

**Butyrylcholinesterase as a Potential Depression  
Biomarker: Insights from a Translational Study**

by

Berkan Bozkurt

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in

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# **Butyrylcholinesterase as a Potential Depression Biomarker: Insights from a Translational Study**

Koç University

Graduate School of Health Sciences

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**Berkan Bozkurt**

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and that any and all revisions required by the final  
examining committee have been made.

Committee Members:

---

Assoc. Prof. Dr. HALE YAPICI ESER (Advisor)

---

Assoc. Prof. Dr. SAFACAN KÖLEMEN (Co-Advisor)

---

Prof. Dr. YASEMİN GÜR SOY ÖZDEMİR

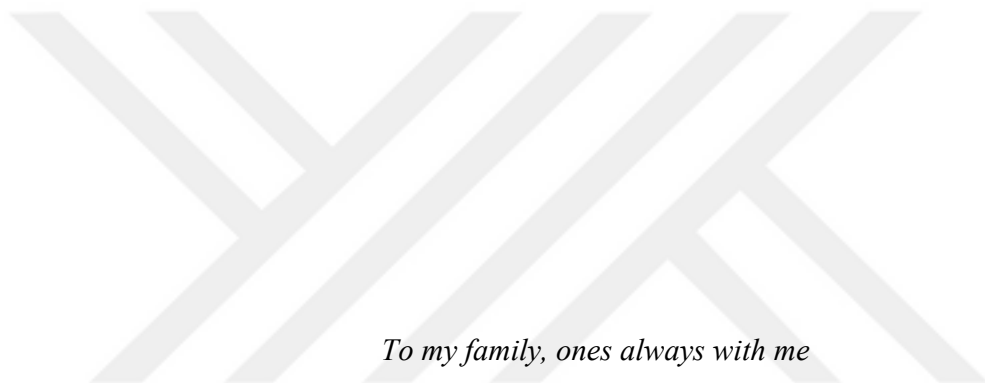
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Assoc. Prof. Dr. ATAY VURAL

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Assoc. Prof. Dr. NEŞE DİREK TECİRLİ

Date: \_\_\_\_\_



*To my family, ones always with me*

## ABSTRACT

### **Butyrylcholinesterase as a Potential Depression Biomarker: Insights from a Translational Study**

**Berkan Bozkurt**

**Master of Science in Neuroscience**

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Major depressive disorder, a prevalent psychiatric disorder, significantly impacts quality of life. Although several biological pathways and related biomarker candidates try to explain its complex pathomechanism and assess objectively its diagnosis and onset, biomarker research in depression is still in infancy. Recent studies showed that depression may be influenced by dysregulation of cholinergic transmission. Within these findings, while the role of acetylcholinesterase is commonly studied in depression pathomechanism, the role of butyrylcholinesterase (BChE) remains unclear. Recently, we developed a BChE-activatable chemiluminescent molecular sensor (BCC) for real-time visualization of BChE activity, demonstrating efficiency *in vitro* and in tumor-bearing mice. In this translational study, we employed BCC to investigate the association between BChE activity and depression. We first assessed BChE activity in patients with depression (32 unipolar depression, 20 bipolar disorders with depressive episodes) compared to healthy controls (n=48) and reassessed changes with respect to remission (n=15). Second, we evaluated BChE activity in rats exposed to chronic unpredictable mild stress (CUMS), CUMS with concurrent fluoxetine treatment, and controls. Lastly, we established cell models for psychological stress by mimicking the hypothalamic-pituitary-adrenal (HPA) axis outcomes with 200 $\mu$ M corticosterone for 24 hours and the sympatho-adrenomedullary (SAM) system outcomes with 1mM norepinephrine treatments for 24 hours and assessed their BChE activity compared to controls in PC-12 Adh cell. Results indicated that individuals with depression exhibited significantly lower BChE activity compared to controls ( $p<.001$ ), with increased activity correlating with remission ( $p<.001$ ). Rats exposed to CUMS showed a significant decrease in BChE levels ( $p=.011$ ), while fluoxetine treatment showed an increasing trend but could not achieve significance ( $p=.345$ ). Additionally, BChE activity significantly decreased with corticosterone exposure ( $p=.002$ ) and increased with norepinephrine exposure ( $p=.002$ ).

Subsequent, 5 $\mu$ M fluoxetine treatment cell exposed to those psychological stresses showed increased BChE activity ( $p=.002$  for both). This translational study demonstrates a decrease in BChE activity in both clinical populations and chronic stress animal models, and psychological stress model established cell models, with levels reversing upon symptom alleviation. Our findings ultimately suggest that BChE activity can be a promising biomarker for depression diagnosis and treatment outcome.



# ÖZETÇE

**Yüksek Lisans Tez Başlığı**

**Berkan Bozkurt**

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Yaygın bir psikiyatrik bozukluk olan majör depresif bozukluk yaşam kalitesini önemli ölçüde etkilemektedir. Çeşitli biyolojik yollar ve ilgili biyobelirteç adayları her ne kadar depresyonun karmaşık patomekanizmalarını açıklamaya çalışıp teşhisini ve tedavisini objektif olarak değerlendirmeye çalışsa da, depresyondaki biyobelirteç araştırmaları henüz başlangıç aşamasındadır. Son çalışmalar depresyonun kolinergik iletimdeki düzensizlikten etkilenebileceğini göstermiştir. Bu bulgular kapsamında, depresyon patomekanizmasında asetilkolinesterazın rolü yaygın olarak çalışılırken, butirilkolinesterazın (BChE) rolü belirsizliğini koruyor. Yakın zamanda, BChE aktivitesinin gerçek zamanlı görselleştirilmesi için, *in vitro* ve tümör taşıyan farelerde etkinliğini gösteren, BChE ile aktiveleştirilebilen bir kemilüminesans moleküler sensör (BCC) geliştirdik. Bu translasyonel çalışmada, BChE aktivitesi ile depresyon arasındaki ilişkiyi araştırmak için BCC'yi kullandık. İlk olarak depresyonlu hastalarda (32 tek kutuplu depresyon, depresif dönemleri olan 20 iki uçlu bozukluk) sağlıklı kontrollerle (n=48) karşılaştırıldığında BChE aktivitesini değerlendirdik ve remisyona (n=15) göre değişiklikleri yeniden değerlendirdik. İkinci olarak, kronik öngörülemez hafif strese (CUMS), eşzamanlı fluoksetin tedavisiyle birlikte CUMS'ye ve sadece tuzlu su enjeksiyonuna maruz control sıçanlarda BChE aktivitesini değerlendirdik. Son olarak, hipotalamik-hipofiz-adrenal (HPA) eksen sonuçlarını taklit etmesi için 24 saat boyunca 200µM kortikosteron, sempo-adrenomedüller (SAM) sistem sonuçlarını taklit etmesi için ise ve 24 saat boyunca 1 mM norepinefrin maruziyetine bırakılmış psikolojik strese yönelik PC-12 Adh hücre modelleri oluşturduk ve BChE aktivitelerini değerlendirdik. Sonuçlar, depresyonlu bireylerin kontrollere kıyasla önemli ölçüde daha düşük BChE aktivitesi sergilediğini ( $p<.001$ ), artan aktivitenin remisyona ilişkili olduğunu ( $p<.001$ ) gösterdi. CUMS'ye maruz kalan sıçanlarda BChE aktivite düzeylerinde anlamlı bir düşüş

