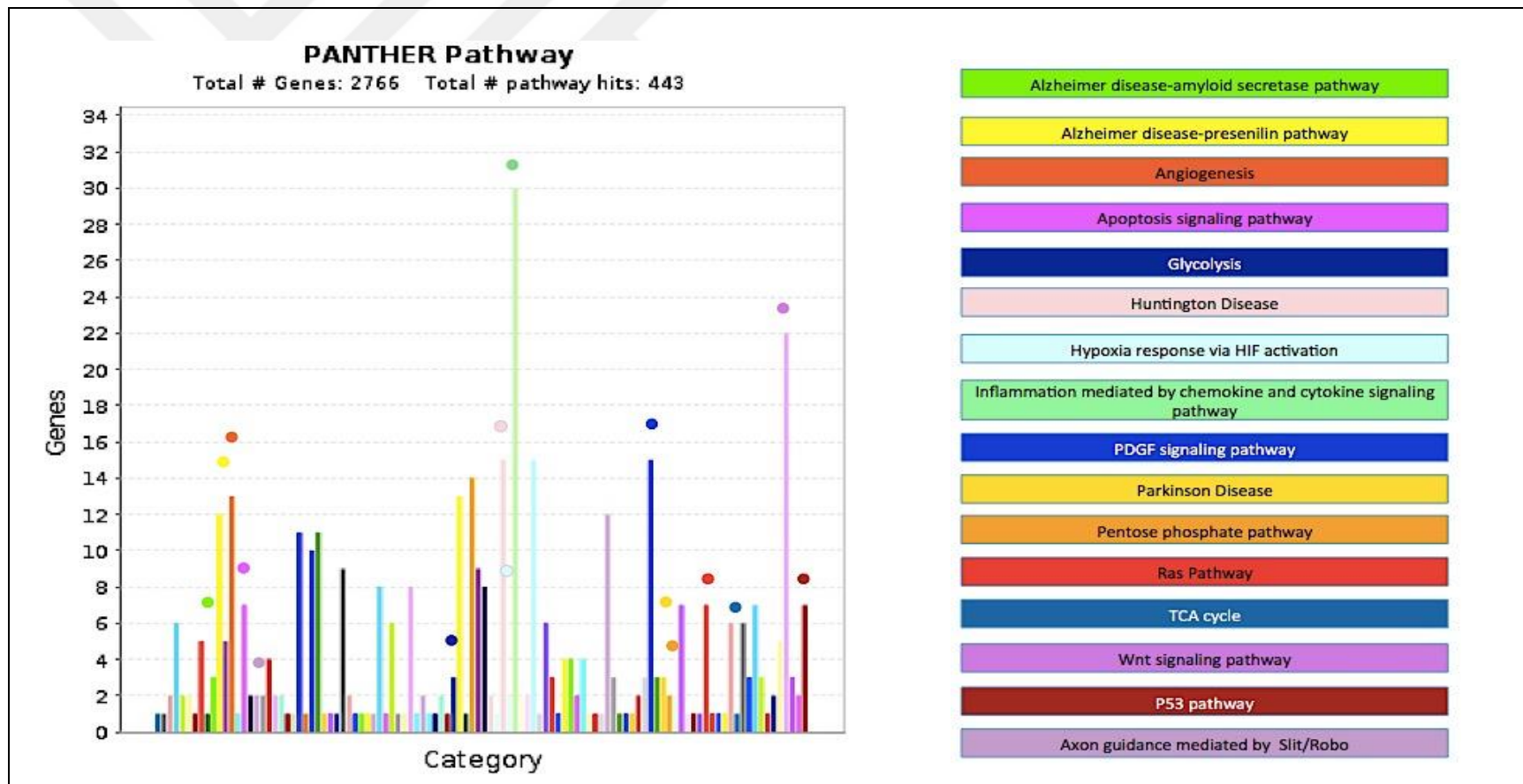


Figure 3.35. Related pathways of the genes that are down-regulated as a result of NaB treatment in the spinal cord

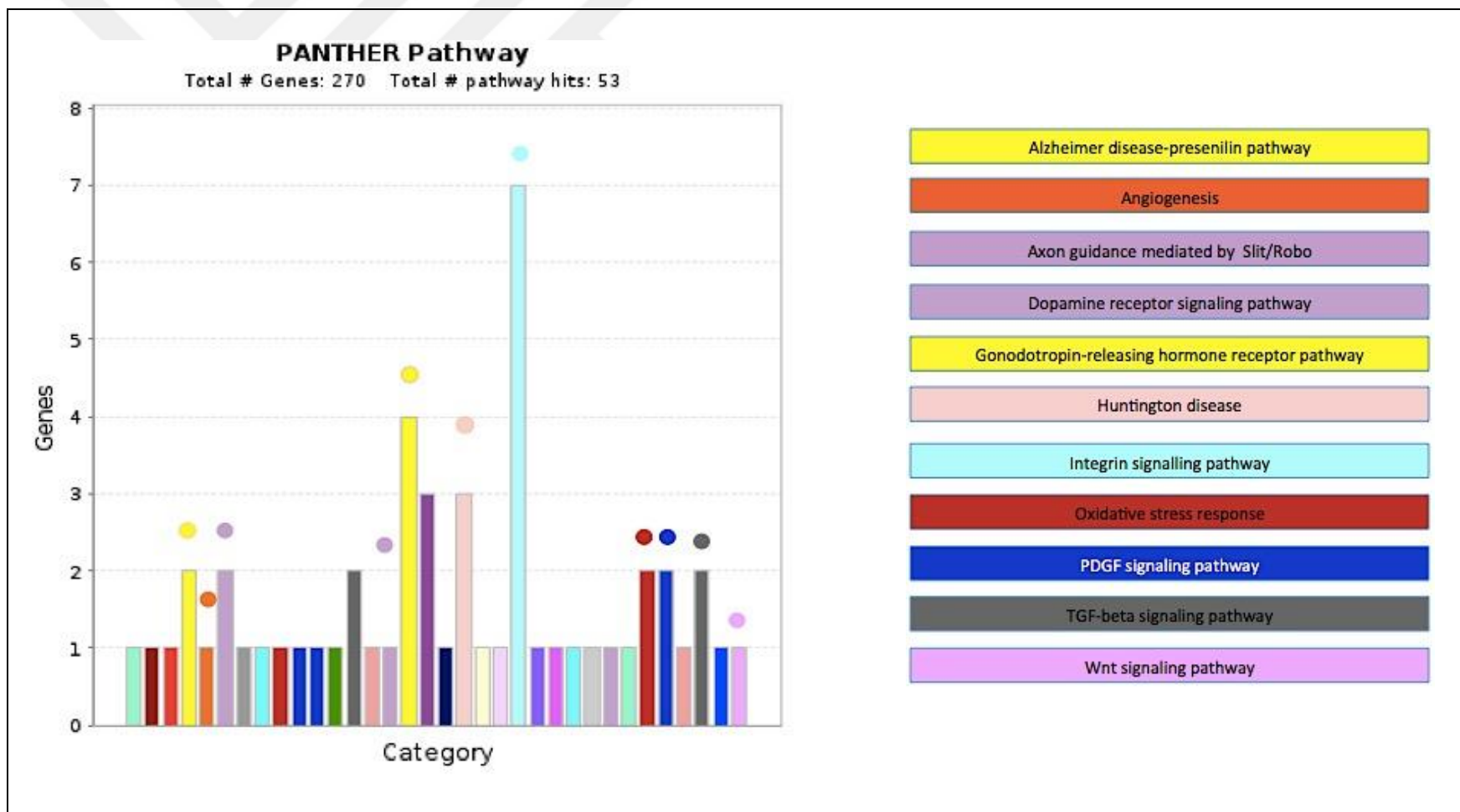


Figure 3.36. Related pathways of the genes that are up-regulated as a result of NaB treatment in the spinal cord

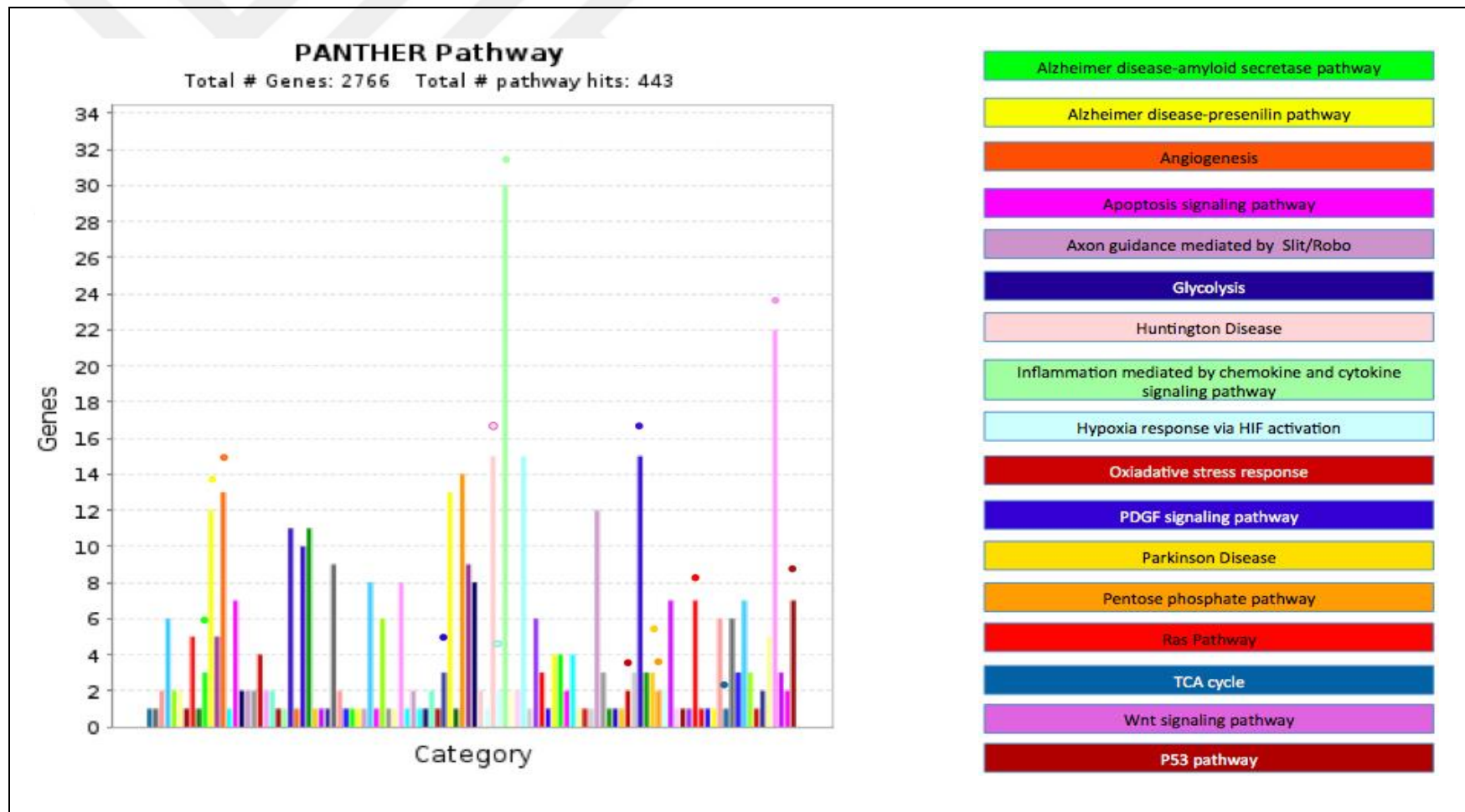


Figure 3.37. Related pathways of the genes that are down-regulated as a result of SPT treatment in the spinal cord

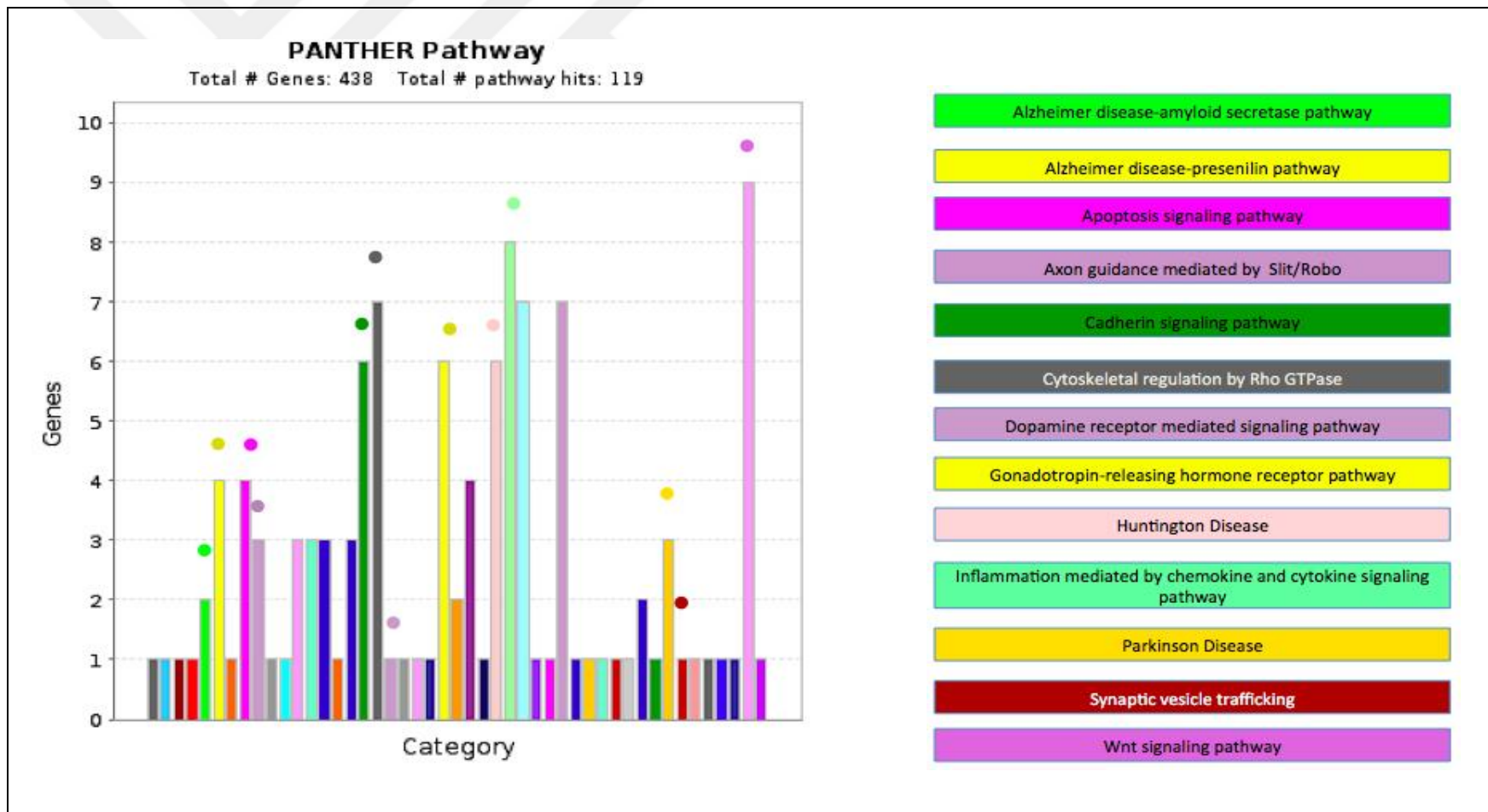


Figure 3.38. Related pathways of the genes that are up-regulated as a result of SPT treatment in the spinal cord

As depicted previously, gene expressions in the NaB and SPT treated and wild-type water treated groups generally show a decreasing trend compared to the transgenic water treated control group. In NaB and SPT treated groups, there was a decrease in the expression of genes involved in pathways such as Alzheimer's disease-amyloid secretase pathway, Alzheimer's disease-presenilin pathway, Huntington disease, Parkinson's disease, and inflammation mediated by chemokine and cytokine signalling pathways, which are related to neurodegenerative diseases and neuroinflammation. In addition, the expressions of genes involved in the energy metabolism pathways such as pentose phosphate pathway, TCA cycle, and glycolysis reached the gene expression profile of the wild-type water treated group (Figure 3.32.-3.37.).

3.5.2.2. Brain Materials

Raw microarray data obtained from brain materials was first uploaded into the GeneSpring GX v14.9 software and then evaluated with Volcano Plot analysis (two-way ANOVA was performed for all groups comparison and two-tailed Student's t-test was performed for dual comparisons). The NaB and SPT treated transgenic groups and water treated wild-type group were compared to the transgenic water group. Fold change ± 2 and more in gene expressions and P-values ≤ 0.05 were accepted as significant.

Initially, data obtained from the overall examination of the groups were extracted as a heatmap. As shown in Figure 3.39., there was no sharp difference in gene expression profiles between the groups in brain samples as in spinal cord materials. There were down-regulated and up-regulated genes in each group, and no clear increase or decrease trends were observed in the overall gene expression profiles of the groups.

Scatter plot analysis was performed to compare the gene expression profiles of the transgenic NaB and SPT treated groups with that of the transgenic water group again (Figure 3.40.). In the groups treated with boron derivatives, although not as many as in spinal cord materials, there were still numerous genes that had a significant alteration in their regulation levels. According to the scatter plot results, 365 genes in the NaB treated group and 993 genes in the SPT treated group were up or down-regulated more than 2 folds (P-values ≤ 0.05). Likewise, the wild-type water group was also compared with the transgenic water group and 804 genes in the wild-type water group were up or down-regulated more than 2 folds comparing the transgenic water group (P-values ≤ 0.05).