görülürken ( $p=.011$ ), fluoksetin tedavisi ise artış eğilimi gösterdi ancak anlamlılığa ulaşamadı ( $p=.345$ ). Ek olarak, BChE aktivitesi kortikosteron maruziyetiyle anlamlı derecede azaldı ( $p=.002$ ) ve norepinefrin maruziyetiyle arttı ( $p=.002$ ). Daha sonra, 5  $\mu\text{M}$  fluoksetin bu psikolojik streslere maruz kalan hücrelerde BChE aktivitesinde artış gösterdi (her ikisi için de  $p=.002$ ). Bu translasyonel çalışma, hem klinik popülasyonlarda hem de kronik stresli hayvan modellerinde BChE aktivitesinde bir azalma olduğunu ve psikolojik stres modelinin oluşturduğu hücre modellerinde semptomların hafifletilmesiyle seviyelerin tersine döndüğünü göstermektedir. Bulgularımız BChE aktivitesinin depresyon tanısı ve tedavisi için umut verici bir biyobelirteç olabileceğini düşündürmektedir.



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## TABLE OF CONTENTS

|                                                                                                       |     |
|-------------------------------------------------------------------------------------------------------|-----|
| List of Tables .....                                                                                  | xiv |
| List of Figures .....                                                                                 | xv  |
| Abbreviations.....                                                                                    | xix |
| Chapter 1: INTRODUCTION .....                                                                         | 1   |
| 1.1 Major Depressive Disorder (MDD) .....                                                             | 1   |
| 1.2 Association between Stress and MDD.....                                                           | 3   |
| 1.3 Biomarker Research in MDD .....                                                                   | 4   |
| 1.4 Butyrylcholinesterase (BChE) .....                                                                | 7   |
| 1.5 Determining BChE Activity .....                                                                   | 8   |
| Chapter 2: MATERIALS AND METHODS.....                                                                 | 10  |
| 2.1 Synthesis of Butyrylcholinesterase Activatable Chemiluminescent Probe (BCC) .....                 | 10  |
| 2.1.1 Materials .....                                                                                 | 10  |
| 2.1.2 Synthesis of BCC.....                                                                           | 10  |
| 2.2 Examination of the Relationship Between BChE Levels and Depression in Human Serum .....           | 11  |
| 2.2.1 Study Sample .....                                                                              | 11  |
| 2.2.2 Collection and Storage of Human Serum Samples.....                                              | 13  |
| 2.2.3 Psychometric Scales .....                                                                       | 14  |
| 2.2.4 Monitoring BChE Activity in Human Serum .....                                                   | 15  |
| 2.2.5 Inhibition of BChE Signal in Human Serum with Donepezil and Tacrine .....                       | 15  |
| 2.2.6 Monitoring BChE Activity in Human Serum from Different Groups .....                             | 16  |
| 2.2.7 Statistical Analysis.....                                                                       | 16  |
| 2.3 Examination of the Relationship Between BChE Levels with Chronic Stress and Animal Behavior ..... | 17  |

|            |                                                                                                                                                         |    |
|------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 2.3.1      | Animal Characteristics and Chronic Unpredictable Mild Stress Model                                                                                      | 17 |
| 2.3.2      | Behavioral Assessments .....                                                                                                                            | 19 |
| 2.3.3      | Plasma Sample Preparation .....                                                                                                                         | 21 |
| 2.3.4      | Monitoring BChE Activity in Rat Plasma .....                                                                                                            | 21 |
| 2.3.5      | Inhibition of BChE Signal in Rat Plasma with Donepezil and Tacrine                                                                                      | 22 |
| 2.3.6      | Monitoring BChE Activity in Rat Plasma from Different Groups .....                                                                                      | 22 |
| 2.3.7      | Statistical Analysis.....                                                                                                                               | 23 |
| 2.4        | Validating the Link Between BChE Levels and Chronic Stress: Utilizing PC-12 Adh Cells and Modulating with Corticosterone, Noradrenaline, and Fluoxetine | 23 |
| 2.4.1      | Cell Culture.....                                                                                                                                       | 23 |
| 2.4.2      | Cell Viability Measurements .....                                                                                                                       | 24 |
| 2.4.3      | Monitoring BChE Activity in PC-12 Adh Cells .....                                                                                                       | 25 |
| 2.4.4      | Monitoring BChE Activity in CORT treated PC-12 Adh Cells .....                                                                                          | 25 |
| 2.4.5      | Monitoring BChE Activity in NE treated PC-12 Adh Cells.....                                                                                             | 26 |
| 2.4.6      | Monitoring BChE Activity in CORT treated PC-12 Adh Cells with Subsequent Fluoxetine Treatment .....                                                     | 26 |
| 2.4.7      | Monitoring BChE Activity in NE treated PC-12 Adh Cells with Subsequent Fluoxetine Treatment .....                                                       | 26 |
| 2.4.8      | Statistical Analysis.....                                                                                                                               | 27 |
| Chapter 3: | RESULTS .....                                                                                                                                           | 28 |
| 3.1        | Synthesis .....                                                                                                                                         | 28 |
| 3.2        | Examination of the Relationship Between BChE Levels and Depression in Human Serum .....                                                                 | 28 |
| 3.2.1      | Optimization of BCC in Human Serum.....                                                                                                                 | 28 |

|            |                                                                                                                                                        |     |
|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 3.2.2      | Monitoring BChE Activity in Clinical and Non-Clinic Human Serums                                                                                       | 31  |
| 3.3        | Examination of the Relationship Between BChE Levels with Chronic Stress and Animal Behavior                                                            | 41  |
| 3.3.1      | Optimization of BCC in Rat Plasma                                                                                                                      | 41  |
| 3.3.2      | Monitoring BChE Activity in CUMS exposed Rat Plasma                                                                                                    | 44  |
| 3.3.3      | Sucrose Preference Test (SPT)                                                                                                                          | 44  |
| 3.3.4      | Forced Swim Test (FST)                                                                                                                                 | 45  |
| 3.3.5      | Weight% Change                                                                                                                                         | 47  |
| 3.3.6      | Age-Behavioral Measurements of Animal Characteristics                                                                                                  | 49  |
| 3.4        | Validating the Link Between BChE Levels and Chronic Stress: Utilizing PC-12 Adh Cells and Modulating with Corticosterone, Noradrenaline and Fluoxetine | 51  |
| Chapter 4: | DISCUSSION                                                                                                                                             | 57  |
| 4.1        | Synthesis                                                                                                                                              | 57  |
| 4.2        | Examination of the relationship between BChE levels and depression in human serum                                                                      | 57  |
| 4.3        | Examination of the Relationship Between BChE Activity with Chronic Stress and Animal Behavior                                                          | 65  |
| 4.4        | Validating the Link Between BChE Levels and Chronic Stress: Utilizing PC-12 Adh Cells and Modulating with Corticosterone, Noradrenaline and Fluoxetine | 70  |
| 4.5        | Overall Discussion                                                                                                                                     | 73  |
| Chapter 5: | CONCLUSION                                                                                                                                             | 76  |
|            | Bibliography                                                                                                                                           | 77  |
|            | Appendix A: NMR Results of BCC                                                                                                                         | 119 |
|            | Appendix B: Total Luminescence Score for each Human Serum Samples                                                                                      | 121 |

|                                                                                                               |     |
|---------------------------------------------------------------------------------------------------------------|-----|
| Appendix C: Detailed Pearson Correlation Table of BChE_0W with Subitems of HDRS .....                         | 123 |
| Appendix D: Detailed Pearson Correlation Table of BChE_0W with Subitems of HDRS for Only Clinical Group ..... | 124 |
| Appendix E: Detailed Pearson Correlation Table of BChE_0W with Subitems of PSS .....                          | 125 |
| Appendix F: Detailed Pearson Correlation Table of BChE_0W with Subitems of PSS for Only Clinical Group .....  | 126 |
| Appendix G: Total Luminescence Score of Each Rat Plasma Samples .....                                         | 127 |



## LIST OF TABLES

|                                                                                                                                                       |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <b>Table 3.1:</b> Groups comparison of sociodemographic data, psychometric data and total BCC luminescence score .....                                | 33 |
| <b>Table 3.2:</b> Multivariate linear regression model to assess predictors of BChE_0W by dividing group with clinical and non-clinical samples ..... | 35 |
| <b>Table 3.3:</b> Multivariate linear regression model to assess predictors of BChE_0W. ....                                                          | 35 |
| <b>Table 3.4:</b> Correlation table of BChE_0W, HDRS, and PSS .....                                                                                   | 36 |
| <b>Table 3.5:</b> Correlation table of BChE_0W, HDRS, and PSS only for clinical group .....                                                           | 36 |
| <b>Table 3.6:</b> Fixed effects parameters estimates for mixed linear analysis of BChE_0W - BChE_8W with HDRS.....                                    | 39 |
| <b>Table 3.7:</b> Fixed effects parameters estimates for mixed linear analysis of BChE_0W - BChE_8W with HDRS suicide item .....                      | 39 |
| <b>Table 3.8:</b> Post Hoc comparison for mixed linear analysis of BChE_0W - BChE_8W with HDRS suicide item .....                                     | 40 |
| <b>Table 3.10:</b> Fixed effects parameters estimates for mixed linear analysis of BChE_0W - BChE_8W with HDRS somatic anxiety item .....             | 40 |
| <b>Table 3.11:</b> Post Hoc comparison for mixed linear analysis of BChE_0W - BChE_8W with HDRS somatic anxiety item.....                             | 40 |
| <b>Table 3.13:</b> Correlation table of BChE total luminescence score with behavioral test parameters .....                                           | 49 |
| <b>Table 3.12:</b> Multivariate linear regression model to assess predictors of BChE score in rat plasma.....                                         | 51 |

## LIST OF FIGURES

|                                                                                                                                                                                                                                                                                                                                                                                                      |    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 2.1: Detailed flow chart for human serum samples. 100 sample were used for the 0 <sup>th</sup> week BChE activity measurement and 15 sample were used for the 8 <sup>th</sup> week BChE activity measurement. ....                                                                                                                                                                            | 13 |
| Figure 2.2: Detailed 35 days CUMS Procedure. For each day, rats were exposed to different stressor in an unpredictable timing. ....                                                                                                                                                                                                                                                                  | 19 |
| Figure 3.1: Proof of principle of BCC in human serum environment. Time dependent (a) and total Luminescence (b) comparison of 0.005% human Serum, 50 $\mu$ M BCC, and 0.005% human serum with 50 $\mu$ M BCC (number of technical replicates= 3). While there is negligible luminescence signal in serum only and probe only, there is a 20-fold increase 50 $\mu$ M BCC in 0.005% human serum. .... | 29 |
| Figure 3.2: Dose-dependent titration in the range of 0.005%-2% human serum with 50 $\mu$ M BCC. Time-dependent luminescence (a), total luminescence score (b), and calibration curve (c) of BCC (number of technical replicates= 3). BCC can perfectly monitor the luminescence signal till 0.5% human serum, then it becomes saturated with increasing concentration. ....                          | 30 |
| Figure 3.3: Total luminescence of 10 $\mu$ M BCC in 0.1% human serum treated with different concentrations (0-10 mM) of donepezil (number of technical replicates= 3). As concentration of donepezil increases, the luminescence signal decreases indicate cholinesterase-derived luminescence signal. ....                                                                                          | 31 |
| Figure 3.4: Total luminescence of 10 $\mu$ M BCC in 0.05% human serum treated with different concentrations (0-100 mM) of tacrine (number of technical replicates= 3). As concentration of tacrine increases, the luminescence signal decreases indicate BChE-derived luminescence signal. ....                                                                                                      | 31 |
| Figure 3.5: Total luminescence score comparison of HC, UD, and BD serum samples. There is a significant difference between HC with both UD ( $p<.001$ ) and BD ( $p=.003$ ), no significant difference between patient groups ( $p=1.00$ ) (number of technical replicates= 3). ....                                                                                                                 | 32 |
| Figure 3.6: Total luminescence score was increased with remission, comparison of 0 <sup>th</sup> Week and 8 <sup>th</sup> week ( $n=15$ , number of replicates=3, $p<.001$ ). ....                                                                                                                                                                                                                   | 38 |