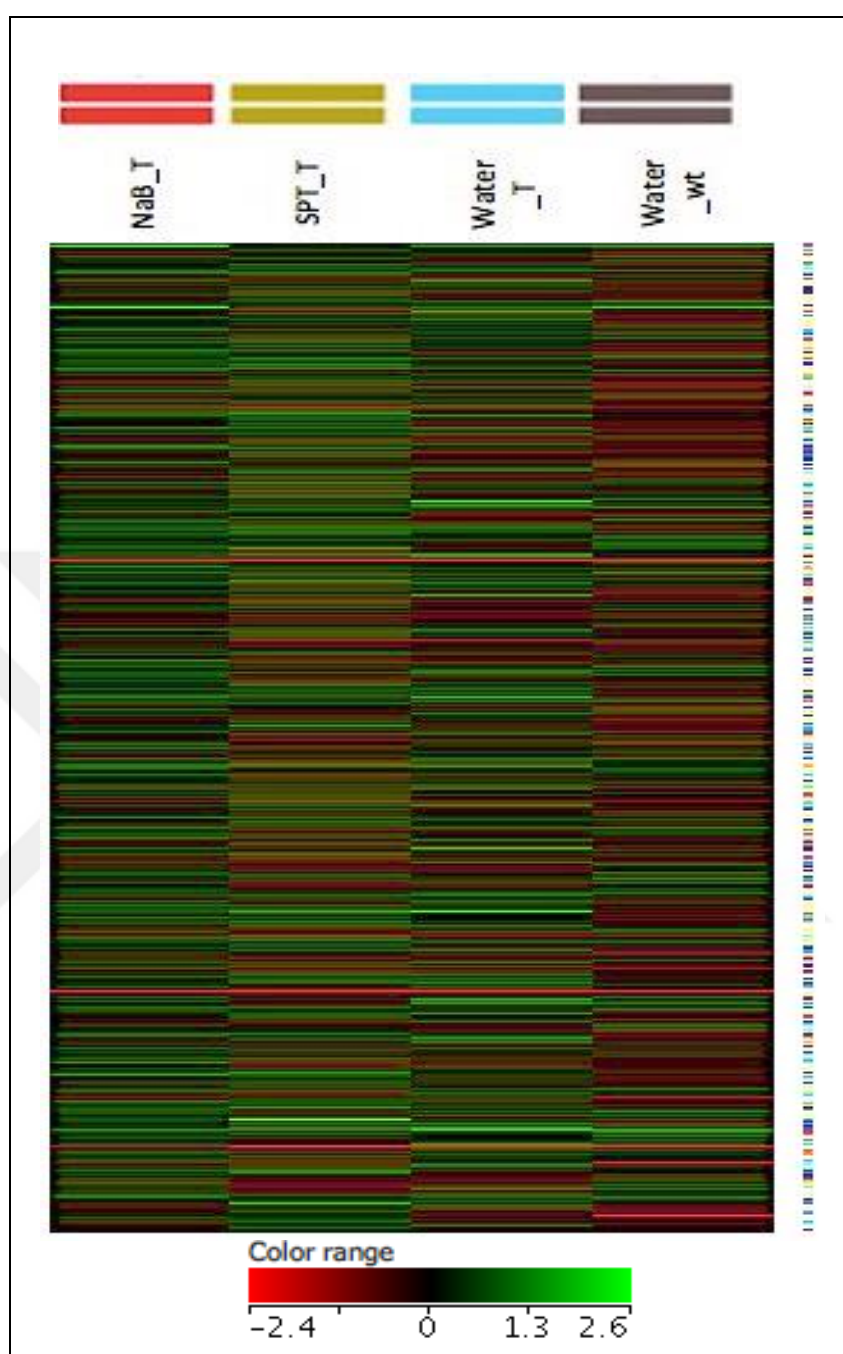


Figure 3.39. Heatmap image of microarray data obtained from brain materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups (n=3). Statistical differences between the groups were compared using Volcano plot analysis (two-way ANOVA; P-values ≤ 0.05 and fold change ± 2 and more were considered statistically significant)

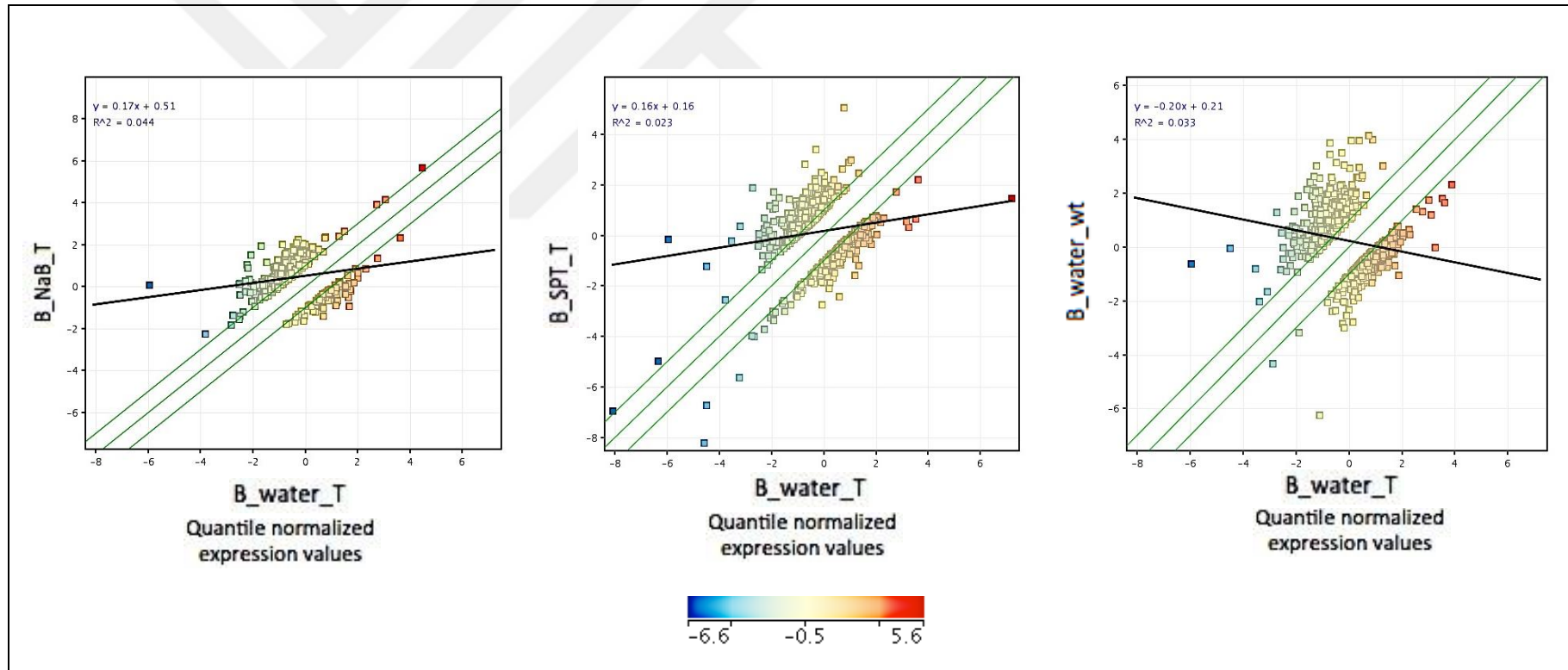


Figure 3.40. Scatter plot demonstration of the up-regulated and down-regulated genes in the (a) transgenic NaB, (b) SPT group, and (c) water treated wild-type group compared to the transgenic water group. The colour range represents $\pm$ fold change is indicated. B: Brain material. Data represents quantile normalized gene expression values (two-way ANOVA, P-values ≤ 0.05 , fold change ± 2 and more, $n=3$)

After the scatter plot analysis, the genes for each group were separated as up-regulated and down-regulated, and then uploaded to the PANTHER Gene Ontology (GO) Classification and Pathway System, and related molecular functions, biological process, and pathway analyses were performed. Some of the prominent gene expression changes in the collected brain materials are listed in Table 3.3. by comparison between groups. Statistical differences between the groups were again compared using Volcano plot analysis (two-way ANOVA was performed; P-values ≤ 0.05 and fold change ± 2 and more were considered statistically significant).

Table 3.3. Regulation of genes in the treatment groups belonging to certain important pathways in samples collected from brain materials. Tg: transgenic, wt: wild-type

Pathways	Gene Symbol	Regulation			
		Wt-Water	Tg-NAB	Tg-SPT	Tg-Water
5HT1 type receptor mediated signalling pathway (P04373), 5HT2 type receptor mediated signalling pathway (P04374), 5HT3 type receptor mediated signalling pathway (P04375), 5HT4 type receptor mediated signalling pathway (P04376)	<i>Slc18a1</i>	Down	Down	Down	Up
5HT2 type receptor mediated signalling pathway (P04374), - Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Plcz1</i>	Up	Down	Down	Down
5HT4 type receptor mediated signalling pathway (P04376), - Inflammation mediated by chemokine and cytokine signalling pathway (P00031), - Dopamine receptor mediated signalling pathway (P05912)	<i>Gnatl</i>	Down	Down	Down	Up
- Alzheimer disease-amyloid secretase pathway (P00003), - Alzheimer disease-presenilin pathway (P00004)	<i>Aph1b</i>	Down	Down	Down	Up
Alzheimer disease-presenilin pathway (P00004)	<i>Mmp2</i>	Down	Up	Up	Up
- Apoptosis signalling pathway (P00006), - FGF signalling pathway (P00021),	<i>Map2k7</i>	Down	Down	Down	Up

Huntington disease (P00029), • Ras Pathway (P04393)					
• Apoptosis signalling pathway (P00006), •	<i>Map3k14</i>	Down	Down	Down	Up
• FGF signalling pathway (P00021)	<i>Ppp2r2b</i>	Down	Down	Down	Up
• Huntington disease (P00029)	<i>Dync1h1</i>	Down	Down	Down	Up
• Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Ccl3</i>	Down	Down	Down	Up
• Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Ccl4</i>	Down	Down	Down	Up
• Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Cxcl10</i>	Down	Down	Down	Up
• Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	<i>Ccl11</i>	Up	Up	Up	Down
• Parkinson's disease (P00049)	<i>Hspa13</i>	Down	Down	Up	Up
• Parkinson's disease (P00049)	<i>Hspa5</i>	Up	Up	Up	Down
• p53 pathway feedback loops 2 (P04398)	<i>Cdkn1a</i>	Up	Up	Up	Down
• Wnt signalling pathway (P00057)	<i>Plcb2</i>	Up	Up	Up	Down

Afterward, the raw data of the selected prominent genes were processed, imported back into the String database v11, and the heatmap related to their regulations were extracted as demonstrated in Figure 3.41.. Statistical differences between the groups were compared using Volcano plot analysis (two-way ANOVA; P-values ≤ 0.05 and fold change ± 2 and more were considered statistically significant).

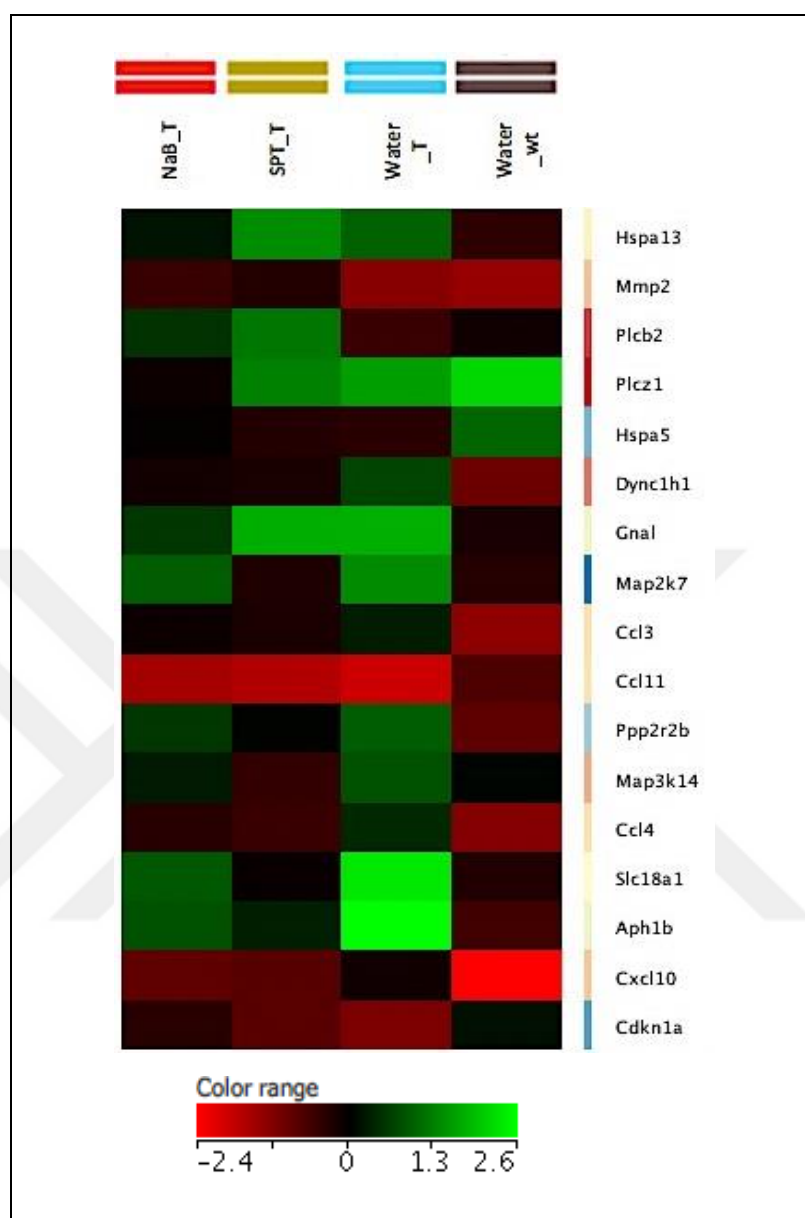


Figure 3.41. Heatmap representation of microarray data of selected genes belonging to brain materials of NaB, SPT (transgenic), and water treated (transgenic and wild-type) groups (n=3)

Then, certain molecular functions, biological processes, and various pathways affected by boron derivatives were represented as pie chart diagrams by uploading the selected genes from GeneSpring GX v14.9 software to PANTHER Gene Ontology (GO) Classification and Pathway System and related molecular functions, biological process and pathway analyses were performed (Figure 3.42.-3.47.).

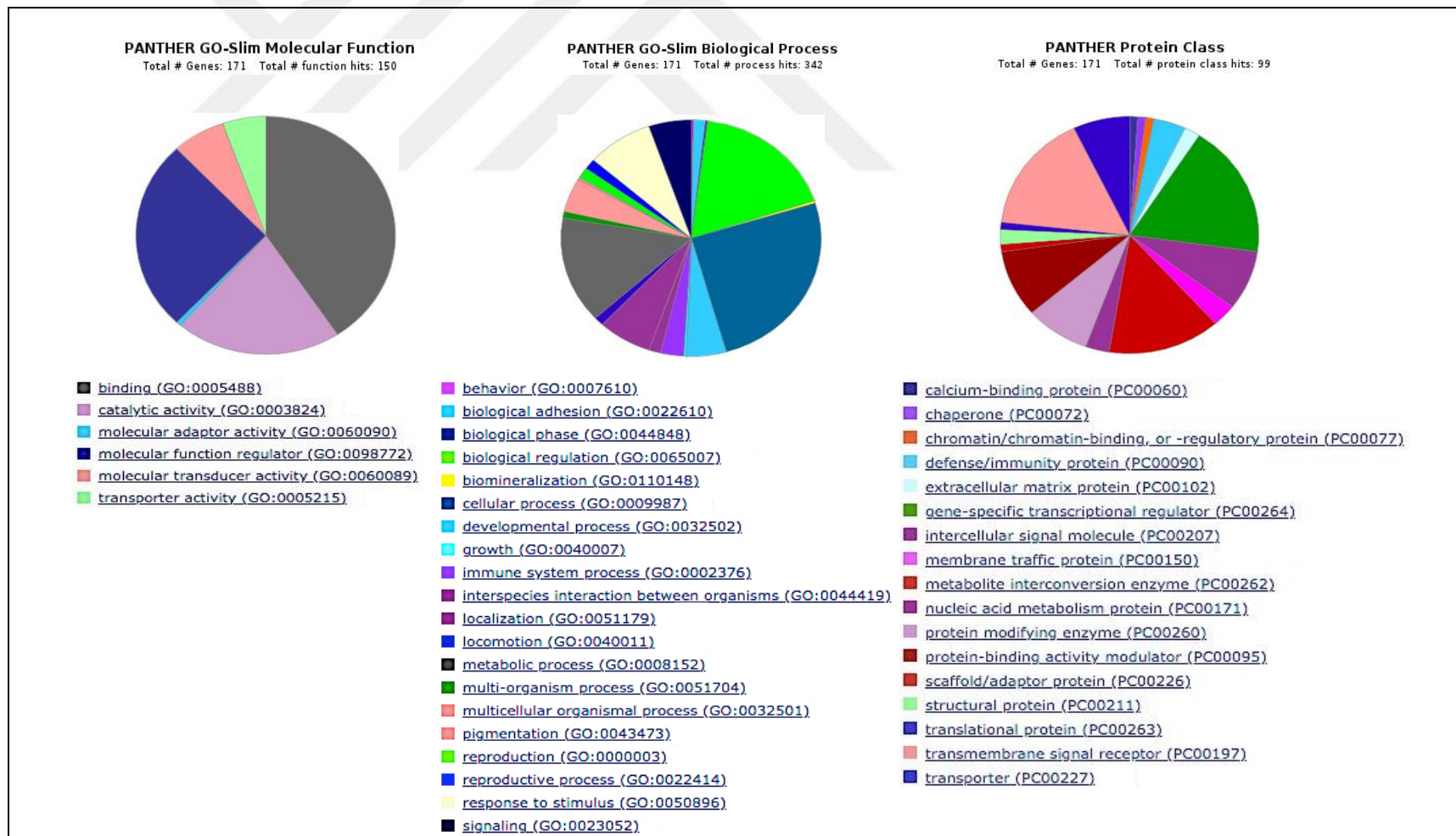


Figure 3.42. Molecular Function, biological processes, and protein classes of the genes, which are up and down-regulated as a result of NaB treatment in the brain

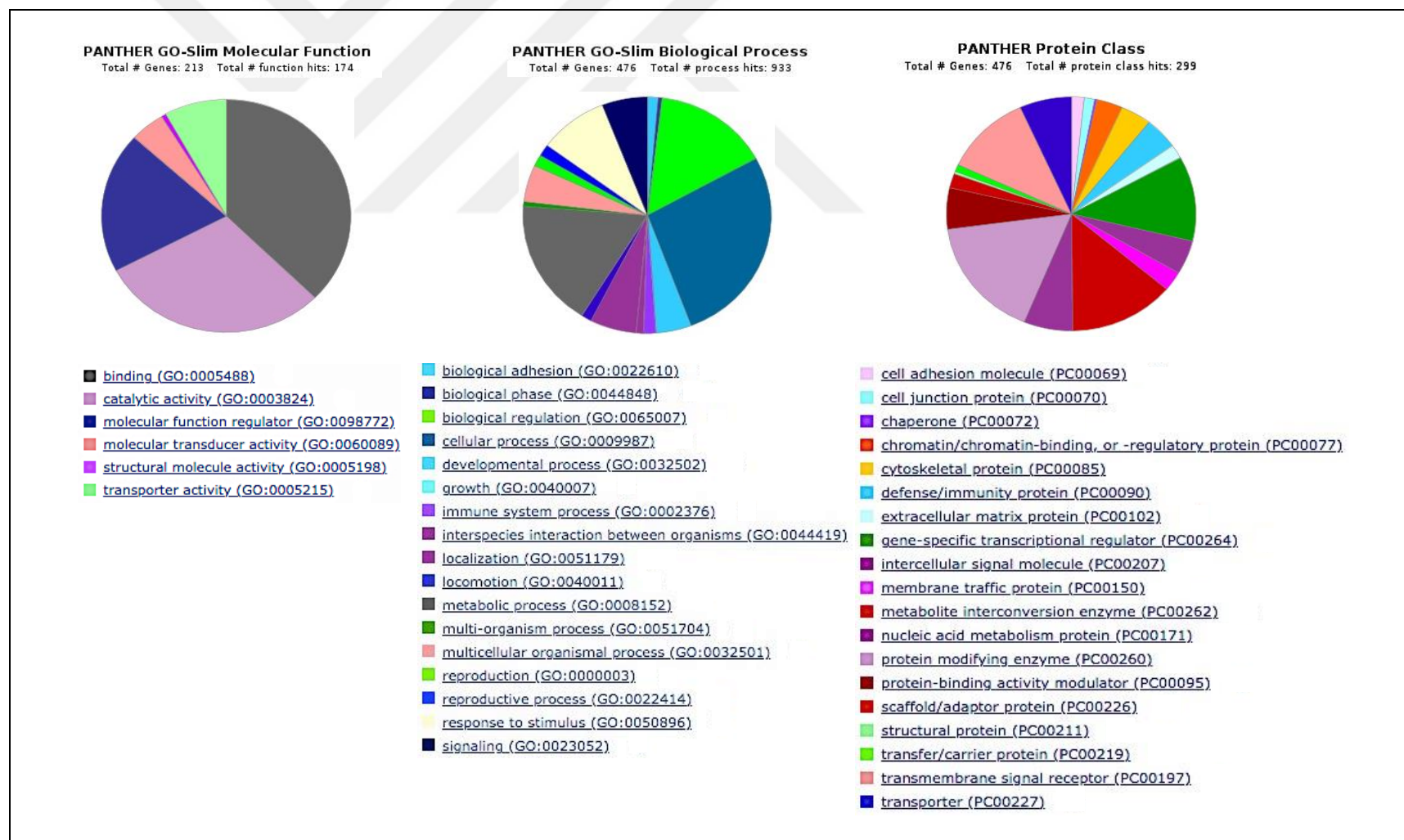


Figure 3.43. Molecular Function, biological processes, and protein classes of the genes, which are up and down-regulated as a result of SPT treatment in the brain