|                                                                                                                                                                                                                                                                                                                                                                                             |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 3.7: Proof of principle of BCC in rat plasma environment. Time dependent (a) and total luminescence (b) comparison of 0.005% rat plasma, 10 $\mu$ M BCC, and 0.005% rat plasma with 10 $\mu$ M BCC (number of technical replicates= 3). While there is negligible luminescence in control groups, there is more than 50-fold increase in their mixture ...                           | 41 |
| Figure 3.8: Dose-dependent titration in the range of 0.005%-2% rat plasma with 10 $\mu$ M BCC. Time-dependent luminescence (a), total luminescence score (b), and calibration curve (c) of BCC (number of technical replicates= 3). BCC is responsive to the rat plasma concentration with high sensitivity.....                                                                            | 42 |
| Figure 3.9: Total luminescence of 10 $\mu$ M BCC in 0.05% rat plasma treated with different concentrations (0-10 mM) of donepezil (number of technical replicates= 3). The fact that decreasing of luminescence signal with increasing of donepezil concentration indicates cholinesterase-derived luminescence signal. ....                                                                | 43 |
| Figure 3.10: Total luminescence of 10 $\mu$ M BCC in 0.05% rat plasma treated with different concentrations (0-100 mM) of tacrine (number of technical replicates= 3). The fact that decreasing of luminescence signal with increasing of tacrine concentration indicates BChE-derived luminescence signal.....                                                                             | 43 |
| Figure 3.11: Total luminescence score comparison of Control+Saline, CUMS+Saline, and CUMS+Fluoxetine plasma samples. There is a significant difference between Control+Saline and CUMS+Saline (p=.011). There is no significant difference between Control+Saline and CUMS+Fluoxetine (p=.323) and CUMS+Saline and CUMS+Fluoxetine (p=.345) (n=28, number of technical replicates= 3) ..... | 44 |
| Figure 3.12: Sucrose preference % comparison by group (n=28). Rats exposed to CUMS have significantly lower sucrose preference% compared to Control+Saline (p=.011).....                                                                                                                                                                                                                    | 45 |
| Figure 3.13: Comparison of immobility time by groups. No significance difference between groups was found (n=28, p=.139).....                                                                                                                                                                                                                                                               | 46 |
| Figure 3.14: Swimming time comparison by group (n=28). Control+Saline group has significant higher swimming time compared to CUMS+Fluoxetine group (p=.001).....                                                                                                                                                                                                                            | 46 |
| Figure 3.15: Climbing Time Comparison by Group (n=28). CUMS+Fluoxetine group has significantly higher climbing time compared to Control+Saline (p=.028) and CUMS+Saline (p=.021). ....                                                                                                                                                                                                      | 47 |
| Figure 3.16: Overall weight% changes of the groups compared to baseline between 2 <sup>nd</sup> week to 5 <sup>th</sup> week. Control+Saline significantly gained more weight than                                                                                                                                                                                                          |    |

|                                                                                                                                                                                                                                                                                                                 |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| CUMS+Fluoxetine group for each week comparison. CUMS+Saline group significantly gained more weight than CUMS+Fluoxetine group for 2 <sup>nd</sup> and 5 <sup>th</sup> week. For * $p < .05$ ; for *** $p < .001$ .....                                                                                          | 48 |
| Figure 3.17:MTT Viability Assay of BCC treated PC-12 Adh (number of technical replicates=6).....                                                                                                                                                                                                                | 51 |
| Figure 3.18: Dose-dependent titration of BCC in PC-12 Adh cells. Time-dependent luminescence (a), total luminescence score (b), and calibration curve (c) of BCC (number of technical replicates= 6). Total luminescence increases with the increasing concentration of BCC, demonstrates great linearity. .... | 52 |
| Figure 3.19: Total luminescence of PC-12 Adh cells treated with tacrine (number of technical replicates=3). The fact that decreasing of luminescence signal with increasing of tacrine concentration indicates BChE-derived luminescence signal. ....                                                           | 53 |
| Figure 3.20: MTT Cell Viability Assay of Tacrine (number of technical replicates=6). The fact that there is no tacrine related cell toxicity in the range of 0-200 $\mu$ M supports BCC's BChE specificity.....                                                                                                 | 53 |
| Figure 3.21: MTT cell viability of 24h CORT treated PC-12 Adh Cells. Increasing concentration of CORT induce cell apoptosis as mimicking the outcome of HPA-axis dysfunction (number of technical replicates=6). ....                                                                                           | 54 |
| Figure 3.22: Comparison of BChE activity control vs 200 $\mu$ M CORT-treated PC-12 Adhc cells. BChE activity significantly decreased in CORT-treated PC-12 Adh cells (number of technical replicates=6, $p = .0022$ ). ....                                                                                     | 54 |
| Figure 3.23: MTT Cell Viability of 24h NE Treated PC-12 Adh cells. Increasing concentration of NE induce cell apoptosis as mimicking the outcome of SAM-axis activation (number of technical replicates=6). ....                                                                                                | 55 |
| Figure 3.24: Comparison of BChE Activity Control vs 1mM NE-treated PC-12 Adh cells. BChE activity significantly increased in NE-treated PC-12 Adh cells (number of technical replicates=6, $p = .0022$ ). ....                                                                                                  | 55 |
| Figure 3.25: Comparison of BChE activity control vs 5 $\mu$ M fluoxetine-treated PC-12 Adh cells after CORT established psychological stress. Fluoxetine significantly increased BChE activity in CORT-established psychological stress model (number of technical replicates=6, $p = .004$ ). ....             | 56 |
| Figure 3.26: Comparison of BChE activity control vs 5 $\mu$ M fluoxetine-treated PC-12 Adh cells after NE established psychological stress. Fluoxetine significantly increased                                                                                                                                  |    |

|                                                                                                                |    |
|----------------------------------------------------------------------------------------------------------------|----|
| BChE activity in NE-established psychological stress model (number of technical replicates=6, $p=.010$ ) ..... | 56 |
|----------------------------------------------------------------------------------------------------------------|----|



## ABBREVIATIONS

|          |                                                          |
|----------|----------------------------------------------------------|
| ACh      | Acetylcholine                                            |
| AChE     | Acetylcholinesterase                                     |
| APOE     | Apolipoprotein                                           |
| BCC      | Butyrylcholinesterase Selective Chemiluminescent Probe   |
| BChE     | Butyrylcholinesterase                                    |
| BD       | Patients with Bipolar Disorder Depressive Episode        |
| BMI      | Body Mass Index                                          |
| CORT     | Corticosterone                                           |
| CUMS     | Chronic Unpredictable Mild Stress                        |
| DMEM/F12 | Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 |
| DMSO     | Dimethyl sulfoxide                                       |
| DPBS     | Dulbecco's Phosphate-Buffered Saline                     |
| DSM-V    | Diagnosis and Statistical Manual of Mental Disorders (V) |
| ELISA    | Enzyme-linked Immunosorbent Assay                        |
| EtOAc    | Ethyl Acetate                                            |
| FBS      | Fatal Bovine Serum                                       |
| FST      | Forced Swim Test                                         |
| GC       | Glucocorticoid                                           |
| HC       | Healthy Control                                          |
| HDRS     | Hamilton Depression Rating Scale Score                   |
| Hex      | Hexane                                                   |
| HPLC     | High-performance Liquid Chromatography                   |
| MTT      | (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium    |
| NE       | DL-norepinephrine hydrochloride                          |
| NKE      | Neuron-derived Exosomes                                  |
| NMR      | Nuclear Magnetic Resonance                               |
| PSS      | Perceived Stress Scale                                   |
| RLU      | Relative Luminescence Unit                               |
| ROS      | Reactive Oxygen Species                                  |
| SCID-I   | Structural Clinical Interview-I                          |

|         |                                                          |
|---------|----------------------------------------------------------|
| SPSS    | Statistical Package for Social Sciences                  |
| SPT     | Sucrose Preference Test                                  |
| Tacrine | 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate |
| TLC     | Thin Layer Chromatography                                |
| UD      | Patients with Unipolar Depression                        |
| WHO     | World Health Organization                                |
| YMRS    | Young Mania Rating Scale                                 |



## Chapter 1: INTRODUCTION

### ***1.1 Major Depressive Disorder (MDD)***

Major Depressive Disorder (MDD), also known as depression, is one of the most prevalent mental disorders globally, affecting approximately 1 in 20 individuals at any given time and 1 in 6 over a lifetime.<sup>1</sup> The World Health Organization (WHO) reported a 25% increase in anxiety and MDD prevalence due to the COVID-19 pandemic.<sup>2</sup> Despite regional differences, reports show that MDD remains a leading cause of disability worldwide, significantly impacting the global economy.<sup>3-5</sup> In the United States, the annual economic burden of MDD amounts to billions of dollars, attributed to healthcare costs and decreased productivity.<sup>5,6</sup>

According to the *Diagnosis and Statistics of Mental Disorders-V (DSM-V)*, MDD is characterized by a persistent depressive mood and the loss of pleasure (anhedonia), accompanied by a range of emotional, behavioral, and cognitive symptoms lasting more than two weeks.<sup>7</sup> Complementary symptoms include significant weight changes, sleep disturbances, psychomotor agitation or retardation, fatigue, feelings of worthlessness or excessive guilt, diminished cognitive functions, and recurrent thoughts of death or suicide.<sup>7</sup> The diagnosis process is mainly clinical and based on the patient's history, symptomatology, and exclusion of other medical illnesses.<sup>1,7</sup> The diagnosis is made when a person shows five or more depressive symptoms for at least two weeks including one of the core symptoms (either a depressed mood or anhedonia), having significant distress or impairment, and without any manic or hypomanic behavior.<sup>7</sup> Patient Health Questionnaire (PHQ-9) and the Hamilton Depression Rating Scale (HDRS) are the two of many assessment instruments that are used to help in track the severity of the MDD.<sup>8,9</sup>

Treatment of MDD comes in a variety of forms, such as medication, psychotherapy, behavioral changes, or a mix of them. Remission rates in clinical trials range from 22% to 40%. However, in effectiveness studies involving representative samples of depressed patients more reflective of clinical practice, remission rates range from 11% to 30%.<sup>10,11</sup> Also, even after multiple attempts at treatment, over 30% of people with MDD do not experience remission.<sup>1</sup> Furthermore, a meta-analysis of clinical trials by Krøgsboll et al.

found that 24% of the improvement in MDD severity was attributed to placebo effects, highlighting the significant role of placebo in remission.<sup>12</sup>

Due to the high comorbidity with other diseases including cancer, inflammatory, metabolic, cardiovascular, and neurodegenerative disorders, MDD frequently exhibits higher prevalence rates than in the general population.<sup>1,13</sup> Moreover, MDD is quite associated with a significant risk of suicide, with patients being nearly 20 times more likely to die by suicide than the general population,<sup>14</sup> highlights its substantial public health burden.

The etiology of MDD is multifaceted, with heritability estimated at around 35%.<sup>1</sup> Environmental factors, particularly childhood adversity, are strongly associated with an increased risk of developing MDD.<sup>1,15</sup>

Cumulative research on MDD shows that the neurobiological underpinnings of MDD involve complex, interacting systems; therefore, it is not possible to explain MDD in one theory. The monoamine hypothesis suggests deficiencies in brain neurotransmitters like serotonin, and catecholamines like norepinephrine, and dopamine contribute to MDD.<sup>1,16–18</sup> In order to restore the levels of these monoamines, the majority of antidepressants, including tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), target this system as a therapeutic goal.<sup>18</sup> However, the delayed therapeutic effects of these drugs and low remission rate indicate additional biological pathways are involved.<sup>1,19</sup>

The dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is implicated in the pathogenesis of MDD. Hyperactivity or dysfunction of the HPA axis is a consistent neuroendocrinological findings in MDD. On top of that, stress and trauma are important contributors for this dysregulation.<sup>1,19–23</sup> The oxidative stress pathway suggests that an imbalance between reactive oxygen species (ROS) production and antioxidant defenses leads to neuronal damage. This is evidenced by elevated oxidative markers and reduced antioxidant capacity in individuals with depression.<sup>24–26</sup> Inflammation related pathways proposes that chronic inflammation and immune dysregulation play crucial roles in MDD, with elevated levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- $\alpha$ ), found in depressed patients.<sup>1,27–29</sup> These cytokines can disrupt neurotransmitter metabolism, reduce neurogenesis, and induce neurotoxic effects, with anti-inflammatory treatments showing promise in alleviating symptoms.<sup>20,28</sup>