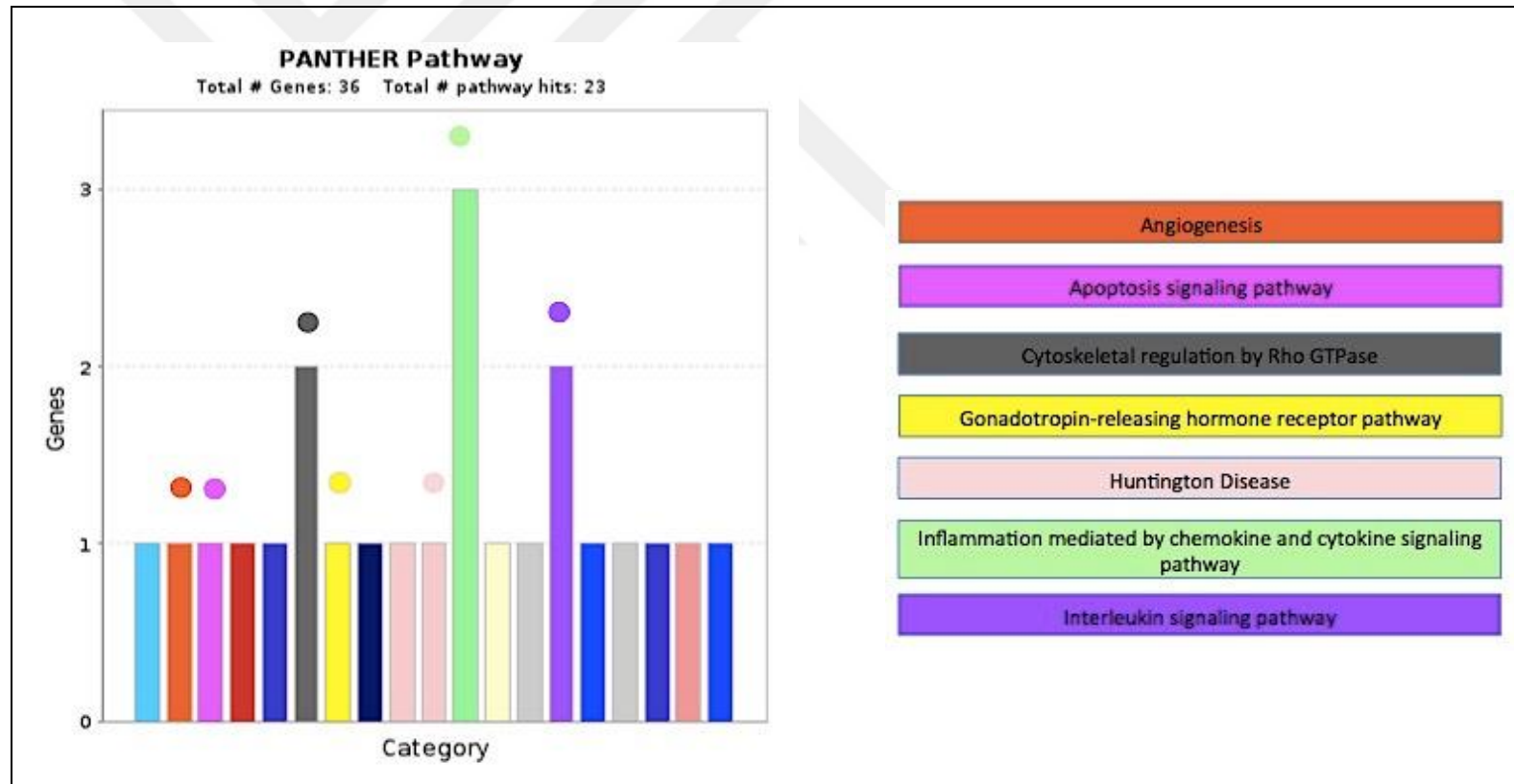


Figure 3.44. Related pathways of the genes that are down-regulated as a result of NaB treatment in the brain

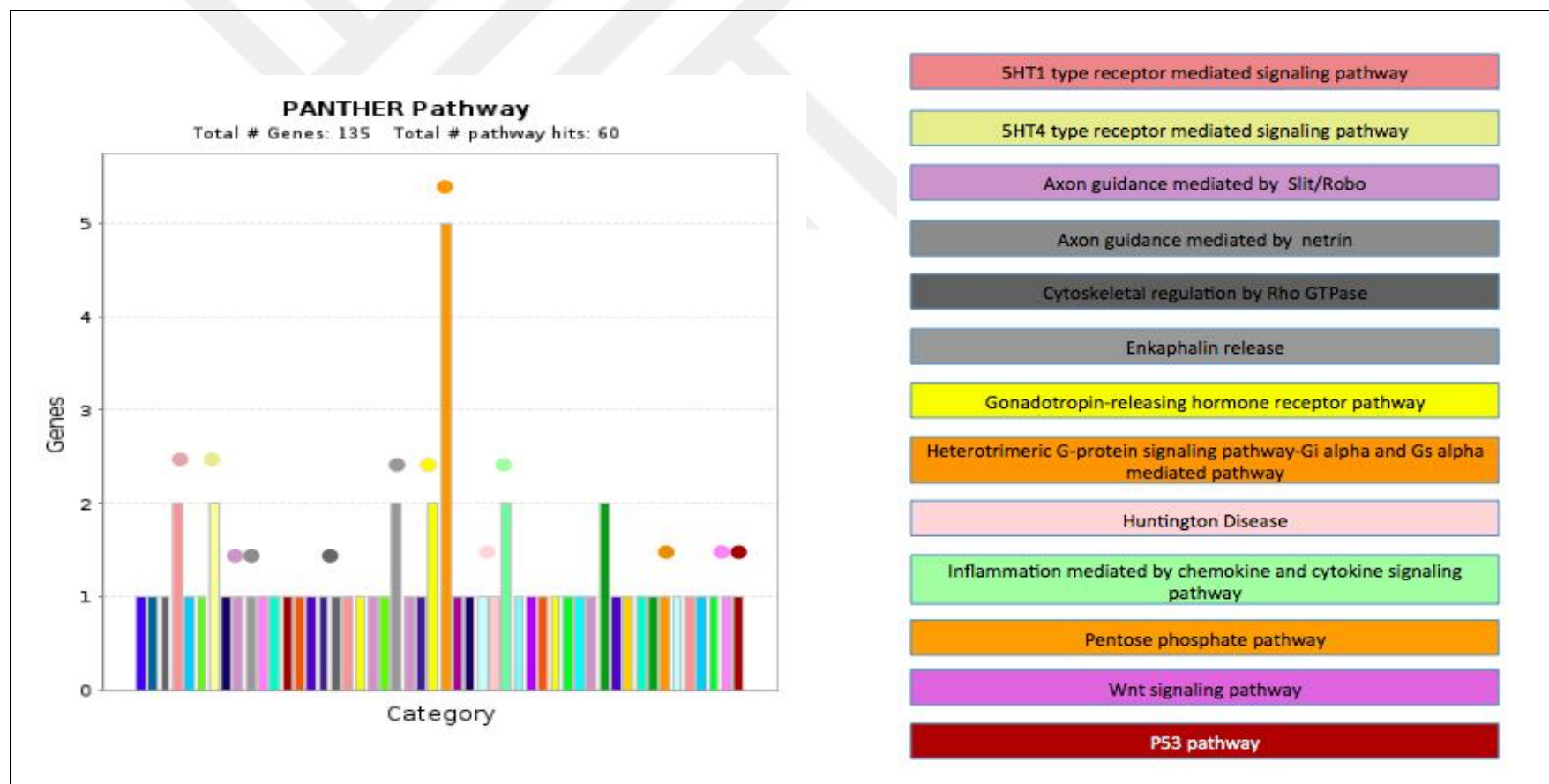


Figure 3.45. Related pathways of the genes that re up-regulated as a result of NaB treatment in the brain

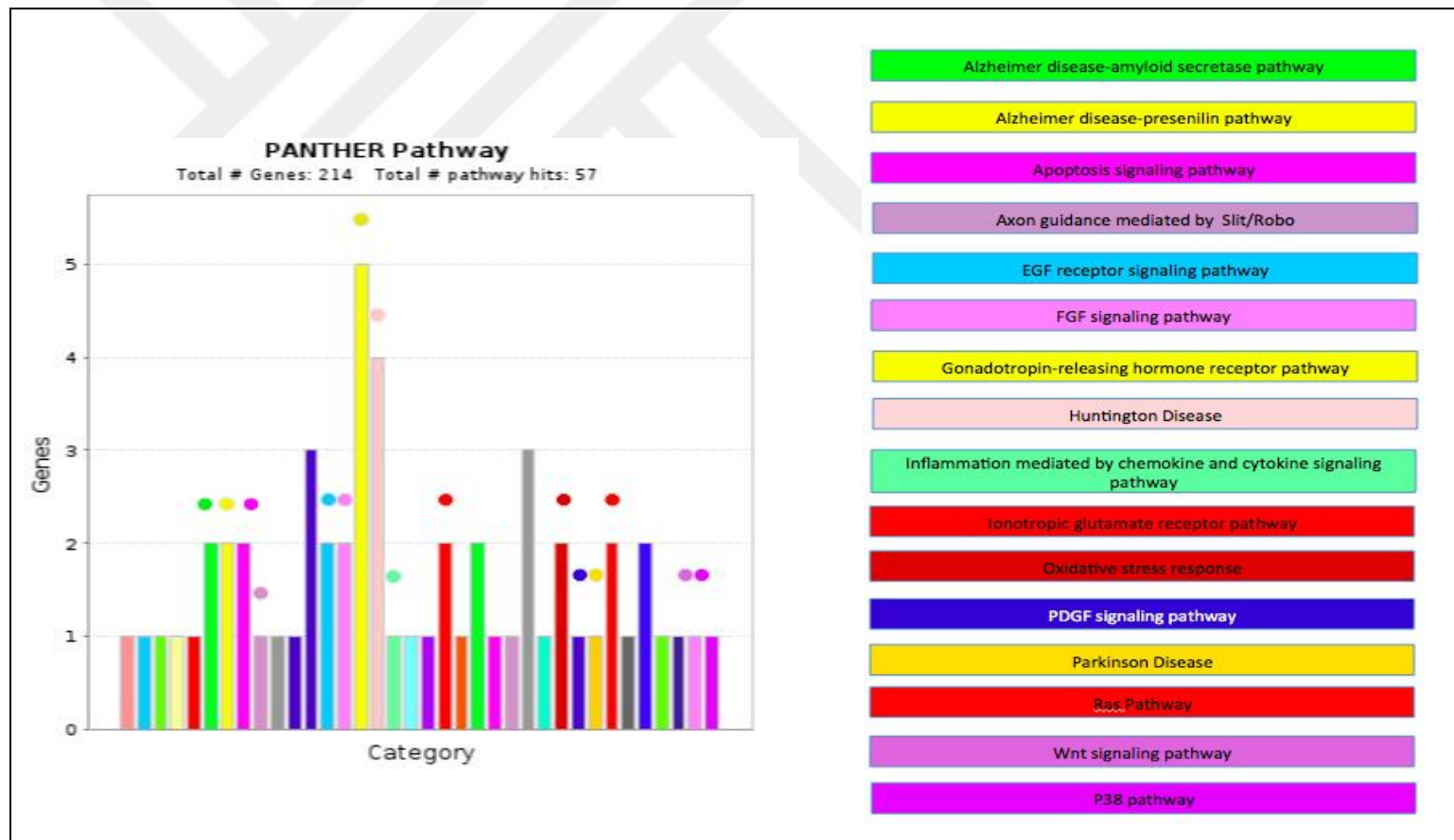


Figure 3.46. Related pathways of the genes that are down-regulated as a result of SPT treatment in the brain

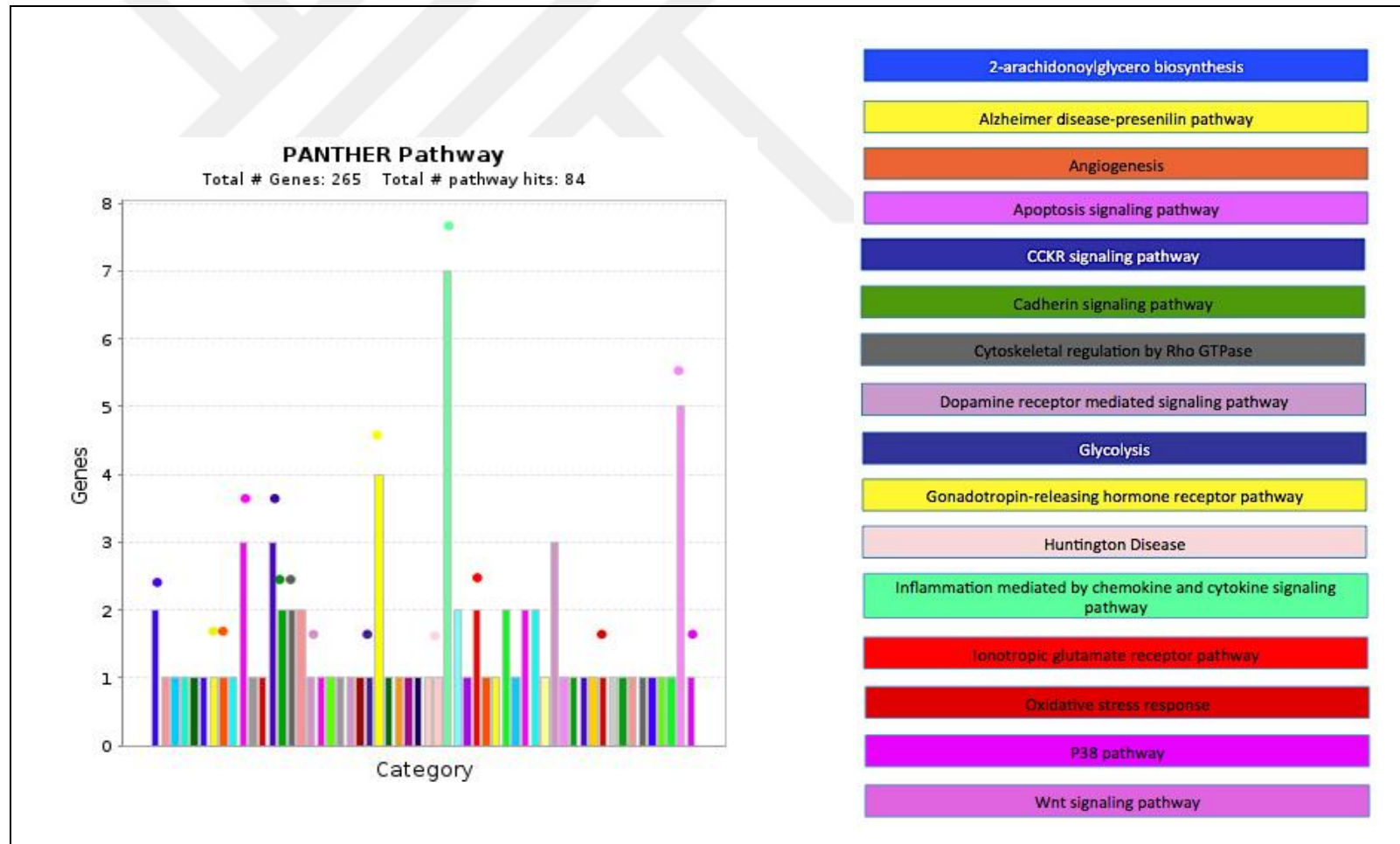


Figure 3.47. Related pathways of the genes that are up-regulated as a result of SPT treatment in the brain

As mentioned before, gene expressions in the NaB and SPT treated and wild-type water treated groups did not show a clear up-regulation or down-regulation trend compared to the transgenic water treated control group (Figure 3.39.). However, just as in the spinal cord materials, in the brain materials collected from NaB and SPT treated groups, genes involved in the pathways such as Alzheimer's disease-amyloid secretase pathway, Alzheimer's disease-presenilin pathway, Huntington Disease, Parkinson's Disease and Inflammation mediated by chemokine and cytokine signalling pathways, which are related to neurodegenerative diseases and neuroinflammation showed the similar regulation trend with the wild-type water group. In addition, the expressions of genes involved in the energy metabolism pathways such as pentose phosphate pathway, oxidative stress response, and glycolysis reached the gene expression profile of the wild-type water treated group (Figure 3.42.-3.47.).

4. DISCUSSION

Amyotrophic lateral sclerosis (ALS) belonging to the wider group of diseases known as motor neuron disorders is a fatal illness, which causes the gradual degeneration of upper and lower MNs. The disease results in bulbar palsy, progressive muscle waste and weakness of the upper and lower limbs, and eventually death within 3–5 years owing to respiratory failure. Despite the mechanisms lying behind the motor neuron degeneration caused by ALS remain vague, numerous abnormal conditions including disturbances of degradative mechanisms of protein, nuclear and cytosolic protein aggregation, aberrant RNA accumulation, mitochondrial dysfunction, ER stress, defective nucleocytoplasmic trafficking, abnormal axonal transport, disrupted neuronal excitability, and excessive oxidative stress have been proposed to be linked with ALS pathogenesis [11,232].

Considering that only 10 percent of overall ALS cases are hereditary (fALS), there are still many unanswered questions regarding the etiology of the sporadic cases that make up the remaining 90 percent [10].

Although genetic factors are believed to be rather associated with fALS, some certain genes have been revealed to be involved in both fALS and sALS cases [16]. The most prevalent fALS mutations are found in genes *SOD1* (copper-zinc superoxide dismutase 1) and *TARDBP* (encodes transactive response DNA binding protein 43) [19,20] (DNA binding protein 43 [20]) and also GGGGCC repeat in the non-coding region of *C9ORF72* gene.

In transgenic fALS mouse models, even in the presence of endogenous, intact SOD1, mutant SOD1 leads to the development of an ALS-like phenotype, pointing out the role of toxic gain of functions of SOD1 in the pathology of ALS. In concordance with that, knockdown of mutant SOD1 in astrocytes was reported to completely ameliorate motor neuron deterioration [30]. In addition to the toxicity generated by mutant SOD1 originating from fALS, wild-type SOD1 protein is also reported to be accumulated by undergoing conformational change, presumably due to abnormal posttranslational modifications, and plays an additional role in motor neuron loss, besides other toxic elements emerged in sALS [31,32]. This evidence proposes a common astrocyte-dependent disease mechanism in sporadic and familial ALS and points out the critical role of glial cells, which is one of

the main claims we took as a reference in our study, in the formation of ALS [2].

Up to date, the most widely used transgenic ALS model, SOD1-G93A mouse line possesses *SOD1* mutation which is the base change corresponding to the conversion of glycine 93 to alanine that one of the two animal models we used in our study (B6SJLTg (SOD1*G93A)1Gur/J mouse line), purchased from Jackson Laboratory. In order to estimate the disease stages when early and late symptoms started to appear following birth, we observed SOD1 transgenic animals' locomotor behaviours as described in a study using the SOD1-G93A mouse line. Therefore, as indicated in the literature, the disease progression was graded according to a scoring system called NeuroScore (NS) [207], which included various motor activity tests and daily assessment of hindlimb function, the first manifestation of the disease occurring in the SOD1-G93A transgenic mouse. In accordance with the literature, we found that the onset of the disease was postnatal day 90 and accepted as the symptomatic phase for collecting CNS material. We also chose postnatal day 60 to collect pre-symptomatic CNS materials. As for Tg(C9orf72_3) 112 line, unlike SOD1-G93A animals, they exhibit no significant motor dysfunction or cognitive deterioration, consequently grading systems have not been developed to rate the severity of the symptoms. In an attempt to understand if excessive C9ORF72 expression leads to any sort of abnormal motor function, we analysed C9ORF72 mice's locomotor activity via rotarod test using 7, 17, and 19 months old (MO) mice. Unfortunately, no significant difference was found in the rotarod performances at the three selected time points. In the 19th month, however, C9ORF72 mice showed a trend-level decrease in performance compared to wild-type 17 MO controls. According to the Jackson Laboratory's statement, no phenotypic alteration in this line is expected until the 16th month [207]. In support of this information, it is later reported that C9ORF72 mice line do not exhibit the ALS and/or FTD-like phenotype during their life, presumably owing to the lack of 5' and 3' regulatory elements required to maintain the adequate sense and antisense transcripts expressions [234–236]. In this context, the fact that we could not observe any dominant phenotypic decline in C9ORF72 animals is consistent with the literature. As a result, the 17th month was chosen as the symptomatic stage for CNS material collection, nevertheless, the pre-symptomatic stage could not be determined due to some emerging difficulties in the maintenance of the transgenic line.

The CNS and peripheral nervous systems consist of glia (oligodendrocytes, microglia, and astrocytes) and neurons. Among these cells, glia is the most excessively found cell type in the brain with a rate of 90 percent. Even though the brain only accounts for two percent of the total body weight, it is responsible for 20 percent of the total oxygen consumption at rest in the body. A decrease in the oxygen level in the blood (hypoxia) stimulates neurons and glia and initiates a series of intracellular regulations [77]. Only one percent of total body glycogen is found in the brain, and this amount is not sufficient to be evaluate as a carbohydrate reserve by neuronal cells [78]. Therefore, brain cells require exogenous glucose to maintain their vital metabolic activities. Glucose deprivation, caused by the decrease in its transfer from the blood to the brain, may rapidly cause damages in cerebral functions and respiration since brain regions are particularly vulnerable to hypoglycemia compared to the other organs and tissues. While brain cells take up glucose from the blood and converted it to pyruvate and lactate in the cytoplasm, oxidative respiration takes place in the mitochondria [75,79]. However, under specific conditions, other substrates can also contribute to brain energy demands [68]. ATP is converted into energy in the mitochondria via pyruvate metabolism in two steps; TCA cycle and the following oxidative phosphorylation. The cells convert pyruvate into lactate by lactate dehydrogenase enzyme (LDH) when the rates of glycolysis exceed that of oxidative phosphorylation. The lactate can be released from the cells through MCTs and be transferred to various cells carrying MCTs as well. [69]. According to the conventional suggestions, together with astrocytes, neurons use glucose obtained through oxidative metabolism as a carbon source. Lately, however, evidence implies that this compartmentalization of carbon source utilization should not be limited to cytoplasm and mitochondrion only, but could also include the overall cell metabolism. According to Astrocyte-Neuron Lactate Shuttle Hypothesis (ANLSH), initially suggested by Luc Pellerin and Pierre j. Magistretti in 1994, glutamate; besides its receptor-mediated activity on neuronal excitability, induces glycolysis and lactate production in astrocytes, and the generated lactate is transferred to neurons, especially during neurotransmission (Figure 1.3.) [74,79]. In 2011, Suzuki and his colleagues including P. Magistretti also reported that, in the hippocampus of rat brain, memory formation triggered a remarkable rise in extracellular lactate concentration generated through glycogen via glycogenic astrocytes. Deterioration in the glial lactate transporters' expression (MCT4 or MCT1) leads to long-term potentiation disruption and amnesia, which is recovered by not glucose, but notably L-lactate [73]. In 2012, Lee et al.

investigated how oligodendrocytes metabolically support axons by rather focusing on lactate, released by monocarboxylate transporter-1 (MCT1) [14]. According to the evidence, MCT1 expression decreased in ALS and, therefore, axons are shown to be incompetent to meet their energetic demands during ALS progression [14,75]. These findings reinforce the ideas that lactate has a crucial role in synaptic plasticity maintenance and is involved in the rescue mechanism in hypoxic brain injuries or diseases arising from impairment in the energy supplementation of the neuronal cells. In our study, inspired by these latest findings regarding possible disruptions in bioenergetic metabolism in ALS, in order to understand the individual contribution of monocarboxylate transporters, which are responsible for stable lactate trafficking in the CNS, to ALS pathogenesis, we investigated the changes in gene and protein expressions of oligodendroglial MCT1, neuronal MCT2, and astrocytic MCT4 in the CNS of transgenic mice carrying mutant genes that cause ALS, including SOD1 and C9ORF72. However, unlike the work of Lee et al. (2012), which we took as a reference; instead of examining MCT expression in organotypic spinal cord cultures, we preferred to perform our study in a cell-type-specific manner by utilizing the magnetic cell sorting (MCS) technique using whole-brain materials. Since the number of cells we obtained as a result of MCS, especially for neuronal cells, was considerably low, even for whole-brain samples, we could not apply the same method for spinal cord samples. However, with qPCR analysis, we could estimate the overall MCT expression status in the whole-spinal cord materials. First of all, in order to measure the efficiency of our sorting method, using PND 90 wild-type whole-brain samples, we performed the characterization of the cell fractions separated after MCS, using neuronal and glial markers by qPCR and the Simple Wes method, which allows us to perform protein analysis in low amounts. According to our results, although we succeeded in separating astrocytes and oligodendrocytes, we failed in obtaining pure neuronal cell fractions, possibly due to the fact that while positive selection was performed by using cell-specific antibodies during glial cells separation, negative selection was performed in neuronal cell extraction as the manufacturer recommends. In addition, the non-neuronal cells biotin-antibody cocktail used in the procedure might be insufficient in terms of antibody affinity to separate glial cells from neurons. However, in the whole-brain materials, the fact that expressions of glial and neuronal markers, which we used as normalization samples in qPCR and protein analyses were significantly lower, compared to those of the cell fractions proved that we achieved our goal in cell enrichment. Then, we examined the relevant MCT gene and

protein expressions in the sorted neuronal and glial cells extracted from symptomatic PND 90 SOD1-G93A mice and their wild-type counterparts. According to qPCR and protein analysis results, contrary to Lee's findings, MCT1 expressions were interestingly higher in both sorted oligodendrocytes extracted from whole-brain and whole-spinal cord materials compared to wild-type controls. MCT4 gene and protein expressions are surprisingly lower in brain-derived sorted astrocytes of SOD1 transgenic mice, and its gene expression was higher in whole-spinal cord materials. Protein expression of MCT2 was examined in sorted neurons and no significant change was observed compared to age-matched wild-type controls. Likewise, MCT gene and protein expressions in pre-symptomatic PND 60 transgenic mice did not exhibit any significant alterations compared to that of age-matched wild-type controls, indicating the disruption in MCT expressions becomes evident with the symptomatic stage. With respect to transgenic C9ORF72 mice, increased gene expression of both glial MCTs (*MCT1* and *MCT4*) was observed in the brain-derived sorted astrocytes and oligodendrocytes. Considering motor neurons' massive size, long axonal processes, and weak calcium-buffering abilities, [236] in parallel with this, their energy demand could be higher. Since the energy produced in the cell body may not spread adequately through such long cell projections, possible energy deprivations that occur in the long axons of motor neurons can be considered as one of the hypotheses for ALS development [14,98]. In normal conditions, lactate obtained through aerobic glycolysis could be an additional, sufficient energy source for such large neurons, especially in cases where neuronal activity increased, as Pellerin and Magistretti hypothesized [75,80]. Oligodendrocytes, located along the long axons just like gas stations provide lactate to the neurons through their MCT1s [105], while astrocytes, also located along the axon and at tripartite synapses [69] and provide lactate to neurons through their MCT4s. Any deterioration in this functional system may play a causal role in the pathogenesis of ALS, as previously described [14]. Dysfunction of MCT1 may theoretically result in decreased capacity of efflux or influx of lactate in oligodendroglia since lactate transport direction depends on the concentration gradient [237]. In Lee's study, it has been proved that exogenous lactate prevents MN degeneration in organotypic spinal cord slices isolated from SOD1-G93A in low-glucose media[14], which points out lactate could still be adopted and used by MNs. In that case, disruption in MCT1 function may have occurred in the ability to release lactate rather than import it to the cell [88]. In addition to that, the decreased expression of MCT4 in the motor cortices of ALS patients [14] implies that motor neuron degeneration may result

from a disruption in low-affinity MCT4, the general lactate provider for neurons. This supports the idea that; the failure of lactate supply to neurons may be due to disruptions in low-affinity glial transporters rather than high-affinity MCT2, providing a net influx of lactate into neurons. If we evaluate our results in the light of these findings and hypothesizes, the reason lying behind the higher MCT1 expressions in brain-derived sorted oligodendrocytes and whole-spinal cord materials, and higher MCT4 expressions in whole-spinal cord materials in SOD1-G93A mice, can be considered as an attempt to compensate for the depletion of lactate entering the cell. This interpretation can also be considered valid for the increased MCTs genes (*MCT1* and *MCT4*) expressions in brain-derived sorted glial cells in transgenic C9ORF72 mice. The lower MCT4 expressions in brain-derived astrocytes from SOD1-G93A transgenic mice are notably consistent with the finding that MCT4 expressions reduced in the motor cortices of patients with ALS. Moreover, the fact that there was no significant difference in MCT2 expressions in symptomatic and pre-symptomatic transgenic SOD1-G93A mice compared to wild-type controls reinforces the idea that neuronal high-affinity MCT2 transporters may not contribute to ALS pathology, as mentioned above. Disruptions in energy metabolism have recently been associated with other neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's Disease (AD) [238–240] and moreover, in this disrupted energy metabolism, interestingly, impaired MCT functions as one of the contributory role players and/or their overexpression as a therapeutic option again come to the fore. In the study conducted by Xiaorong et al. (2021), using a rat model of stroke, it has been observed that a post-stroke cognitive decline similar to what occurs in AD emerged and MCT2 expression decreases. However, behavioural tests have been showed that MCT2 overexpression promotes the amelioration of cognitive decline following stroke in the rat model [240]. These studies highlight the importance of intact MCT2 function for the maintenance of neuronal metabolism. In 2011, Suzuki and his colleagues showed that decreased expression of MCT1 or MCT4 in astrocytes causes impaired long-term memory formation that was ameliorated by exogenous lactate but not glucose, as mentioned before. However, in the same study, it is found that decreased expression of neuronal MCT2 also leads to loss of long-term memory and was not recovered by exogenous lactate or glucose [73], implying that transport of lactate into neurons is required for long-term memory formation and MCT2 play a more critical role during this process. In another study carried out by Zhang et al. (2018), lactate concentration and cerebral MCT1, MCT2, and MCT4 expressions were shown to reduce in

APP/PS1 AD mice model. This study suggests that the decreases of cerebral lactate content and lactate transporters may result in the inhibition of lactate transport from glia to neurons, causing depletion of neuronal lactate [241]. In our study, the lack of any significant change in MCT2 expressions in mice with ALS compared to wild-type controls can be evaluated as a characteristic that distinguishes ALS from Alzheimer's, underlying causes of which shows resemblance to a certain level in this sense. However, future studies with CNS tissues isolated from the patients will provide more precise information about the expression status of MCT2 in ALS.

Dysfunctional mitochondria has been shown to cause ALS development, as well [238–240]. Therefore, in addition to MCTs, the expression of the mitochondrial *COX10* gene was investigated in whole-spinal cord materials, in PND 90 SOD1-G93A transgenic mice, and it was observed that the gene expression was decreased significantly in the transgenic mice when compared to wild-type counterparts, highlighting the involvement of the aberrant mitochondrial gene functions in ALS pathology. *COX10* encodes heme A: farnesyltransferase, which is not a structural subunit, however, is essential for the endurance of functional COX (cytochrome c-oxidase) expression, which is an inner mitochondrial membrane marker and the terminal component of respiratory chain in mitochondria, mediating the transfer of the electrons from reduced cytochrome c to oxygen [245]. Hashimoto et al. (2008) and Newington et al. (2013) showed that MCT1 and MCT2 co-localize with COX in cortical, hippocampal, and thalamic neurons in rat brain and also co-precipitated with COX from isolated mitochondria [246,247]. It is quite remarkable that these proteins, which play a key role in energy metabolism, are co-localized in the rat brain, and the expression of their encoding genes changes in mice with ALS as demonstrated in our study. In addition to the possible association of the COX protein with MCTs, in a study carried out by Kirkinezos *et al.* (2005) a reduced activity of COX within the spinal cords of paralyzed G93A-SOD1 mice were observed [248]. Furthermore, according to the study conducted by Son and his colleagues using G93A-SOD1 transgenic mice overexpressing CCS (copper chaperone for superoxide dismutase) protein, an accelerated disease course that was associated with enhanced mitochondrial pathology and increased mitochondrial localization of mutant SOD1 of which wild-type form is mainly cytosolic were observed. SDS-PAGE analysis elicited that spinal cords from CCS/G93A-SOD1 transgenic mice showed significant decreases in the levels of two structural subunits

of COX; *COX1* and *COX5b*. Our study showed that aside from these structural subunit-encoding genes, the expression of non-structural *COX10* gene was also decreased in the spinal cord of SOD1-G93A mice [249]. In future studies, it will be extremely useful to examine the possible co-localizations of MCTs and COX in the brain and spinal cord of mouse models with ALS and whether they play a synergistic role in the pathogenesis of ALS in these animal models.

Numerous *in-vitro* culture models have been generated to investigate the pathology of neurodegenerative diseases in greater depth and to mimic the neurotoxic environment emerging in the disease. While primary culture models are mostly prepared with organotypic and differentiated embryonic or neonatal tissues, the main advantage of these models is that cells can undergo morphological and physiological changes over time and resemble their in-situ forms. However, the development of the neuronal characters of these cells requires time, and then the cells cannot be subcultured. Thus, primary neurons were hybridized with tumor lines in order to develop cells that can divide and be serially subculture while preserving their neuronal characteristics [250]. NSC-34 is a cell line generated by the fusion of neuroblastoma cells obtained from mice and mouse embryonic spinal MN cells [242]. These cells can be differentiated and acquire morphological and physiological properties very close to the motor neuron cells, nevertheless, without being manipulated by various inducing agents, they already possess some of the physiological and metabolic characteristics specific to MNs, including synthesis of neurofilament triplet proteins, acetylcholine and choline acetyltransferase generation and storage, elongation of neurites and expression of the basal lamina adhesion molecule specific to neuromuscular junction, S-laminin [212–214,251,252]. Based on this information, we designed *in-vitro* experiments using NSC-34 cell line due to its motor neuron cell-like properties and investigated the consumption of lactate as an energetic fuel, its importance for cell survival, and how its depletion affects cell viability in a glucose-free environment. Then we chemically blocked the function of MCTs in an attempt to observe the extent of the possible degeneration of cells without MCT function and thereby the consequences of loss of lactate trafficking. For the glucose-starvation experiment, DMEM/low glucose media with a glucose content of 5.5 mM were used and mixed with 80, 40, 20, and 10 mM Sodium L-lactate, respectively to estimate the optimal lactate concentration for NSC-34 cells. As an initial step to measure cell survival, MTS assay was performed every other day

for 12 days for long-term observation of cell growth in lactate-containing media. Cell survival of the low-glucose and low-glucose + lactate treated groups was relatively evaluated to the high glucose treated control group, where cell survival was considered maximum. According to the results, the group treated with 20 mM L-lactate + low-glucose media gave the closest average cell viability to the group treated with classic high glucose complete media between day six and day 10, when cell proliferation peaked. Due to the fact that 80 mM Na L-lactate was most probably toxic for the cells, the group treated with 80 mM lactate + low-glucose showed the lowest cell viability. As for Hoechst/PI nuclear staining, quite interestingly, the high glucose treated group exhibited quite a low cell viability rates—even lower than lactate treated groups throughout the experiment in contrast with its MTS assay results. Nevertheless, except for this inconsistency, the cell viability of the group treated with 20 mM L-lactate + low-glucose media bore a close resemblance to its MTS measurement and possess the highest average cell survival rates among lactate treated groups. The questionable finding of this experiment, contrary to expectations, is the inconsistency in cell viability results obtained from MTS and nuclear (Hoechst/PI) staining in NSC-34 cells treated with high glucose. According to the results of nuclear staining, lactate has even become a more preferable energy source for NSC-34 cells than glucose. In MTS experiments, however, appropriate concentration of lactate still comes right after glucose. The underlying reason behind this contradiction could be the fact that although the experiment initiated with a small number of cells, as a result of long-term observation without subculturing, the cells might enter the death phase. Nevertheless, the remaining viable cells showed more reduction activity of the MTS tetrazolium compound in the high glucose treated group, which MTS assay is based on [244]. However, in the nuclear staining experiment, in which we relatively measured only the number of dead cells since cell proliferation might be slower in cells fed with lactate, and they might not reach the death phase yet, they outnumbered the glucose-treated group in terms of the number of viable cells. The definitive conclusion that can be drawn from these two data is that NSC-34 cells can use an appropriate dose of lactate as an energy source and that cell viability rates are higher than the low glucose-treated group used as negative control.

In the second part of our *in-vitro* experiments, we used AR-C155858 in order to chemically block MCT function and observe the effect of the consequent lactate depletion on cells. AR-C155858 is a cell-permeable thieno[2, 3 d]pyrimidinedione compound, which

behaves as a highly effective inhibitor of MCT1 ($K_i=2.3$ nM for lactate uptake in rat erythrocytes) and has been proven to inhibit MCT2 function when associated with basigin ($IC_{50} = 10$ nM) [216]. However, although the general distribution of MCT isoforms in CNS cells was demonstrated via *in-vitro* and *in-vivo* studies, and NSC-34 cells were also designed to mimic mouse motor neurons, undifferentiated NSC-34 cells might have exhibited a different MCT expression profile from adult neurons. Therefore, we examined the expression profile of neuronal and glial cell markers and MTCs by qPCR in undifferentiated NSC-34 cells. According to the results; *MBP* was the highest expressed marker gene comparing *GLAST* and *NeuN*, moreover, the *MCT1* gene appeared to be the most abundant isoform expressed among others, implying that; undifferentiated NSC-34 cells have glial cell characteristics rather than neuronal, and more specifically, they resemble oligodendrocyte cells with its considerably high *MBP* and *MCT1* gene expressions. After it became clear that NSC-34 cells mainly express MCT1 and therefore AR-C155858 was a suitable inhibitor, it was added to each experimental dish with a final concentration of 200 nM in cell culture media as described before [217]. Media and cell pellets were collected at the end of various time points (0, 2, 4, 8, 16, and 32 minutes) in order to analyse lactate amount in both samples by HPLC analysis to examine the inhibition level of lactate entry into the cell. According to the results, the amount of L-lactate exhibited a decrease in the media depending on time, pointing out the fact that lactate has been used at certain levels, regardless of the presence of AR-C155858 inhibitor. Despite this, when the positive controls and the negative controls (without inhibitor) belonging to the same time points were examined, it was clearly seen that the amount of lactate in the media was higher than in their negative controls, meaning the inhibitor prevents the lactate entry by the cell to a certain level. Specifically, this difference was even more pronounced between the media of negative and positive controls collected at two and eight minutes. The amount of lactate in the cells could not be determined, as the lactate in the cell pellet could not be measured by HPLC method. However, the amount of lactate measured in the media allows us to make a rough estimation regarding the influx of lactate and its usage in the cell indirectly. Interestingly, it was observed that the amount of lactate in the negative control and positive control media converged at the following time points. Hypothetically, it can be resulted from the lactate export to the media by uninhibited MCT1, as a consequence of metabolic activities inside the cell in the negative control, which again highlights oligodendroglial characteristic of undifferentiated NSC-34

cells and the involvement of its resident MCT1 in both influx and efflux of lactate [254]. Once it was revealed that AR-C155858 inhibitor prevented lactate entry into the cell to a certain level, in an attempt to investigate the effect of the long-term presence of the inhibitor on lactate consumption and its reflection on cell survival, we incubated cells with AR-C155858 for 12, 24, 48, and 72 hours and measured their cell viability by nuclear staining method. According to the results, the rate of cell viability in the control group treated with high glucose media was the highest at the end of 72 hours, while this rate was reduced by more than fifty percent in cells treated with AR-C155858 + 20 mM L-lactate. DMEM/no glucose media treated cells showed the lowest survival rate as anticipated. On the other hand, the cell viability rate of cells treated with DMEM/no glucose media + 20 mM L-lactate was approximately 60 percent, closest to the high glucose treated control group. Another remarkable finding to emerge from the data is that, unlike the previous nuclear staining experiment, the highest cell viability rate was observed in control cells treated with high glucose, which could be resulted from the fact that the observation period of the cells was kept relatively shorter, and therefore the cells did not enter the death phase. In summary, L-lactate was a preferable energy source after glucose for undifferentiated NSC-34 cells and AR-C155858 inhibitor clearly blocked the entry of lactate into the cell to a certain level, which significantly reduced cell survival compared to the control group. Although studies on bioenergetic mechanisms in the brain and this study mostly focus on the use of lactate by neurons, Sanchez-Abarca et al. (2001) demonstrated elevated levels of lactate utilization in cultured oligodendrocytes isolated from forebrains of 1-2 day-old Wistar rats [92] and also Rinholm et al. (2011) showed that lactate supports oligodendrocyte development and myelination in cultured slices of developing cerebral cortex from mice [93]. Given that the undifferentiated NSC-34 cells we used in this study have rather oligodendroglial properties, their ability to use lactate is compatible with the literature. In future studies, all MCT isoforms, including MCT4, can be inhibited in differentiated NSC-34 cells or in primary motor neuron cultures prepared using spinal motor neurons, and possible degenerations of motor neurons, including disruption in axon elongation, can be examined in more detail. While providing an alternative approach regarding motor neuron degenerations occurring in ALS, these motor neuron damages induced by *in-vitro* MCT inhibitions highlight the importance of the development of therapeutic agents mitigating overexpression of MCTs in ALS and similar neurodegenerative diseases.