The neurotrophic-based model focuses on impaired synaptic plasticity, especially in the hippocampus and prefrontal cortex, with reduced levels of brain-derived neurotrophic factor (BDNF) implicated in MDD.<sup>1,24,30</sup> Antidepressants have been shown to increase BDNF levels and promote neurogenesis.<sup>24,31</sup>

These current literatures represent different facets of MDD's complex pathophysiology, with the interplay between monoaminergic systems, HPA axis dysfunction, oxidative stress, inflammation, and neuroplasticity underscoring its multifactorial nature. Chronic stress can trigger inflammatory responses, leading to increased oxidative stress and impaired neuroplasticity, all contributing to depressive symptoms.<sup>24</sup> MDD's heterogeneity in symptomatology, severity, comorbid conditions, and treatment responses necessitates personalized management strategies.

## ***1.2 Association between Stress and MDD***

Studies have shown that psychological stress in humans operates primarily through the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenal medullary (SAM) system.<sup>32</sup> Acute stress activates the SAM system, releasing catecholamines (adrenaline and noradrenaline) that increase heart rate and blood pressure, indicated by elevated salivary alpha-amylase levels.<sup>33–35</sup> Concurrently, the HPA axis is activated, beginning with the release of corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN) and resulting in cortisol release from the adrenal cortex.<sup>35</sup> The activation of both systems is essential for preparing the body to handle stress and restoring homeostasis afterward.

Under normal circumstances, homeostasis is maintained by feedback mechanisms that lessen the effects of stress. However, prolonged, severe, or repeated stress can become pathological, leading to higher increases in norepinephrine and cortisol levels compared to acute stress.<sup>36</sup> Chronic mild stress temporarily suppresses the HPA axis, reducing cortisol responses to ACTH.<sup>37</sup> Animal studies show that chronic stress can lead to weight loss, disrupted HPA axis feedback, persistently high cortisol levels, reduced CRH receptor numbers, and adrenal hypertrophy.<sup>38,39</sup>

These findings are corresponding with cumulative results in human research. Major depressive disorder is highly associated with stressful life events, which has led to research on patients with these diagnoses to better understand the impact of prolonged

stress.<sup>40</sup> MDD patients exhibit increased CRH mRNA and neuron numbers in the hypothalamic PVN, elevated CRH in cerebrospinal fluid, and higher blood cortisol levels.<sup>39,41–43</sup> They also show a reduced adrenocorticotrophic hormone (ACTH) response to CRH release due to desensitization of CRH receptors, with complementary adrenal and pituitary gland enlargement.<sup>43–45</sup> Elevated cortisol levels also modify neural pathways related to emotion processing and adaptability. Elevated cortisol levels also modify neural pathways related to emotion processing and adaptability. For example, in a study, increasing in amygdala excitability and decreasing long-term potentiation in projections from the basolateral amygdala to the medial prefrontal cortex (mPFC) and eventually stress reactivity and impaired cognitive functioning were observed.<sup>46</sup>

In summary, psychological stress primarily was operated through the HPA axis and the SAM system, activating physiological responses crucial for managing acute stress and restoring homeostasis. Chronic stress, however, disrupts these mechanisms, leading to pathological conditions such as MDD. Elevated cortisol levels not only alter cellular functions but also impact brain connectivity, exacerbating stress reactivity and impairing cognitive processes. Understanding these mechanisms highlights the importance of addressing chronic stress to prevent its detrimental effects on mental and physical health.

### **1.3 Biomarker Research in MDD**

Biomarkers are important tools for monitoring normal or pathologic biological process as well as treatment response in respond to therapeutic interventions. As mentioned above, the complex etiology of MDD, which includes genetic, biological, environmental, and psychological factors, prevents the drawing of a general theory that will explain all aspects. In addition, possible issues in the diagnosis such as subjective nature of evaluation, categorical nature of diagnosis result in a heterogeneous patient profile which could explain some part of low remission rates although several treatment options. This complexity underscores the necessity for biomarker research to unravel the pathomechanisms of MDD and develop objective diagnostic tools, potentially transforming therapeutic approaches and prognostic evaluations. Some of the promising biomarkers studied to characterize MDD pathomechanism summarized as below:

As an inflammatory system based MDD biomarkers, proinflammatory cytokines are great example. Meta-analyses indicate elevated levels of proinflammatory cytokines IL-

1, IL-6, and TNF- $\alpha$  in the peripheral blood of individuals with MDD, along with increased C-reactive protein (CRP), an essential inflammation marker.<sup>47–49</sup> Potential mechanisms for cytokine-induced MDD include the induction of extrahypothalamic corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), glucocorticoid resistance, activation of indoleamine 2,3-dioxygenase (IDO), and enhanced expression of the serotonin transporter.<sup>47,50,51</sup> Given that inflammation diminishes with antidepressant treatment and appearing to be more irregular in individuals who do not respond to treatment and those who remain untreated indicates the inflammatory contribution into MDD pathomechanism.<sup>52,53</sup> Also, therapies targeting inflammation have a potential to alleviate the severity of MDD.<sup>48,49,52,53</sup> also, However, these markers lack definitive specificity and sensitivity for MDD, as variability in cytokine studies and MDD subtypes complicates the replication of findings.<sup>54</sup> Improved diagnostic accuracy may be achieved through cerebrospinal fluid (CSF) inflammatory profiles and neuroimaging.<sup>54</sup>

As a glutamate system-based MDD biomarkers, the N-methyl-D-aspartate (NMDA) receptor, one of the four member of ionotropic glutamate receptors is a great example. Research shows that its subunits particularly GluN1, GluN2, and GluN3 plays a crucial role in synaptic plasticity and neuroplasticity.<sup>55</sup> According to the glutamate hypothesis, decreased NMDA receptor activity leads to defective synaptic plasticity and reduced neuroplasticity, both of which contribute to depressive symptoms.<sup>56</sup> Additionally, several preclinical and postmortem studies of MDD have identified overexpression of NR2B subunits and downregulation of NR2A subunits.<sup>57</sup> Also, NMDA receptor antagonists, like ketamine, exhibit rapid antidepressant effects.<sup>58</sup> However, there is more research is needed for especially the association between MDD subtypes, individual differences, and determining suitable protocols for maintenance ketamine therapy and its discontinuation.<sup>58</sup>