Boron is a type of naturally occurring trace element [115] with the symbol B and atomic number five [126]. It is an essential nutrient for many organisms. In healthy tissues, boron exists in the form of borate or boric acid. In the body, the total amount of boron is approximately 3-20 mg; however, it varies according to the structure of the tissues. Accumulated evidence proved that boron shows diverse pleiotropic effects, ranging from antimicrobial, antiviral [122,123] anti-inflammatory [124], antioxidant [121,123] wound healing effects [122], to the mediating various body systems. In the light of these developments, the designs of boron-based drugs have come to the fore [124,125]. Boron is employed in various metabolic pathways, such as bone formation and sustenance [128], hormonal activity [129], and psychological functions [116]. It is recently revealed that boron supports short and long-term weight loss when consumed as a dietary supplement [98, 99]. Boron derivatives are also known for their antioxidant effects [123]. As evidence accumulated to support that boron is crucial for human health, in the early 90s, the possible relationship between boron nutrition and brain and psychological activity began to be explored. The first study conducted with mature rats showed that a gradual increase of boron supplement in a low boron-containing diet enhances brain electrical activity [146]. Based on the findings obtained from this study, three separate human studies were conducted assessing the effects of boron compounds on brain electrical activity and the various mental and motor tasks performance. Two studies have shown that boron supplementation has led to the improvement of psychomotor skills of elderly men and women, elimination of drowsiness, short-term memory improvement, and mental alertness [118]. Similar results were observed in rats [148]. In a study carried out by Ozansoy et al. [166], the effects of boron derivatives, boric acid (BA), and sodium borate decahydrate (borax decahydrate) on amyloid beta ($A\beta$) toxicity were investigated in the SH-SY5Y neuroblastoma cell line, and findings indicate that BA provides protection against $A\beta_{1-42}$ toxicity, and besides its therapeutic nature, it can be considered as a preventive treatment due to its ability to increase the expression of Sirt1, which has been shown to guard against oxidative stress. In another study, Sorout et al.. (2020) examined the effects of boron nitride nanotubes (BNNTs), which resemble carbon nanotubes (CNTs), on the secondary structure of $A\beta_{1-42}$ trimer recently in Alzheimer's disease (AD). It is confirmed that since BNNTs are not as hydrophobic as CNTs, they keep peptides of $A\beta_{1-42}$ trimer apart and appear to limit self-aggregation. In association with this finding, an inspiring study regarding functionalized BNNTs highlighted that they possess the capability to enter human

umbilical vein endothelial cells [191], suggesting that these structures could be used specifically for drug delivery in the CNS, in addition to their ability to behave as therapeutic option themselves for AD treatment as reported in this research. All these findings strengthened the view that boron is an essential supplement for brain metabolism and psychological functions in the body and served as a basis for the investigation of the possible neuroprotective role of boron in many studies including ours. In this context, in the second part of our study, in accordance with the use of some boron-based compounds and structures as an alternative therapeutic candidates for neurodegenerative disease treatment as mentioned above, we investigated the effect of some boron derivatives on locomotor behaviours and molecular mechanisms including MCT expressions in animal models with ALS. Sodium pentaborate pentahydrate and boric acid, antimicrobial [255], anti-inflammatory, and wound-healing properties [124] of which have been proven in previous studies, and sodium perborate tetrahydrate (SPT; $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$), with no published studies of its usage in any medical field, were preferred for boron administration. Nowadays, SPT is still being used as a whitening agent and washing powder [256–258], and NaB, on the other hand, has not yet been tested on neurodegenerative diseases, although there are important studies on its usage in the medical field [124,255]. Pre-symptomatic 80 days old female SOD1-G93A mice and their wild-type counterparts were used for the application. Boron derivatives dissolved in their drinking water were administered to mice by gavage every other day for 3 weeks. Only water was given to the wild-type and transgenic control groups. Brain and spinal cord materials were collected from the animals after the treatment aiming to explore the possible effects of applied boron derivatives on the onset and course of ALS symptoms. In order to understand whether boron derivatives cause an alteration in the motor coordination of animals, especially transgenic mice, the animals were subjected to certain motor function tests before and after boron applications. Neurological scoring for each mouse was assessed along with the experiment, and no significant effect of boron derivatives on the onset of pathology has been observed. At the end-stage of the experiment, the neuroscores of the mice, neuroscore levels of which were measured regularly were again scored as NS1. Nonetheless, mice in the NaB and SPT treated groups exhibit notably milder NS1 symptoms (mild or moderate hindlimb retraction, ataxia, and tremor) as the experiment progressed and at the end of the experiment comparing the transgenic water group. As a result of the comparison of the motor functions of these transgenic animals; it was observed that SPT increased

significantly ($P \leq 0.01$) the performance of the rotarod test by 88.4 percent in the transgenic SOD1-G93A mice when compared to the SOD1-G93A water control group. In the NaB applied group, the increase in the rotarod performance (62,45 percent) was determined as the trend-level compared to the transgenic water group, but no statistically significant result could be obtained. Although a motor activity improvement was also observed at the trend-level in the BA treated group (26,6 percent), it was even subtler than NaB provided. In the transgenic control group, it was observed that the symptoms intensified over time and the rotarod performance decreased. In this respect, our study is the first to show that boron-based compounds increase locomotor activity in an animal model, except for the studies published by Penland (1994 and 1998) in which boron diet was reported to strengthen the psychomotor skills in human subjects [120,148]. The remarkable improvement provided by NaB and, especially SPT on NeuroScore test and rotarod performance led us to examine the possible effects of these derivatives on molecular mechanisms including MCT expressions in SOD1-G93A mice by microarray analysis, which enables to provide valuable information regarding the transcriptome of the collected materials. Spinal cord and brain materials ($n=3$ per material type) collected from groups treated with NaB and SPT and water treated wild-type and transgenic controls groups were subjected to microarray analysis. Volcano plot analysis and scatter plot analysis using GeneSpring GX v14.9 software has revealed that thousands of genes expressions profiles in the spinal cord and hundreds of genes in brain materials were affected by the treatment with boron derivatives. However, more strikingly, as we understand from the heatmap representation, especially in spinal cord samples (Figure 3.29.), NaB and SPT derivatives approximate the general gene expression profile of transgenic animals to that of wild-type control mice after about three weeks of the application. While in the spinal cord, the overall gene expression profile tended to be up-regulated in the transgenic water control group, the derivatives down-regulated most of these genes, providing a picture similar to the wild-type gene profile. These genes were then downloaded into the PANTHER Gene Ontology (GO) Classification and Pathway System and separated into their respective functions and pathways according to the number of hits they received. At this stage, genes that received no hits were eliminated. Later, from this narrowing gene pool, the expressions of the genes that are relatively more critical for our study than the others were extracted as a heatmap (Figure 3.31. and 3.41.) with the GeneSpring GX v14.9, and also shown as a table (Table 3.2. and 3.3.) with the pathways they involved. In NaB and SPT

treated groups, in spinal cord materials, there was a significant down-regulation in the expression of genes associated with pathways including Alzheimer's disease-amyloid secretase pathway, Alzheimer's disease-presenilin pathway, Huntington Disease, Parkinson's Disease and inflammation mediated by chemokine and cytokine signaling pathways, which are related to neurodegenerative diseases and neuroinflammation. In addition, the expressions of genes involved in the energy metabolism pathways such as the pentose phosphate pathway, TCA cycle, and glycolysis pathway reached the gene expression profile of the wild-type water-treated control group. In brain materials, gene expressions in the NaB and SPT treated and wild-type water treated groups did not show a clear up-regulation or down-regulation trend comparing the transgenic water treated control group (Figure 3.39.). However, just as in the spinal cord materials, in the brain materials collected from NaB and SPT treated groups, genes linked with the pathways such as Alzheimer's disease-amyloid secretase pathway, Alzheimer's disease-presenilin pathway, Huntington Disease, Parkinson's Disease and inflammation mediated by chemokine and cytokine signaling pathways, which are related to neurodegenerative diseases and neuroinflammation showed a similar regulation trend with the wild-type control group. In addition, the expressions of genes associated with the energy metabolism pathways including pentose phosphate pathway, oxidative stress response, and glycolysis also approximated to that of the wild-type water treated group. In a study published by Herrero-Mendez et al. (2009), it is elicited that an important activator of glycolysis, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase isoform 3 (Pfkfb3), is constantly degraded in neurons [259]. This implies that glucose metabolism in neurons tends to direct towards the pentose phosphate pathway and antioxidant generation rather than glycolysis, and neurons possess a low glycolytic rate, pointing out the necessity of consuming lactate for aerobic respiration, contrary to general belief. These findings also propose that antioxidant vulnerability of neurons can be compensated by pentose phosphate pathway, even if it leads to bioenergetic glycolysis inhibition [260]. Tefera et al. (2019) detected impairments in the pentose phosphate and glycolytic pathways in SOD1-G93A mice CNS tissues at the onset of disease, which caused increased oxidative stress [261] In this context, that boron derivatives are effective in the expression of some genes involved in the pentose phosphate pathway according to our microarray results is an important finding of our study. According to our results, one of the genes involved in this pathway and spinal cord expression of which decreases and approaches the wild-type profile is the *Hk1* gene,

encoding the glycolytic hexokinase-1 (Hk1) enzyme [262]. The Hk1 is known to be expressed at low levels in the mouse spinal cord [263]. In a study conducted by Magri et al. (2016) using NSC-34-SOD1-G93A ALS cell model, it is revealed that Hk1 competes with mutant SOD1-G93A for binding VDAC1 (voltage-dependent anion channel isoform 1 in the outer mitochondrial membrane, a docking site for SOD1-G93A mutant in ALS mice and the physiological receptor of Hk1), indicating that in ALS spinal cord the available Hk1-binding sites could be used by SOD1 mutants for docking mitochondria, thereby causing organelle dysfunction [263]. In our study, the fact that the expression of the *Hk1* gene in the transgenic control group was higher (~ 2.5 fold) compared to that of boron-treated groups (closer to wild-type control) can be interpreted as a defense mechanism, aiming to increase the expression of the Hk1 protein in the spinal cord of transgenic control mice to compete better with mutant SOD1. The reduction of *Hk1* expression to that of wild-type level in boron-treated groups may be due to the general alleviation of ALS-induced pathology by the derivatives. Although abnormal SOD1 accumulation is associated with fALS, as previously mentioned; misfolded wild-type SOD1 has been found to accumulate in mouse models [31,32] and postmortem tissue of patients with sALS [264]. Thus, Hk1, which plays an important role in bioenergetics metabolism and acts as a plug to prevent aberrant SOD1 binding to mitochondrial membrane, could be a promising therapeutic target for ALS treatment.

Neuroinflammation, characterized by the reactivation of glia cells—especially astrocytes and microglia—and T cells is a common reaction seen in ALS and also other different neurodegenerative diseases [265]. In patients and animal models with ALS, in addition to chronically induced astrocytes and microglia, infiltrating immune cells are also involved in affected areas of the CNS. They are the typical pathological manifestations of ALS [266]. Over-activated glial cells cause inflammation and subsequent enhancement of reactive oxygen species (ROS). The ROS accumulation in the CNS leads to neuronal disruption and eventually cell death [159]. It is proved that misfolded mutant SOD1 protein aggregates in the mitochondria in both ALS patients and SOD1-G93A transgenic mice also causes physiological disruptions such as impaired energy homeostasis, ATP, and ROS release, and abnormal activation of apoptosis [242]. Reactive microglia secretes proinflammatory cytokines (eg, $\text{TNF}\alpha$, $\text{IL1}\beta$, IL12 , $\text{IFN}\gamma$), neurotrophic factors (eg, IGF-1), anti-inflammatory factors (eg, $\text{TGF-}\beta$), and mitogenic factors (eg, MCP-1, M-CSF) [267]. In

this study, SPT and NaB, which are already known for their anti-inflammatory effects, approximate the expression of the genes engaged with neuroinflammation to that of wild-type in the brain and spinal cord of SOD1-G93A transgenic mice. This reinforces the idea that these derivatives could be promising therapeutic candidates for the treatments aspired to reduce neuroinflammation, which is one of the underlying causes of ALS and other CNS diseases. *Mapk8*, involved in Alzheimer disease-amyloid secretase, Parkinson's disease, T-cell activation, TGF- β signalling, oxidative stress response, and apoptosis signaling pathways, is one of the genes which is down-regulated after boron treatment in the spinal cord. *Pik3c2g*, linked with axon guidance mediated by netrin, T-cell activation, inflammation mediated by chemokine and cytokine signalling, and VEGF signalling pathways, and *Map2k3*, involved in insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase (MAPKKK) cascade, and oxidative stress response are also among these genes. Aside from that, *Cxcl10* and *Bcl3*, associated only with inflammation mediated by chemokine and cytokine signalling pathways, and *Cd28*, associated only with T-cell activation in our analysis, and *Map2k7*, involved in FGF signaling, Huntington disease, apoptosis signalling, and T cell activation pathways, are some of the genes that down-regulated after boron administration and have been already found to be engaged with neuroinflammation in ALS, according to a transcriptome study conducted by Morello et al. (2017) [268]. A proinflammatory mediator and involved in axon guidance mediated by Slit/Robo and inflammation mediated by chemokine and cytokine signaling pathways; *Cxcr4* is one of those down-regulated genes. It is reported that an FDA-approved *Cxcr4* antagonist; AMD3100 enhanced survival and eased ALS pathology in SOD1-G93A mice. Carbone et al. [269] described the depletion of EAAT1 in patients with ALS. Expression of the *SLC1A3* (GLAST) gene encoding EAAT1 protein is observed to be elevated in SOD1-G93A mice, according to our microarray results and boron derivatives again reduced the expression to the wild-type level. Theoretically, that could result from the fact that GLAST, as a neurotrophic factor [252], played a compensative role and increased in the transgenic control group, and for the boron treated groups, reduced to normal levels with the decrease of inflammation as a result of boron application. Another remarkable result of the application of boron derivatives is the down-regulation of the *MCT4* gene, which is one the main target of our study, after boron derivatives, again in spinal cord materials. This result is consistent with the observation that *MCT4* expression increased in the spinal cords of transgenic SOD1 animals, which we confirmed with qPCR analysis in spinal cord

materials. In this way, after boron administration, *MCT4* expression was also down-regulated and approached the wild-type profile in NaB and SPT treated groups. There was no significant alteration in the regulation of other MCTs in the spinal cord and brain samples according to microarray analysis results.

As for the brain materials, there was no clear trend of gene regulation when comparing wild-type and transgenic controls, as previously mentioned. Again, boron derivatives were able to bring the regulation of many genes to a level close to that of transgenic animals. On the other hand, the number of genes with altered regulation is considerably low compared to the spinal cord material. Hypothetically, this could arise from the difficulty for the derivatives to pass the blood-brain barrier and be effective in the brain compared to the spinal cord. Some of the notable pathways, aside from those associated with neurodegenerative diseases, in which boron derivatives are particularly effective in the brain are the 5HT1, 5HT2, 5HT3, and 5HT4 type receptor-mediated signaling pathways. 5-HT receptors and transmitters take a major part in many neuropsychic processes in the CNS and constitute the main drug target in many brain disorders including mood disorders, schizophrenia, and migraine. With the exception of the 5-HT3 receptor, the 5-HT receptors belong to G-protein-coupled receptors (GPCRs) family [271,272]. In this context, it is not coincidental that boron derivatives were also effective in the heterotrimeric G-protein signaling pathway in our study. As a consequence of the early discovery of its encoding gene and the availability of selective ligands, 5-HT1A is the best-characterized serotonin receptor comparing other 5-HT receptors. 5-HT1A receptors constitute one of the main elements that can change synaptic physiology by altering the membrane potential and excitatory and inhibitory tones. Induced 5-HT1A receptors play an activating role in the neural precursor development into adult neurons by increasing the proliferation of neuronal progenitors, especially in the dentate gyrus's subgranular region [270–272]. Noto et al. (2016) reported an elevated neurogenesis in adult mice with a permanent overexpression of the postsynaptic 5-HT1A receptor [273]. Polter et al. (2010) also demonstrated a neuroprotective role of 5-HT1A receptors in both *in-vivo* cultures and in animal models of Parkinson's disease and ischemia [273]. Down-regulated in boron treated groups, *Slc18a1*, interestingly involved in all 5HT- type (1-4) receptor-mediated signaling pathways in our study. This gene is also one of the common central genes that is regulated both [275] in obsessive-compulsive disorder and bipolar disorder, according to the study of Hamidian et

al. in 2020. Down-regulated *Gnatl* is also associated with 5HT4 type receptor-mediated signaling pathway, and additionally, inflammation mediated by chemokine and cytokine signaling and dopamine receptor-mediated signaling pathway. Down-regulated *Hspa13* and up-regulated *Hspa5* are associated with Parkinson's disease pathway. Along with that, down-regulated *Ccl3*, engage with inflammation-mediated by chemokine and cytokine signaling pathway, and down-regulated *Map2k7*, involved in apoptosis signaling pathway have been previously found to be associated with neuroinflammation in ALS, according to Morello et al. [249].

To sum up, in this study, by providing clear information regarding cell type-specific MCT expressions in the brain and overall spinal cord, especially in transgenic SOD1-G93A mice models, we have reinforced the idea that MCTs may take part in the development of ALS, and in this context, in addition to the abnormalities described so far, disruptions in energy metabolism may also cause ALS pathology. We also demonstrated *in-vitro* that lactate can be used as an energetic fuel, besides glucose, proving once again that it can be utilized as a carbon source for the survival of brain cells in the presence or absence of glucose in CNS. Finally, by applying NaB and SPT boron derivatives to the SOD1-G93A model, we demonstrated that the derivatives significantly approximate the gene expressions engaged with neurodegenerative disease and neuroinflammation to that of wild-type levels in transgenic mice, also resulting in a considerable improvement in the motor activity of the animals.