As a neuroendocrine system-based MDD biomarkers, there are several biomarker candidates such as brain-derived neurotrophic factor (BDNF), nerve growth factor (VGF), and fibroblast growth factor (FGF).<sup>29,59</sup> Brain-derived neurotrophic factor (BDNF) is one of the most extensively studied growth factors in MDD. It is primarily released from neurons and subsequently transported by platelets, leukocytes, and other cells.<sup>60</sup>

Findings emphasize the role of brain-derived neurotrophic factor (BDNF) in the stress response and its protective effects against alterations in brain stimulation. For

instance, in animal studies, both physical and behavioral stress have been shown to reduce BDNF expression in the hippocampus.<sup>61,62</sup> Additionally, chronic stress has been demonstrated to diminish neurogenesis and resilience by inhibiting BDNF-related pathways in animals.<sup>61</sup> In humans, multiple studies and meta-analyses indicate that individuals with depression exhibit higher levels of BDNF in their blood.<sup>63–66</sup> Moreover, patients with MDD who respond positively to treatment have been found to have higher baseline BDNF levels, suggesting that BDNF levels may be useful in predicting antidepressant response.<sup>64</sup>

Oxidative stress indicators, glucagon-like peptide-1 (GLP-1), and ghrelin may be administered as metabolic system-based MDD biomarkers. As previously mentioned, oxidative stress refers to the disproportion between the body's capacity to eliminate free radicals and their formation, which can cause harm to cells and ultimately be associated with disorders such as MDD.<sup>26</sup> To determine if oxidative stress and MDD pathology are related, researchers looked at several oxidative markers, including malondialdehyde (MDA), nitric oxide (NO), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD) for antioxidant enzymes and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and nitric oxide (NO) for ROS species and malondialdehyde (MDA) for oxidative damage product.<sup>67,68</sup> Furthermore, besides from its primary role in nutrient intake and glucagon release inhibition, GLP-1 has also gained attention as having anti-inflammatory and mood-stabilizing effects on the central nervous system in recent years.<sup>69</sup> Research shows that GLP-1 and GLP-1 receptor agonists (GLP-1 RAs) could be a MDD biomarker as their possible antioxidant role and therapy of MDD.<sup>70</sup> Lastly, ghrelin, another important hormone for food intake and lipid metabolism, has been implicated in the several pathomechanism of psychiatric disorders.<sup>71,72</sup>

In summary, there are many biomarker candidates related to different systems, which points out the complex nature of MDD. However, it could be said that the clinical usage of biomarkers is still in infancy for psychiatry. There are several challenges to obtain a set of biomarkers to predict diagnosis, subtyping, and predicting treatment outcome. Inconsistent protocols for specimen collection and storage, ignoring some of the important patient parameters such as sex, age, medication/supplement use, menstrual cycle, timing of the study, smoking status, or BMI, and inconsistency in patient population

could be seen as major drawbacks<sup>68</sup> to underlies the need for more biomarker research in MDD.

#### **1.4 Butyrylcholinesterase (BChE)**

Cholinergic transmission is vital for the healthy nervous system functioning in both central and peripheral as having role in arousal, attention, memory, and motivation.<sup>73</sup> Acetylcholine (ACh) is the main part of this transmission could act as both neurotransmitter and neuromodulator. Recent studies show that its impairment, specifically ACh level dysregulation, may also be associated with major depressive disorder pathology.<sup>74–76</sup> There are two main enzymes to degrade acetylcholine: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).<sup>77</sup> Because of AChE has a substantial presence in the brain and shows specificity towards ACh compared to BChE, most of the studies looked the association between cholinergic dysfunction and MDD based on AChE perspective<sup>78–84</sup> and BChE was partly ignored. However, BChE is still a substantial constituent of both central and peripheral nervous system.<sup>77,85–87</sup> It can be defined as other (pseudo) part of the esterase family, can catalyze the hydrolysis of choline and non-choline esters such as cocaine and heroin due to having larger acyl pocket in its active side compared to AChE.<sup>85</sup> Rather than AChE, it is found in various tissues, but it is concentrated more in the liver, plasma, and serum; therefore, it is commonly referred to as plasma/serum cholinesterase as well.<sup>86,87</sup> However, although its presence in the brain is generally low, cholinergic transmission is carried out by BChE in some regions of important parts of the brain, especially some of the proximal dendrites of the amygdala, hippocampus, and thalamus.<sup>85,88–90</sup> It also has a role in neurite growth and cellular proliferation during the development of the nervous system.<sup>91–93</sup> Also, it was reported that in the absence of AChE due to pathological conditions such as Alzheimer Disease, its presence increased and compensate for some of the functions of AChE.<sup>94–96</sup> On the other hand, it has an essential part of lipid metabolism, such as inhibiting ghrelin,<sup>97</sup> an important hormone for energy metabolism and protein functions such as ability to enhance the proteolytic activity of trypsin.<sup>98</sup> Although BChE has a broad role in homeostasis, its role in pathomechanism of MDD has not been investigated in detail as AChE.

### 1.5 Determining BChE Activity

Several assays based on different perspectives such as spectrophotometric, fluorometric, and electrochemical were established to measure BChE activity. Among them, a photometric called as Ellman's colorimetric assay is the most employed method. Briefly, its working mechanism as following: hydrolysis of a thiocholine derivative by the desired cholinesterase type (AChE or BChE) and the reaction with thiol reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), leading to the formation of 5-thio-2-nitrobenzoic acid (TNB) anion.<sup>99</sup> Although its popularity and less expensive feature, Ellman's colorimetric assay has low accuracy, may provide inconsistent results due to simply its indirect measuring principle, and less sensitivity.<sup>100,101</sup> Another popular one based on the pH change according to enzyme activity-sourced acetic acid release has similar problems.<sup>102</sup>