5. CONCLUSION

Amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease, is a fatal, progressive neurodegenerative disease affecting the motor neurons (MNs) [1]. The disease is characterized by degeneration of MNs in the brain stem, motor cortex, and spinal cord, causing muscle paralysis and eventually death [2].

In this study, in an attempt to understand the possible contribution of MCTs to ALS pathogenesis, the changes in expression levels of MCT1, MCT2, and MCT4 in the CNS of the transgenic mouse models were examined in a cell-type-specific manner. According to the results, a significant increase in MCT1 expressions was determined in both whole-spinal cord materials and brain-derived oligodendrocytes, while a significant decrease in MCT4 expressions was detected in brain-derived astrocytes, and no significant change was observed in MCT2 expressions in transgenic SOD1-G93A mice, offering a compensatory role for MCT1 and a causal role for MCT4 in ALS.

In-vitro experiments using NSC-34 MN-like cells once again proved that lactate at appropriate doses can be used as an energy source, as an alternative to glucose, and when lactate transporters were chemically blocked, lactate trafficking was partially interrupted, negatively affecting cell survival.

Additionally, inspired by the introduction of some boron derivatives for the treatments of neurodegenerative diseases, the effect of various boron derivatives on locomotor behaviors and molecular mechanisms were tested in the SOD1-G93A mouse model. It was observed that especially sodium pentaborate pentahydrate (NaB) and sodium perborate tetrahydrate (SPT) led to a considerable improvement in motor activities of transgenic mice, and moreover, according to microarray data, they positively changed the expression of genes associated with numerous metabolic pathways involved in neurodegenerative diseases, neuroinflammation, and neuroprotection.

In our study, we provided further evidence for the role of MCTs in the pathogenesis and progression of ALS. In future studies, examining whether the manipulated up-regulation of MCT4, the expression of which appears to be decreased in *ex-vivo* models of astrocytes originating from ALS tissue and in the motor cortex of patients with ALS, or

transplantation of healthy astrocyte will improve survival in mouse models with ALS will be a tremendously beneficial step for the treatment of ALS. It will also provide further insight into the role of MCT4 in ALS.

This study was also the first to reveal possible neuroprotective effects of NaB and SPT for the treatment of ALS and other related neurodegenerative diseases. Future work that will focus on the investigation of the longer-term applications of combined boron derivatives on transgenic mouse models will allow us to attain more detailed knowledge about the neuroprotective role of boron derivatives. These studies could pave the way for clinical trials to test the therapeutic potential of boron derivatives in the treatment of ALS or other neurodegenerative diseases and assess the possibility for the inclusion of these compounds in future standard of care.

.

REFERENCES

1. Kim C, Lee HC, Sung J-J. Amyotrophic lateral sclerosis - cell based therapy and novel therapeutic development. *Exp Neurobiol.* 2014;23(3):207–14.
2. Pirooznia SK, Dawson VL, Dawson TM. Motor neuron death in ALS: programmed by astrocytes?. *Neuron.* 2014;81(5):961–3.
3. ALS Association. Genetics. 2021 [cited 2021 Nov 14]. Available from: <https://www.als.org/research/research-we-fund/scientific-focus-areas/genetics>
4. Aktekin MR, Uysal H. Epidemiology of amyotrophic lateral sclerosis-Amyotrofik lateral skleroz epidemiyolojisi. *Turkish J Neurol.* 2020;26:187–96.
5. ALS MNH Derneği. Türkiye’de ALS MNH hastalığı. 2021 [cited 2021 Nov 14]. Available from: <https://als.org.tr/turkiyede-als-mnh-hastaligi/>
6. Stifani N. Motor neurons and the generation of spinal motor neuron diversity. *Front Cell Neurosci.* 2014;81(5):1001–8.
7. Jara JH, Genç B, Cox GA, Bohn MC, Roos RP, Macklis JD, et al. Corticospinal motor neurons are susceptible to increased ER stress and display profound degeneration in the absence of UCHL1 function. *Cereb Cortex.* 2015;25(11):4259–72.
8. Işık A, Fidan U, Özkan N. Design and acquisition of EOG based interactive communications for ALS patients. *Int J Appl Math Electron Comput.* 2017;1(Special Issue-1):1–4.
9. How To Relief. Lower Motor Neuron Lesions Sign & Symptom. [cited 2021 Nov 16]. Available from: <https://www.howtorelief.com/lower-motor-neuron-lesions-sign-symptom/>
10. Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. *Ann Neurol.* 2009;65(Suppl):3–9.
11. Amyotrophic lateral sclerosis. Centers for Disease Control and Prevention. 2017

[cited 2021 Nov 16]. Available from: <https://www.cdc.gov/als/WhatIsALS.html>

12. Pellerin L, Bergersen LH, Halestrap AP, Pierre K. Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. *J Neurosci Res.* 2005;79(1–2):55–64.
13. Philips T, Rothstein JD. Glial cells in amyotrophic lateral sclerosis. *Exp Neurol.* 2014;262PB(Part B):111.
14. Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature.* 2012;487(7408):443–8.
15. The ALS Association. FDA-Approved Drugs. [cited 2021 Oct 27]. Available from: <https://www.als.org/navigating-als/living-with-als/fda-approved-drugs>.
16. ALS News Today. Radicava (Edaravone) for Amyotrophic Lateral Sclerosis (ALS). [cited 2021 Oct 27]. Available from: <https://alsnewstoday.com/radicava-edaravone-for-als/>.
17. Bensimon G, Lacomblez L, Delumeau J, Bejuit R, Truffinet P. A study of riluzole in the treatment of advanced stage or elderly patients with amyotrophic lateral sclerosis. *J Neurol.* 2002;249(5):609–15.
18. Gu J, Chen F, Iqbal K, Gong C-X, Wang X, Liu F. Transactive response DNA-binding protein 43 (TDP-43) regulates alternative splicing of tau exon 10: implications for the pathogenesis of tauopathies. *J Biol Chem.* 2017;292(25):10600–12.
19. Prasad A, Bharathi V, Sivalingam V, Girdhar A, Patel BK. Molecular mechanisms of TDP-43 misfolding and pathology in amyotrophic lateral sclerosis. *Front Mol Neurosci.* 2019;12(25).
20. Bunton-Stasyshyn RKA, Saccon RA, Fratta P, Fisher EMC. SOD1 function and its implications for amyotrophic lateral sclerosis pathology: new and nascent themes. *Neuroscientist.* 2015;21(5):519–29.
21. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al.

- Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*. 1993;362(6415):59–62.
22. Saccon RA, Bunton-Stasyshyn RKA, Fisher EMC, Fratta P. Is SOD1 loss of function involved in amyotrophic lateral sclerosis?. *Brain*. 2013;136(8):2342–58.
 23. Bunton-Stasyshyn RKA, Saccon RA, Fratta P, Fisher EMC. SOD1 function and its implications for amyotrophic lateral sclerosis pathology: new and nascent themes. *Neuroscientist*. 2015;21(5):519–29.
 24. Frey D, Schneider C, Xu L, Borg J, Spooren W, Caroni P. Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *J Neurosci*. 2000;20(7):2534–42.
 25. Hegedus J, Putman CT, Gordon T. Time course of preferential motor unit loss in the SOD1 G93A mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis*. 2007;28(2):154–64.
 26. Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, Khan J, Polak MA GJ. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp Neurol*. 2004;185(232):240.
 27. Fischer LR, Glass JD. Axonal degeneration in motor neuron disease. *Neurodegener Dis*. 2007;4(6):431–42.
 28. Clement AM, Nguyen MD, Roberts EA, Garcia ML, Boillée S, Rule M, et al. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science*. 2003;302(5642):113–7.
 29. Haidet-Phillips AM, Hester ME, Miranda CJ, Meyer K, Braun L, Frakes A, et al. Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. *Nat Biotechnol*. 2011;29(9):824–8.
 30. Rotunno MS, Bosco DA. An emerging role for misfolded wild-type SOD1 in sporadic ALS pathogenesis. *Front Cell Neurosci*. 2013;7(253).
 31. Deng H-X, Shi Y, Furukawa Y, Zhai H, Fu R, Liu E, et al. Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked

- insoluble aggregates of SOD1 in mitochondria. *Proc Natl Acad Sci.* 2006;103(18):7142–7.
32. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron.* 2011;72(2):245–56.
 33. Van Blitterswijk M, DeJesus-Hernandez M R. How do C9ORF72 repeat expansions cause amyotrophic lateral sclerosis and frontotemporal dementia: can we learn from other noncoding repeat expansion disorders. *Curr Opin Neurol.* 2012;25(6):689–700.
 34. Nguyen L, Cleary JD, Ranum LPW. Repeat Associated Non-ATG translation: molecular mechanisms and contribution to neurologic disease. *Annu Rev Neurosci.* 2019;42:227–47.
 35. Mori K, Weng SM, Arzberger T, May S, Rentzsch K, Kremmer E, Schmid B, Kretzschmar HA, Cruts M, Van Broeckhoven C, Haass C ED. The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. *Science.* 2013;339(6125):1335–8.
 36. Ash PE, Bieniek KF, Gendron TF, Caulfield T, Lin WL, DeJesus-Hernandez M, van Blitterswijk MM, Jansen-West K, Paul JW, Rademakers R, Boylan KB, Dickson DW PL. Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. *Neuron.* 2013;77(4):639–46.
 37. Polymenidou M, Lagier-Tourenne C, Hutt KR, Bennett CF, Cleveland DW, Yeo GW. Misregulated RNA processing in amyotrophic lateral sclerosis. *Brain Res.* 2012;1462:3–15.
 38. Higelin J, Catanese A, Semelink-Sedlacek L, Oeztuerk S, Lutz A, Bausinger J, et al. NEK1 loss-of-function mutation induces DNA damage accumulation in ALS patient-derived motoneurons. *Stem Cell Res.* 2018;30:150–62.
 39. Kenna KP, van Doormaal PTC, Dekker AM, Ticozzi N, Kenna BJ, Diekstra FP, et al. NEK1 variants confer susceptibility to amyotrophic lateral sclerosis. *Nat Genet.*

2016;48(9):1037–42.

40. Borroni B, Archetti S, Del Bo R, Papetti A, Buratti E, Bonvicini C, et al. TARDBP mutations in frontotemporal lobar degeneration: frequency, clinical features, and disease course. *Rejuvenation Res.* 2010;13(5):509–17.
41. Buratti E, Baralle FE. The multiple roles of TDP-43 in pre-mRNA processing and gene expression regulation. *RNA Biol.* 2010;7(4):420–9.
42. Sreedharan J, Blair IP, Tripathi VB, Hu X, Vance C, Rogelj B, et al. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science.* 2008;319(5870):1668–72.
43. Suk TR, Rousseaux MWC. The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. *Mol Neurodegener.* 2020;15(1):1–16.
44. Lagier-Tourenne C, Cleveland DW. Rethinking ALS: the FUS about TDP-43. *Cell.* 2009;136(6):1001–4.
45. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science (80-).* 2006;314(5796):130–3.
46. Wegorzewska I, Bell S, Cairns NJ, Miller TM, Baloh RH. TDP-43 mutant transgenic mice develop features of ALS and frontotemporal lobar degeneration. *Proc Natl Acad Sci U S A.* 2009;106(44):18809–18814.
47. Gitcho M, Baloh R, Chakraverty S, Mayo K, Norton J, Levitch D, et al. TDP-43 A315T mutation in familial motor neuron disease. *Ann Neurol.* 2008;63(4):535–8.
48. 017919 - STOCK Tg(CAG-EGFP,-dsRed2/RNAi:Tardbp)6Zxu/J. [cited 2021 Oct 27]. Available from: <https://www.jax.org/strain/017919>
49. Yang C, Wang H, Qiao T, Yang B, Aliaga L, Qiu L, et al. Partial loss of TDP-43 function causes phenotypes of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A.* 2014;111(12):1121–9.
50. Zakaryan RP, Gehring H. Identification and characterization of the nuclear localization/retention signal in the EWS proto-oncoprotein. *J Mol Biol.*

2006;363(1):27–38.

51. Daoud H, Valdmanis PN, Kabashi E, Dion P, Dupré N, Camu W, et al. Contribution of TARDBP mutations to sporadic amyotrophic lateral sclerosis. *J Med Genet.* 2009;46(2):112–4.
52. Kim HJ, Kim NC, Wang Y-D, Scarborough EA, Moore J, Diaz Z, et al. Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature.* 2013;495(7442):467–473.
53. Sharma A, Lyashchenko AK, Lu L, Nasrabady SE, Elmaleh M, Mendelsohn M, et al. ALS-associated mutant FUS induces selective motor neuron degeneration through toxic gain of function. *Nat Commun.* 2016;7(1):1–14.
54. TP73 - Tumor protein p73 - Homo sapiens (Human) - TP73 gene & protein. [cited 2021 Oct 28]. Available from: <https://www.uniprot.org/uniprot/O15350>
55. Lucena-Araujo AR, Kim HT, Thomé C, Jacomo RH, Melo RA, Bittencourt R, et al. High $\Delta Np73/TAp73$ ratio is associated with poor prognosis in acute promyelocytic leukemia. *Blood.* 2015;126(20):2302–6.
56. Pozniak CD, Barnabé-Heider F, Rymar V V., Lee AF, Sadikot AF, Miller FD. p73 is required for survival and maintenance of CNS neurons. *J Neurosci.* 2002;22(22):9800.
57. Russell KL, Downie JM, Gibson SB, Tsetsou S, Keefe MD, Duran JA, et al. Pathogenic effect of TP73 gene variants in people with amyotrophic lateral sclerosis. *Neurology.* 2021;97(3):225–35.
58. Wood H. TP73 variants implicate apoptosis in amyotrophic lateral sclerosis pathogenesis. *Nat Rev Neurol.* 2021;17(8):462.
59. Chia R, Chiò A, Traynor BJ. Novel genes associated with amyotrophic lateral sclerosis: diagnostic and clinical implications. *Lancet Neurol.* 2018;17(1):94–102.
60. Watanabe Y, Nakagawa T, Akiyama T, Nakagawa M, Suzuki N, Warita H, et al. An amyotrophic lateral sclerosis-associated mutant of C21ORF2 is stabilized by NEK1-mediated hyperphosphorylation and the inability to bind FBXO3. *iScience.*

2020;23(9):101491.

61. Association A. Genetics. 2021 [cited 2021 Nov 14]. Available from: <https://www.als.org/research/research-we-fund/scientific-focus-areas/genetics>
62. Kang SH, Li Y, Fukaya M, Lorenzini I, Cleveland DW, Ostrow LW, et al. Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. *Nat Neurosci*. 2013;16(5):571–9.
63. Kang SH, Fukaya M, Yang JK, Rothstein JD, Bergles DE. NG2+ CNS glial progenitors remain committed to the oligodendrocyte lineage in postnatal life and following neurodegeneration. *Neuron*. 2010;68(4):668–81.
64. Halestrap AP. The monocarboxylate transporter family-structure and functional characterization. *IUBMB Life*. 2012;64(1):1–9.
65. Poole R, Halestrap A. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol*. 1993;264(4):761–82.
66. Friesema EC, Ganguly S, Abdalla A, Manning Fox JE, Halestrap AP VT. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. *J Biol Chem*. 2003;278(41):40128–35.
67. Halestrap AP, Wilson MC. The monocarboxylate transporter family-role and regulation. *IUBMB Life*. 2012;64(2):109–19.
68. Garcia C, Goldstein J, RK P, Anderson R, Brown M. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. *Cell*. 1994;76(5):865–73.
69. Haven N. Expression of monocarboxylate transporter mRNAs in mouse brain: support for a distinct role of lactate as an energy substrate for the neonatal vs. adult brain. *Proc Natl Acad Sci*. 1998;95(7):3990–5.
70. Bergersen LH. Lactate transport and signaling in the brain: potential therapeutic targets and roles in body-brain interaction. *J Cereb Blood Flow Metab*. 2015;35(2):176–85.
71. de Castro Abrantes H, Briquet M, Schmuziger C, Restivo L, Puyal J, Rosenberg N,

- et al. The lactate receptor HCAR1 modulates neuronal network activity through the activation of G α and G $\beta\gamma$ subunits. *J Neurosci*. 2019;39(23):4422–33.
72. Lauritzen KH, Morland C, Puchades M, Holm-Hansen S, Hagelin EM, Lauritzen F, et al. Lactate receptor sites link neurotransmission, neurovascular coupling, and brain energy metabolism. *Cereb Cortex*. 2014;24(10):2784–95.
 73. Suzuki A, Stern SA, Bozdagi O, Huntley GW, Walker RH, Magistretti PJ, et al. Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell*. 2011;144(5):810–23.
 74. Schurr A, Payne RS, Miller JJ, Rigor BM. Brain lactate, not glucose, fuels the recovery of synaptic function from hypoxia upon reoxygenation: an in vitro study. *Brain Res*. 1997;744(1):105–11.
 75. Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A*. 1994;91(22):10625–9.
 76. Martinez BA. Lactate-starved neurons in ALS. *Dis Model Mech*. 2012;5(6):711–2.
 77. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J*. 1999;343(2):281–99.
 78. Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. Energy on demand. *Science*. 1999;283(5401):496–7.
 79. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends Neurosci*. 2013;36(10):587–97.
 80. Genc S, Kurnaz I, Ozilgen M. Astrocyte - neuron lactate shuttle may boost more ATP supply to the neuron under hypoxic conditions - in silico study supported by in vitro expression data. *BMC Syst Biol*. 2011;5(1):1–13.
 81. Halestrap A. The SLC16 gene family - structure, role and regulation in health and disease. *Mol Aspects Med*. 2013;34(2–3):337–49.
 82. Vijay N, Morris M. Role of monocarboxylate transporters in drug delivery to the

- brain. *Curr Pharm Des.* 2014;20(10):1487–98.
83. Halestrap AP, Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch Eur J Physiol.* 2004;447(5):619–28.
 84. Ovens MJ, Davies AJ, Wilson MC, Murray CM, Halestrap AP. AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10. *Biochem J.* 2010;425(3):523–30.
 85. Philp NJ, Yoon H G. Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE. *Am J Physiol.* 1998;274(6):1824–8.
 86. Wilson MC, Meredith D, Bunnun C, Sessions RB, Halestrap AP. Studies on the DIDS-binding site of monocarboxylate transporter 1 suggest a homology model of the open conformation and a plausible translocation cycle. *J Biol Chem.* 2009;284(30):20011–21.
 87. Wilson MC, Meredith D, Manning Fox JE, Manoharan C, Davies AJ, Halestrap AP. Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is embigin (gp70). *J Biol Chem.* 2005;280(29):27213–21.
 88. Fisel P, Schaeffeler E, Schwab M. Clinical and functional relevance of the monocarboxylate transporter family in disease pathophysiology and drug therapy. *Clin Transl Sci.* 2018;11(4):352–64.
 89. Dalsgaard MK. Fuelling cerebral activity in exercising man. *J Cereb Blood Flow Metab.* 2006;26(6):731–50.
 90. Bergersen L, Waerhaug O, Helm J, Thomas M, Laake P, Davies AJ, et al. A novel postsynaptic density protein: the monocarboxylate transporter MCT2 is co-localized with delta-glutamate receptors in postsynaptic densities of parallel fiber-Purkinje cell synapses. *Exp Brain Res.* 2001;136(4):523–34.
 91. Bradl M, Lassmann H. Oligodendrocytes: biology and pathology. *Acta Neuropathol.*

- 2010;119(1):37–53.
92. Sánchez-Abarca LI, Tabernero A MJ. Oligodendrocytes use lactate as a source of energy and as a precursor of lipids. *Glia*. 2001;36(3):321-329.
 93. Rinholm J, Hamilton N, Kessaris N, Richardson W, Bergersen L, Attwell D. Regulation of oligodendrocyte development and myelination by glucose and lactate. *Neurosci*. 2011;31(2):538-548.
 94. Nehlig A, Pereira de Vasconcelos A. Glucose and ketone body utilization by the brain of neonatal rats. *Prog Neurobiol*. 1993;40(2):163-221.
 95. Ouardouz M, Coderre E, Basak A, Chen A, Zamponi GW, Hameed S, et al. Glutamate receptors on myelinated spinal cord axons: I. GluR6 kainate receptors. *Ann Neurol*. 2009;65(2):151–9.
 96. Rinholm JE, Bergersen LH. Neuroscience: the wrap that feeds neurons. *Nature*. 2012;487(7408):435–6.
 97. Nave KA. Myelination and the trophic support of long axons. *Nat Rev Neurosci*. 2010;11(4):275–83.
 98. Kirk P, Wilson M, Heddle C, Brown M, Barclay A, Halestrap A. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J*. 2000;19(15):3896–904.
 99. Lasienė J, Yamanaka K. Glial cells in amyotrophic lateral sclerosis. *Neurol Res Int*. 2011;2011.
 100. Baltan S. Can lactate serve as an energy substrate for axons in good times and in bad, in sickness and in health?. *Metab Brain Dis*. 2014;30(1):25–30.
 101. Pierre K, Chatton J, Parent A, Repond C, Gardoni F, Di Luca M, et al. Linking supply to demand: the neuronal monocarboxylate transporter MCT2 and the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid receptor GluR2/3 subunit are associated in a common trafficking process. *Eur J Neurosci*. 2009;29(10):1951–63.
 102. Bergersen LH, Magistretti PJ PL. Selective postsynaptic co-localization of MCT2

- with AMPA receptor GluR2/3 subunits at excitatory synapses exhibiting AMPA receptor trafficking. *Cereb Cortex*. 2005;15:361–370.
103. Dienel G. Fueling and imaging brain activation. *ASN Neuro*. 2012;4(5):267–321.
 104. Brown AM, Tekkök SB, Ransom BR. Glycogen regulation and functional role in mouse white matter. *J Physiol*. 2003;549(2):501–12.
 105. Fünfschilling U, Supplie LM, Mahad D, Boretius S, Saab AS, Edgar J, et al. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature*. 2012;485(7399):517–21.
 106. Vernadakis A. Glia-neuron intercommunications and synaptic plasticity. *Prog Neurobiol*. 1996;49(3):185–214.
 107. Vallejo R, Tilley DM, Vogel L, Benyamin R. The role of glia and the immune system in the development and maintenance of neuropathic pain. *Pain Pract*. 2010;10(3):167–84.
 108. Mangia S, Simpson IA, Vannucci SJ, Carruthers A. The in vivo neuron-to-astrocyte lactate shuttle in human brain: evidence from modeling of measured lactate levels during visual stimulation. *J Neurochem*. 2009;109:55–62.
 109. Sofroniew M, Vinters H. Astrocytes: biology and pathology. *Acta Neuropathol*. 2010;119(1):7–35.
 110. Siracusa R, Fusco R, Cuzzocrea S. Astrocytes: role and functions in brain pathologies. *Front Pharmacol*. 2019;10:1114.
 111. Peters A, Palay SL WH. The fine structure of the nervous system 3rd ed. Oxford; 1991.
 112. Bröer S, Schneider HP, Bröer A, Rahman B, Hamprecht B, Deitmer JW. Characterization of the monocarboxylate transporter 1 expressed in *Xenopus laevis* oocytes by changes in cytosolic pH. *Biochem J*. 1998;333(1):167–74.
 113. Rafiki A, Boulland J, Halestrap A, Ottersen O, Bergersen L. Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. *Neuroscience*. 2003;122(3):677–88.

114. Aghajanian GK, Bloom FE. The formation of synaptic junctions in developing rat brain: a quantitative electron microscopic study. *Brain Res.* 1967;6(4):716–27.
115. World Health Organization, Agency IAE, Nations & F and AO of the U. Trace elements in human nutrition and health World Health Organization. World Health Organization. Geneva, Switzerland; 1996.
116. Das BC, Thapa P, Karki R, Schinke C, Das S, Kambhampati S, et al. Boron chemicals in diagnosis and therapeutics. *Future Med Chem.* 2013;5(6):653.
117. Nielsen FH. Boron in human and animal nutrition. *Plant Soil.* 1997;193(1):199–208.
118. Penland J. The importance of boron nutrition for brain and psychological function. *Biol Trace Elem Res.* 1998;66(1–3):299–317.
119. Sutherland B, Strong P, King J. Determining human dietary requirements for boron. *Biol Trace Elem Res.* 1998;66(1–3):193–204.
120. Altinoz MA, Topcu G, Elmaci İ. Boron's neurophysiological effects and tumoricidal activity on glioblastoma cells with implications for clinical treatment. *Int J Neurosci.* 2019;129(10):963–77.
121. Devirian T, Volpe S. The physiological effects of dietary boron. *Crit Rev Food Sci Nutr.* 2003;43(2):219–31.
122. Brittingham A, Wilson WA. The antimicrobial effect of boric acid on *Trichomonas vaginalis*. *Sex Transm Dis.* 2014;41(12):718–22.
123. Trippier P, McGuigan C. Boronic acids in medicinal chemistry: anticancer, antibacterial and antiviral applications. *Medchemcomm.* 2010;1(3):183–98.
124. Demirci S, Doğan A, Karakuş E, Halıcı Z, Topçu A, Demirci E, et al. Boron and poloxamer (F68 and F127) containing hydrogel formulation for burn wound healing. *Biol Trace Elem Res.* 2015;168(1):169–80.
125. Barranco WT, Kim DH, Stella SL, Eckhert CD. Boric acid inhibits stored Ca^{2+} release in DU-145 prostate cancer cells. *Cell Biol Toxicol.* 2009;25(4):309–20.
126. Das BC, Thapa P, Karki R, Schinke C, Das S, Kambhampati S, et al. Boron

- chemicals in diagnosis and therapeutics. *Future Med Chem.* 2013;5(6):653–76.
127. Fernandes G, Denny W, Dos Santos J. Boron in drug design: recent advances in the development of new therapeutic agents. *Eur J Med Chem.* 2019;179:791–804.
 128. Nielsen FH. Update on human health effects of boron. *J Trace Elem Med Biol.* 2014;28:383–7.
 129. Sheng MH, Janette Taper L, Veit H, Qian H, Ritchey SJ, William Lau KH. Dietary boron supplementation enhanced the action of estrogen, but not that of parathyroid hormone, to improve trabecular bone quality in ovariectomized rats. *Biol Trace Elem Res.* 2001;82(1):109–23.
 130. Aysan E, Sahin F, Telci D, Yalvac M, Emre S, Karaca C, et al. Body weight reducing effect of oral boric acid intake. *Int J Med Sci.* 2011;8(8):653–8.
 131. Kucukkurt I, Akbel E, Karabag F, Ince S. The effects of dietary boron compounds in supplemented diet on hormonal activity and some biochemical parameters in rats. *Toxicol Ind Health.* 2015;31(3):255–60.
 132. Demirci S, Kaya MS, Doğan A, Kalay Ş, Altin NÖ, Yarat A, et al. Antibacterial and cytotoxic properties of boron-containing dental composite. *Turkish J Biol.* 2015;39(3):417–26.
 133. Türkez H, Geyikoğlu F, Tatar A, Keleş S, Özkan A. Effects of some boron compounds on peripheral human blood. *Zeitschrift fur Naturforsch - Sect C J Biosci.* 2007;62(11–12):889–96.
 134. Lawler A. Brookhaven prepares for boron trials. *Science.* 1995;267(5200):956.
 135. Ferreira T, Marino A, Rocca A, Liakos I, Nitti S, Athanassiou A, et al. Folate-grafted boron nitride nanotubes: possible exploitation in cancer therapy. *Int J Pharm.* 2015;481(1–2):56–63.
 136. Gonzalez A, Peters U, Lampe JW, White E. Boron intake and prostate cancer risk. *Cancer Causes Control.* 2007;18(10):1131–40.
 137. Yinghuai Z, Hosmane N. Applications and perspectives of boron-enriched nanocomposites in cancer therapy. *Future Med Chem.* 2013;5(6):705–14.

138. Portu A, Molinari A, Thorp S, Pozzi E, Curotto P, Schwint A, et al. Neutron autoradiography to study boron compound microdistribution in an oral cancer model. *Int J Radiat Biol*. 2015;91(4):329–35.
139. Scorei R, Ciubar R, Ciofrangeanu CM, Mitran V, Cimpean A, Iordachescu D. Comparative effects of boric acid and calcium fructoborate on breast cancer cells. *Biol Trace Elem Res* 2007 1223. 2008;122(3):197–205.
140. Tülüce Y, Lak P, Koyuncu İ, Kılıç A, Durgun M, Özkol H. The apoptotic, cytotoxic and genotoxic effect of novel binuclear boron-fluoride complex on endometrial cancer. *Biometals*. 2017;30(6):933–44.
141. Dick L, Fleming P. Building on bortezomib: second-generation proteasome inhibitors as anti-cancer therapy. *Drug Discov Today*. 2010;15(5–6):243–9.
142. Schabath M, Wu X, Vassilopoulou-Sellin R, Vaporciyan A, Spitz M. Hormone replacement therapy and lung cancer risk: a case-control analysis. *Clin Cancer Res*. 2004;10(1):113–23.
143. Wang Y, Zhao Y, Chen X. Experimental study on the estrogen-like effect of boric acid. *Biol Trace Elem Res*. 2007;121(2):160–70.
144. Mahabir S, Spitz M, Barrera S, Dong Y, Eastham C, Forman M. Dietary boron and hormone replacement therapy as risk factors for lung cancer in women. *Am J Epidemiol*. 2008;167(9):1070–80.
145. Scorei R, Popa R. Boron-containing compounds as preventive and chemotherapeutic agents for cancer. *Anticancer Agents Med Chem*. 2010;10(4):346–51.
146. Doğan A, Demirci S, Çağlayan AB, Kılıç E, Günel MY, Uslu Ü, et al. Sodium pentaborate pentahydrate and pluronic containing hydrogel increases cutaneous wound healing in vitro and in vivo. *Biol Trace Elem Res*. 2014;162(1–3):72–9.
147. Şen Ö, Emanet M, Çulha M. Stimulatory effect of hexagonal boron nitrides in wound healing. *ACS Appl Bio Mater*. 2019;2(12):5582–96.
148. Penland JG. Dietary boron, brain function, and cognitive performance. *Environ Health Perspect*. 1994;102(suppl 7):65–72.

149. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. *Alexandria J Med.* 2019;54(4):287–93.
150. DeKosky S, Taffe K, Abrahamson E, Dixon C, Kochanek P, Ikonovic M. Time course analysis of hippocampal nerve growth factor and antioxidant enzyme activity following lateral controlled cortical impact brain injury in the rat. *J Neurotrauma.* 2004;21(5):491–500.
151. Huber KR, Sridhar R, Griffith EH, Amma EL, Roberts J. Superoxide dismutase-like activities of copper(II) complexes tested in serum. *Biochim Biophys Acta (BBA)/Protein Struct Mol.* 1987;915(2):267–76.
152. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev.* 2014;2014.
153. Ho E, Karimi Galougahi K, Liu C, Bhindi R, Figtree G. Biological markers of oxidative stress: applications to cardiovascular research and practice. *Redox Biol.* 2013;1(1):483–91.
154. Mao C, Yuan J-Q, Lv Y-B, Gao X, Yin Z-X, Kraus VB, et al. Associations between superoxide dismutase, malondialdehyde and all-cause mortality in older adults: a community-based cohort study. *BMC Geriatr.* 2019;19(1):1–9.
155. Sarica Z, Eren M, Şentürk M. Effect of boron on the potassium dichromate induced oxidative damage in brain tissue of sprague dawley rats. *Pak J Zool.* 2019;51(5):1905–10.
156. Chen G, Shi J, Hu Z, Hang C. Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: a potential neuroprotective mechanism of N-acetylcysteine. *Mediators Inflamm.* 2008;2008.
157. Dohi K, Satoh K, Nakamachi T, Yofu S, Hiratsuka K, S, Nakamura S, et al. Does edaravone (MCI- 186) act as an antioxidant and a neuroprotector in experimental traumatic brain injury?. *Antioxid Redox Signal.* 2007;9(2):281–7.
158. PW, Huh P, Belayev L, Zhao W, Clemens J, Panetta J, R, Busto R, et al.

- Neuroprotection by LY341122, a novel inhibitor of lipid peroxidation, against focal ischemic brain damage in rats. *Eur J Pharmacol.* 2000;389(1):79–88.
159. Wilson J, Gelb A. Free radicals, antioxidants, and neurologic injury: possible relationship to cerebral protection by anesthetics. *J Neurosurg Anesthesiol.* 2002;14(1):66–79.
 160. Ataizi ZS, Ozkoc M, Kanbak G, Karimkhani H, Donmez DB, Ustunisik N, et al. Evaluation of the neuroprotective role of boric acid in preventing traumatic brain injury-mediated oxidative stress. *Turk Neurosurg.* 2021;31(4):493–9.
 161. Colak S, Geyikoglu F, Keles O, Türkez H, Topal A, Unal B. The neuroprotective role of boric acid on aluminum chloride-induced neurotoxicity. *Toxicol Ind Health.* 2011;27(8):700–10.
 162. Hacıoğlu C, Kar F, Şentürk H, Kanbak G. Neuroprotective effects of boric acid against fluoride toxicity on rat synaptosomes. *Med Sci Discov.* 2018;5(7):260–6.
 163. Yokel R. The toxicology of aluminum in the brain: a review. *Neurotoxicology.* 2000;21(5):813–28.
 164. Rondeau V, Jacqmin-Gadda H, Commenges D, Helmer C, Dartigues J. Aluminum and silica in drinking water and the risk of Alzheimer's disease or cognitive decline: findings from 15-year follow-up of the PAQUID cohort. *Am J Epidemiol.* 2009;169(4):489–96.
 165. Miu A, Olteanu A, Miclea M. A behavioral and ultrastructural dissection of the interference of aluminum with aging. *J Alzheimers Dis.* 2004;6(3):315–28.
 166. Küçük M, Kalayci R, Cevik A, Elmas I, Kaya M. Effect of aluminum on the blood-brain barrier permeability in acute and chronically hyperglycemic rats. *Biol Trace Elem Res.* 2001;80(2):181–9.
 167. Howe PD. A review of boron effects in the environment. *Biol Trace Elem Res.* 2013;66(1):153–66.
 168. Ozansoy M, Altıntaş M, Ozansoy M, Günay N, Kiliç E, Kiliç Ü. Two boron-containing compounds affect the cellular viability of SH-SY5Y cells in an in vitro

- amyloid-beta toxicity model. *Turkish J Biol.* 2020;44(4):208–14.
169. Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, et al. Amyloid- β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med.* 2008;14(8):837–42.
 170. Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J. Amyloid-beta: a crucial factor in Alzheimer's disease. *Med Princ Pract.* 2015;24(1):1–10.
 171. Selkoe D, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med.* 2016;8(6):595–608.
 172. BrightFocus Foundation. Alzheimer's Disease Progression in the Brain. [cited 2021 Oct 19]. Available from: <https://www.brightfocus.org/alzheimers-disease/infographic/progression-alzheimers-disease>
 173. Silbert L. Does statin use decrease the amount of Alzheimer disease pathology in the brain?. *Neurology.* 2007;69(9):8–11.
 174. Rahman S, Islam R. Mammalian Sirt1: insights on its biological functions. *Cell Commun Signal.* 2011;9(1):1–8.
 175. Lu C, Hu J, Wang Z, Xie S, Pan T, Huang L, et al. Discovery of boron-containing compounds as A β aggregation inhibitors and antioxidants for the treatment of Alzheimer's disease. *Medchemcomm.* 2018;9(11):1862–70.
 176. Jana MK, Cappai R, Pham CLL, Ciccotosto GD. Membrane-bound tetramer and trimer A β oligomeric species correlate with toxicity towards cultured neurons. *J Neurochem.* 2016;136(3):594–608.
 177. Kreutzer AG, Yoo S, Spencer RK, Nowick JS. Stabilization, Assembly, and Toxicity of Trimers Derived from A β . *J Am Chem Soc.* 2017;139(2):966–75.
 178. Bitan G, Kirkitadze MD, Lomakin A, Vollers SS, Benedek GB, Teplow DB. Amyloid β -protein (A β) assembly: A β 40 and A β 42 oligomerize through distinct pathways. *Proc Natl Acad Sci.* 2003;100(1):330.
 179. Sorout N, Chandra A. Effects of boron nitride nanotube on the secondary structure

- of A β (1–42) trimer: possible inhibitory effect on amyloid formation. *J Phys Chem B*. 2020;124(10):1928–40.
180. Merlo A, Mokkapati VRSS, Pandit S, Mijakovic I. Boron nitride nanomaterials: biocompatibility and bio-applications. *Biomater Sci*. 2018;6(9):2298–311.
 181. Ciofani G, Raffa V, Mencias A, Cuschieri A. Boron nitride nanotubes: an innovative tool for nanomedicine. *Nano Today*. 2009;4(1):8–10.
 182. Ciofani G, Danti S, D'Alessandro D, Ricotti L, Moscato S, Bertoni G, et al. Enhancement of neurite outgrowth in neuronal-like cells following boron nitride nanotube-mediated stimulation. *ACS Nano*. 2010;4(10):6267–77.
 183. Ciofani G, Danti S, Nitti S, Mazzolai B, Mattoli V, Giorgi M. Biocompatibility of boron nitride nanotubes: an up-date of in vivo toxicological investigation. *Int J Pharm*. 2013;444(1–2):85–8.
 184. Ciofani G, Ricotti L, Danti S, Moscato S, Nesti C, D'Alessandro D, et al. Investigation of interactions between poly-L-lysine-coated boron nitride nanotubes and C2C12 cells: up-take, cytocompatibility, and differentiation. *Int J Nanomedicine*. 2010;5(1):285–98.
 185. Ciofani G, Raffa V, Yu J, Chen Y, Obata Y, Takeoka S, et al. Boron nitride nanotubes: a novel vector for targeted magnetic drug delivery. *Curr Nanosci*. 2009;5(1):33–8.
 186. Li C, Adamcik J, Mezzenga R. Biodegradable nanocomposites of amyloid fibrils and graphene with shape-memory and enzyme-sensing properties. *Nat Nanotechnol*. 2012;7(7):421–7.
 187. Yang Z, Ge C, Liu J, Chong Y, Gu Z, Jimenez-Cruz CA, et al. Destruction of amyloid fibrils by graphene through penetration and extraction of peptides. *Nanoscale*. 2015;7(44):18725–37.
 188. Mahmoudi M, Akhavan O, Ghavami M, Rezaee F, Ghiasi SMA. Graphene oxide strongly inhibits amyloid beta fibrillation. *Nanoscale*. 2012;4(23):7322–5.
 189. Song M, Zhu Y, Wei G, Li H. Carbon nanotube prevents the secondary structure

- formation of amyloid- β trimers: an all-atom molecular dynamics study. *Mol Simul.* 2017;43(13–16):1189–95.
190. Sun Y, Qian Z, Wei G. The inhibitory mechanism of a fullerene derivative against amyloid- β peptide aggregation: an atomistic simulation study. *Phys Chem Chem Phys.* 2016;18(18):12582–91.
 191. Ciofani G, Del Turco S, Genchi GG, D'Alessandro D, Basta G, Mattoli V. Transferrin-conjugated boron nitride nanotubes: protein grafting, characterization, and interaction with human endothelial cells. *Int J Pharm.* 2012;436(1–2):444–53.
 192. Kalivendi S V, Kotamraju S, Cunningham S, Shang T, Hillard CJ, Kalyanaraman B. 1-Methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis and mitochondrial oxidant generation: role of transferrin-receptor-dependent iron and hydrogen peroxide. *Biochem J.* 2003;371(1):151–64.
 193. Huang Y, Chen J, Chen Y, Zhuang Y, Sun M, Behnisch T. The neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) alters hippocampal excitatory synaptic transmission by modulation of the GABAergic system. *Front Cell Neurosci.* 2015;9:299.
 194. Küçükdoğru R, Türkez H, Arslan M, Tozlu Ö, Sönmez E, Mardinoğlu A, et al. Neuroprotective effects of boron nitride nanoparticles in the experimental Parkinson's disease model against MPP⁺ induced apoptosis. *Metab Brain Dis.* 2020;35(6):947–57.
 195. Hayashi Y, Homma K, Ichijo H. SOD1 in neurotoxicity and its controversial roles in SOD1 mutation-negative ALS. *Adv Biol Regul.* 2016;60:95–104.
 196. Polański R, Hodgkinson CL, Fusi A, Nonaka D, Priest L, Kelly P, et al. Activity of the monocarboxylate transporter 1 inhibitor AZD3965 in small cell lung cancer. *Clin Cancer Res.* 2014;20(4):926–37.
 197. Cassina P, Cassina A, Pehar M, Castellanos R, Gandelman M, León A de, et al. Mitochondrial dysfunction in SOD1G93A-bearing astrocytes promotes motor neuron degeneration: prevention by mitochondrial-targeted antioxidants. *J Neurosci.* 2008;28(16):4115.

198. Chen Y, Qin C, Huang J, Tang X, Liu C, Huang K, et al. The role of astrocytes in oxidative stress of central nervous system: a mixed blessing. *Cell Prolif.* 2020;53(3):e12781.
199. Subramanian V, Crabtree B, Acharya KR. Human angiogenin is a neuroprotective factor and amyotrophic lateral sclerosis associated angiogenin variants affect neurite extension/ pathfinding and survival of motor neurons. *Hum Mol Genet.* 2008;17(1):130–49.
200. Wu D, Yu W, Kishikawa H, Folkerth RD, Iafrate AJ, Shen Y, et al. Angiogenin loss-of-function mutations in amyotrophic lateral sclerosis. *Ann Neurol.* 2007;62(6):609–17.
201. Miyake M, Goodison S, Lawton A, Gomes-Giacoa E, Rosser CJ. Angiogenin promotes tumoral growth and angiogenesis by regulating matrix metalloproteinase-2 expression via the ERK1/2 pathway. *Oncogene.* 2014;34(7):890–901.
202. Ainley AD, Challenger F. CCLXXX.—Studies of the boron-carbon linkage. Part I. The oxidation and nitration of phenyl-boric acid. *J Chem Soc.* 1930;2171–80.
203. Tandarić T, Hok L, Vianello R. From hydrogen peroxide-responsive boronated nucleosides towards antisense therapeutics-a computational mechanistic study. *Croat Chem Acta.* 2019;92(2):287–95.
204. Hoang TT, Smith TP, Raines RT. A boronic acid conjugate of angiogenin that shows ROS-responsive neuroprotective activity. *Angew Chemie Int Ed.* 2017;56(10):2619–22.
205. Thareja S, Zhu M, Ji X, Wang B. Boron-based small molecules in disease detection and treatment (2013–2016). *Heterocycl Commun.* 2017;23(3):137–53.
206. Hatzipetros T, Kidd JD, Moreno AJ, Thompson K, Gill A, Vieira FG. A quick phenotypic neurological scoring system for evaluating disease progression in the SOD1-G93A mouse model of ALS. *J Vis Exp.* 2015;104:e53257.
207. Laboratory J. C57BL/6J-Tg(C9orf72_i3)112Lutzy/J. 2021 [cited 2021 Jun 22]. Available from: <https://www.jax.org/strain/023099>

208. Koppers M, Blokhuis AM, Westeneng H-J, Terpstra ML, Zundel CAC, Vieira De S R, et al. C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits. *Ann Neurol*. 2015;78(3):426–38.
209. Holt LM, Olsen ML. Novel applications of magnetic cell sorting to analyze cell-type specific gene and protein expression in the central nervous system. *PLoS One*. 2016;11(2):e0150290.
210. Harris VM. Protein detection by simple westernTM analysis. *West Blotting*. 2015;465–8.
211. Fink LH, Anastasio NC, Fox RG, Rice KC, Gerard Moeller F, Cunningham KA. Individual differences in impulsive action reflect variation in the cortical serotonin 5-HT 2A receptor system. *Neuropsychopharmacology*. 2015;40(8):1957–68.
212. Gyawali A, Kang YS. Transport alteration of 4-phenyl butyric acid mediated by a sodium- and proton-coupled monocarboxylic acid transporter system in ALS model cell lines (NSC-34) under inflammatory states. *J Pharm Sci*. 2021;110(3):1374–84.
213. Gill AL, Wang MZ, Levine B, Premasiri A, Vieira FG. Primary neurons and differentiated NSC-34 cells are more susceptible to arginine-rich ALS dipeptide repeat protein-associated toxicity than non-differentiated NSC-34 and CHO cells. *Int J Mol Sci*. 2019;20(24):6238.
214. Hounoum BM, Vourc'h P, Felix R, Corcia P, Patin F, Guéguinou M, et al. NSC-34 motor neuron-like cells are unsuitable as experimental model for glutamate-mediated excitotoxicity. *Front Cell Neurosci*. 2016;10(118).
215. Huang C, Sheng S, Li R, Sun X, Liu J, Huang G. Lactate promotes resistance to glucose starvation via upregulation of Bcl-2 mediated by mTOR activation. *Oncol Rep*. 2015;33(2):875–84.
216. Sigma Aldrich. Monocarboxylate Transport Inhibitor, AR-C155858 - CAS 496791-37-8 - Calbiochem. [cited 2021 Jul 13]. Available from: <https://www.sigmaaldrich.com/TR/en/product/mm/533436>
217. Guan X, Rodriguez-Cruz V, Morris ME. Cellular uptake of MCT1 inhibitors AR-C155858 and AZD3965 and their effects on MCT-mediated transport of L-lactate in

- murine 4T1 breast tumor cancer cells. *AAPS J.* 2019;21(2):1–10.
218. Oliván S, Calvo AC, Rando A, Muñoz MJ, Zaragoza P, Osta R. Comparative study of behavioural tests in the SOD1G93A mouse model of amyotrophic lateral sclerosis. *Exp Anim.* 2014;64(2):147–53.
 219. Blacher E, Bashiardes S, Shapiro H, Rothschild D, Mor U, Dori-Bachash M, et al. Potential roles of gut microbiome and metabolites in modulating ALS in mice. *Nature.* 2019;572(7770):474–80.
 220. Bektas S, Öztürk G. Enhancement of cultured adult motor neuron survival with cold pre-incubation. *Neurosci Lett.* 2013;533:23–7.
 221. Scitable by Nature Education. Microarray. [cited 2021 Aug 3]. Available from: <https://www.nature.com/scitable/definition/microarray-202/>
 222. Govindarajan R, Duraiyan J, Kaliyappan K, Palanisamy M. Microarray and its applications. *J Pharm Bioallied Sci.* 2012;4(Suppl 2):S310.
 223. Agilent Technologies. One-color microarray-based gene low input quick amp labeling protocol. Version 6. Santa Clara, CA: Agilent Technologies, Inc.; 2015. 96 p.
 224. Mi H, Thomas P. PANTHER pathway: an ontology-based pathway database coupled with data analysis tools. *Protein Networks Pathw Anal.* 2009;123–40.
 225. Gurney M, Pu H, Chiu A. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science.* 1994;264(5166):1772–5.
 226. Wvu. Institutional Animal Care and Use Committee WVU IACUC Policy and Guidelines: Breeding and Weaning of Mice and Rats.
 227. The Jackson Laboratory. Colony Planning. [cited 2021 Sep 1]. Available from: <https://www.jax.org/jax-mice-and-services/customer-support/technical-support/breeding-and-husbandry-support/colony-planning#>
 228. Gurney ME. The use of transgenic mouse models of amyotrophic lateral sclerosis in preclinical drug studies. *J Neurol Sci.* 1997;152(Suppl. 1):s67–73.

229. Scott S, Kranz J, Cole J, Lincecum J, Thompson K, Kelly N, et al. Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph lateral Scler.* 2008;9(1):4–15.
230. Antonicka H, Leary SC, Guercin GH, Agar JN, Horvath R, Kennaway NG, et al. Mutations in COX10 result in a defect in mitochondrial heme A biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency. *Hum Mol Genet.* 2003;12(20):2693–702.
231. Cedarlane. Mouse Motor Neuron-Like Hybrid Cell Line (NSC-34). [cited 2021 Sep 3]. Available from: <https://www.cedarlanelabs.com/products/detail/clu140>
232. Okada M, Yamashita S, Ueyama H, Ishizaki M, Maeda Y, Ando Y. Long-term effects of edaravone on survival of patients with amyotrophic lateral sclerosis. *ENeurologicalSci.* 2018;11:11–4.
233. Martier R, Liefhebber JM, García-Osta A, Miniarikova J, Cuadrado-Tejedor M, Espelosin M, et al. Targeting RNA-mediated toxicity in C9orf72 ALS and/or FTD by RNAi-based gene therapy. *Mol Ther Nucleic Acids.* 2019;16:26–37.
234. Liu Y, Pattamatta A, Zu T, Reid T, Bardhi O, Borchelt DR, et al. C9orf72 BAC mouse model with motor deficits and neurodegenerative features of ALS/FTD. *Neuron.* 2016;90(3):521–34.
235. O'Rourke JG, Bogdanik L, Muhammad AKMG, Gendron TF, Kim KJ, Austin A, et al. C9orf72 BAC transgenic mice display typical pathologic features of ALS/FTD. *Neuron.* 2015;88(5):892–901.
236. Murray CM, Hutchinson R, Bantick JR, Belfield GP, Benjamin AD, Brazma D, et al. Monocarboxylate transporter MCT1 is a target for immunosuppression. *Nat Chem Biol.* 2005;1(7):371–6.
237. Pierre K, Pellerin L. Monocarboxylate transporters in the central nervous system: distribution, regulation and function. *J Neurochem.* 2005;94(1):1–14.
238. Kori M, Aydin B, Unal S, Arga KY, Kazan D. Metabolic biomarkers and neurodegeneration: a pathway enrichment analysis of Alzheimer's disease, Parkinson's Disease, and amyotrophic lateral sclerosis. *OMICS.* 2016;20(11):645–

- 61.
239. Agnihotri A, Aruoma OI. Alzheimer's Disease and Parkinson's Disease: a nutritional toxicology perspective of the impact of oxidative stress, mitochondrial dysfunction, nutrigenomics and environmental chemicals. *J Am Coll Nutr.* 2020;39(1):16–27.
240. Yu X, Zhang R, Wei C, Gao Y, Yu Y, Wang L, et al. MCT2 overexpression promotes recovery of cognitive function by increasing mitochondrial biogenesis in a rat model of stroke. *Animal Cells Syst (Seoul).* 2021;25(2):93–101.
241. Zhang M, Cheng X, Dang R, Zhang W, Zhang J, Yao Z. Lactate deficit in an Alzheimer Disease mouse model: the relationship with neuronal damage. *J Neuropathol Exp Neurol.* 2018;77(12):1163–76.
242. Muyderman H, Chen T. Mitochondrial dysfunction in amyotrophic lateral sclerosis – a valid pharmacological target?. *Br J Pharmacol.* 2014;171(8):2191.
243. Dupuis L, Gonzalez De Aguilar JL, Oudart H, De Tapia M, Barbeito L, Loeffler JP. Mitochondria in amyotrophic lateral sclerosis: a trigger and a target. *Neurodegener Dis.* 2004;1(6):245–54.
244. Smith EF, Shaw PJ, De Vos KJ. The role of mitochondria in amyotrophic lateral sclerosis. *Neurosci Lett.* 2019;710:132933.
245. NCBI. COX10 cytochrome c oxidase assembly factor heme A:farnesyltransferase COX10 [Homo sapiens (human)]. [cited 2021 Nov 9]. Available from: <https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=1352>
246. Hashimoto T, Hussien R, Cho HS, Kaufer D, Brooks GA. Evidence for the mitochondrial lactate oxidation complex in rat neurons: demonstration of an essential component of brain lactate shuttles. *PLoS One.* 2008;3(8):e2915.
247. Newington JT, Harris RA, Cumming RC. Reevaluating metabolism in Alzheimer's Disease from the perspective of the astrocyte-neuron lactate shuttle model. *J Neurodegener Dis.* 2013;2013:1–13.

248. Kirkinezos IG, Bacman SR, Hernandez D, Oca-Cossio J, Arias LJ, Perez-Pinzon MA, et al. Cytochrome c association with the inner mitochondrial membrane is impaired in the CNS of G93A-SOD1 mice. *J Neurosci*. 2005;25(1):164.
249. Son M, Leary SC, Romain N, Pierrel F, Winge DR, Haller RG, et al. Isolated cytochrome c oxidase deficiency in G93A SOD1 mice overexpressing CCS protein. *J Biol Chem*. 2008;283(18):12267.
250. Vhbio. NSC-34 Cell Line. [cited 2021 Nov 6]. Available from: <https://www.vhbio.com/product/nsc-34-cell-line/>
251. Cashman NR, Durham HD, Blusztajn JK, Oda K, Tabira T, Shaw IT, et al. Neuroblastoma × spinal cord (NSC) hybrid cell lines resemble developing motor neurons. *Dev Dyn*. 1992;194(3):209–21.
252. Matusica D, Fenech MP, Rogers ML, Rush RA. Characterization and use of the NSC-34 cell line for study of neurotrophin receptor trafficking. *J Neurosci Res*. 2008;86(3):553–65.
253. Malich G, Markovic B, Winder C. The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. *Toxicology*. 1997;124(3):179–92.
254. Latif A, Chadwick AL, Kitson SJ, Gregson HJ, Sivalingam VN, Bolton J, et al. Monocarboxylate Transporter 1 (MCT1) is an independent prognostic biomarker in endometrial cancer. *BMC Clin Pathol*. 2017;17(1):1–10.
255. Brittingham A, Wilson WA. The antimicrobial effect of boric acid on trichomonas vaginalis. *Sex Transm Dis*. 2014;41(12):718–22.
256. Weiger R, Kuhn A, Löst C. Effect of various types of sodium perborate on the pH of bleaching agents. *J Endod*. 1993;19(5):239–41.
257. McKillop A, Sanderson WR. Sodium perborate and sodium percarbonate: cheap, safe and versatile oxidising agents for organic synthesis. *Tetrahedron*. 1995;51(22):6145–66.
258. Dugua J, Simon B. Crystallization of sodium perborate from aqueous solutions: I.

- nucleation rates in pure solution and in presence of a surfactant. *J Cryst Growth*. 1978;44(3):265–79.
259. Herrero-Mendez A, Almeida A, Fernández E, Maestre C, Moncada S, Bolaños JP. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol*. 2009;11(6):747–52.
 260. Stobart JL, Anderson CM. Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. *Front Cell Neurosci*. 2013;0(MAR):38.
 261. Tefera TW, Bartlett K, Tran SS, Hodson MP, Borges K. Impaired pentose phosphate pathway in the spinal cord of the hSOD1 G93A mouse model of amyotrophic lateral sclerosis. *Mol Neurobiol*. 2019;56(8):5844–55.
 262. Saito ER, Miller JB, Harari O, Cruchaga C, Mihindukulasuriya KA, Kauwe JSK, et al. Alzheimer's disease alters oligodendrocytic glycolytic and ketolytic gene expression. *Alzheimer's Dement*. 2021;17(9):1474–86.
 263. Magri A, Belfiore R, Reina S, Tomasello MF, Di Rosa MC, Guarino F, et al. Hexokinase I N-terminal based peptide prevents the VDAC1-SOD1 G93A interaction and re-establishes ALS cell viability. *Sci Rep*. 2016;6(1):1–14.
 264. Medinas DB, Rozas P, Traub FM, Woehlbier U, Brown RH, Bosco DA, et al. Endoplasmic reticulum stress leads to accumulation of wild-type SOD1 aggregates associated with sporadic amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*. 2018;115(32):8209–14.
 265. Philips T, Robberecht W. Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *Lancet Neurol*. 2011;10(3):253–63.
 266. Liu J, Wang F. Role of neuroinflammation in amyotrophic lateral sclerosis: cellular mechanisms and therapeutic implications. *Front Immunol*. 2017;8:1005.
 267. Obrador E, Salvador R, Estrela JM, López-Blanch R, Jihad-Jebbar A, Vallés SL. Oxidative stress, neuroinflammation and mitochondria in the pathophysiology of amyotrophic lateral sclerosis. *Antioxidants (Basel, Switzerland)*. 2020;9(9):1–16.

268. Morello G, Spampinato AG, Cavallaro S. Neuroinflammation and ALS: transcriptomic insights into molecular disease mechanisms and therapeutic targets. *Mediators Inflamm.* 2017;2017.
269. Carbone M, Duty S, Rattray M. Riluzole elevates GLT-1 activity and levels in striatal astrocytes. *Neurochem Int.* 2012;60(1):31–8.
270. Philips T, Robberecht W. Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *Lancet Neurol.* 2011;10(3):253–63.
271. Gresch PJ, Sanders-Bush E. Serotonin receptor signaling. *Encycl Biol Chem.* 2004;33–7.
272. Hoyer D. 5-hydroxytryptamine receptors. *xPharm Compr Pharmacol Ref.* 2007;1–7.
273. Polter AM, Li X. 5-HT_{1A} receptor-regulated signal transduction pathways in brain. *Cell Signal.* 2010;22(10):1406.
274. Noto B, Klempin F, Alenina N, Bader M, Fink H, Sander SE. Increased adult neurogenesis in mice with a permanent overexpression of the postsynaptic 5-HT_{1A} receptor. *Neurosci Lett.* 2016;633:246–51.
275. Hamidian S, Pourshahbaz A, Bozorgmehr A, Ananloo S, Dolatshahi B, Ohadi M. How obsessive-compulsive and bipolar disorders meet each other? An integrative gene-based enrichment approach. *Ann Gen Psychiatry.* 2020;19(1):1–10.



T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU
(YÜDHEK)
ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
12.05.2017	600	28.04.2017	Prof. Dr. Fikrettin ŞAHİN

‘ALS fare modellerinde merkezi sinir sistemindeki MCT ekspresyonlarının hücre spesifik değişimleri ve ALS patojenizine etkileri’ adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.		
Etik Onay Geçerlilik Süresi: 3 Yıl	Hayvan Türü ve Cinsiyeti: Fare ♂/♀	Hayvan Sayısı: 30/4

GÖREVİ	ADI SOYADI	
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Vet. Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Soner DOĞAN	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Gamze TORUN KÖSE	
Üye	Yrd. Doç. Dr. Aylin YABA UÇAR	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	



T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2019-805	08.11.2019	2019/11	2019/11-5	Dr. Burcu Kasapoğlu Usta
‘Bor Bileşiklerinin Transgenik Amiyotrofik Lateral Skleroz (ALS) Fare Modelinde Hastalık Seyri, Şiddeti ve Mikrobiyota Üzerine Olan Etkilerinin Araştırılması’ isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				
Hayvan Türü/ırkı		Toplam Hayvan Sayısı		Hayvanın Cinsiyeti
Fare		64		Dişi

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	