Fluorescence imaging presents an attractive method for monitoring butyrylcholinesterase (BChE) activity, allowing for real-time imaging within native biological environments with high spatial and temporal resolution. A range of small activity-based fluorescent probes has been developed for both *in vitro* and *in vivo* imaging of BChE activity.<sup>103–108</sup> Nonetheless, these probes still exhibit chronic drawbacks, including limited penetration of excitation light, susceptibility to photobleaching, autofluorescence interference, and low signal-to-noise ratios, particularly in *in vivo* studies.<sup>118</sup> Phenoxy 1,2-dioxetane-based chemiluminescent probes, which emit light through a chemical reaction without requiring external light excitation, have emerged as a promising alternative to conventional fluorophores following the innovative work of the Shabat group in 2017.<sup>109</sup> Compared to conventional fluorescent based models, it offers heightened sensitivity, lower background noise, and longer signal duration under physiological conditions.<sup>110,111</sup> It's no need to any external irradiation nature enables to overcome the chronic problem of low signal-to-noise ratios.<sup>110,111</sup>

Moreover, by simply masking the phenol group with a cage unit that can be selectively removed by a physiologically relevant analyte, chemiluminescence scaffolds can be easily converted into triggerable activity-based and organelle-targeted probes.<sup>112,113</sup>

Numerous examples of 1,2-dioxetane-based probes have been documented in this approach for monitoring various analytes, including enzymes, oxidative stress markers (ROS- RNS), and other biomolecules.<sup>112–117</sup>

Recently, the first ever BChE-sensitive chemiluminescence based probe (BCC) was developed, considering the important role of BChE in various physiological states mentioned above.<sup>118</sup> Also, its real-time monitoring competency was reported in both *in vitro* and *in vivo*.<sup>118</sup> Usage of cyclopropyl masking unit, which was first proposed by Yang et al.<sup>119</sup> and has been demonstrated in other studies<sup>120–122</sup> combined with chemiluminescent advantages created a highly sensitive monitoring probe with a very low detection limit.<sup>118</sup>

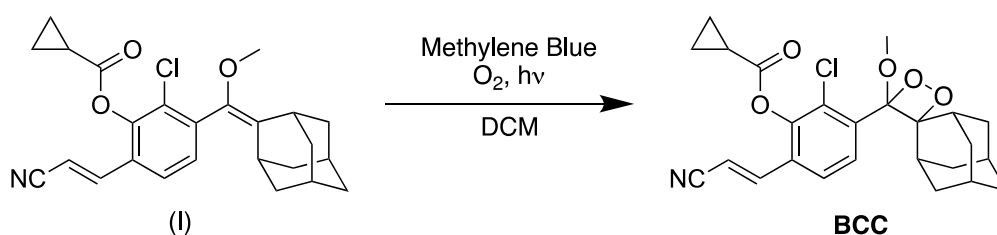
In this study, we employed BCC to investigate the association between BChE activity and major depressive disorder from a translational perspective. First, the competency of BCC to monitor BChE activity in human serum was determined, and BChE activity was compared in people from healthy controls, patients with major depressive disorder, and patients with bipolar disorder with depressive episode. Second, BCC's capability to monitor BChE activity in rat plasma was investigated, and BChE activity was compared in rats from exposed to chronic unpredictable mild stress and saline injection, rats from exposed to chronic unpredictable mild stress and concurrent fluoxetine treatment, and control rats with saline injection. Lastly, BChE activity in psychological stress model established PC-12 Adh cells and their response to fluoxetine was investigated. Our study paved the way for the use of chemiluminescent probes in biomarker research and for understanding the dynamic relationship between BChE activity and major depressive disorder in diagnosis and subsequent treatment.

## Chapter 2:

## MATERIALS AND METHODS

2.1 *Synthesis of Butyrylcholinesterase Activatable Chemiluminescent Probe (BCC)*2.1.1 *Materials*

All commercially available reagents were used without any further purification unless otherwise noted. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded at room temperature by employing a Bruker Avance III Ultrashield (500 MHz) spectrometer, with  $\text{CDCl}_3$  as the solvent and tetramethyl silane (TMS) as the internal standard. Parts per million (ppm) is the recoding unit for chemical shifts, whereas Hz is the unit of expression for coupling constants. The terms s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet) refer to splitting patterns. The NMR spectra were processed using MestReNova program. For column chromatography, thick-walled glass columns filled with silica gel 60 (Merck 230-400 mesh) were utilized. Commercially available 0.25 mm thick silica gel plates (Merck Silica Gel 60 F254) were used for thin layer chromatography (TLC). Volume-to-volume ratios (% v/v) are used to express the relative proportions of solvents in chromatography solvent mixtures.

2.1.2 *Synthesis of BCC*

Scheme 2.1: Synthesis of BCC

Butyrylcholinesterase selective chemiluminescent probe (BCC), which was previously synthesized and reported in KolenLab<sup>118</sup>, was acquired by repeating the last step of the synthesis (formation of dioxetane ring) to be used in this study. 50 mg Compound (I), cyclopropyl bearing chemiluminescent precursor<sup>118</sup>, was obtained from KolenLab. Afterward, BCC was synthesized as following the reported procedure.<sup>118</sup> In brief, methylene blue (2 mg, 0,006 mmol) was dissolved in  $\text{O}_2$ -bubbled DCM in a

Schlenk tube. Next, the compound (I) was added to the tube and the reaction was cooled down to the 0°C with ice-water mixture. Then, the reaction mixture was exposed to a constant 595nm LED light irradiation for 150 min in O<sub>2</sub>-bubbling medium meanwhile the completion of the compound (I) was checked with TLC in a regular 30 min time interval. Upon the depletion of compound (I) signal on the TLC, the reaction was terminated. The reaction mixture was concentrated under reduced pressure, and BCC (26.1 mg, 52.2%) was obtained by purifying the crude solid using column chromatography on silica gel (Hex:EtOAc, 95:5). Structural characterization of BCC was performed *via* proton (<sup>1</sup>H) and carbon (<sup>13</sup>C) Nuclear Magnetic Resonance (NMR) spectroscopy as follows: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.05 (d, J = 8.4 Hz, 1H), 7.57 (d, J = 8.4 Hz, 1H), 7.42 (d, J = 16.7 Hz, 1H), 6.01 (d, J = 16.7 Hz, 1H), 3.20 (s, 3H), 3.01 (q, J = 2.9 Hz, 1H), 2.27 – 2.19 (m, 1H), 2.05 – 1.57 (m, 11H), 1.46 (dq, J = 13.2, 3.0 Hz, 1H), 1.35 (dq, J = 13.5, 2.9 Hz, 1H), 1.29 – 1.27 (m, 2H), 1.16 (dq, J = 7.7, 4.2 Hz, 2H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 171.75, 146.50, 143.19, 136.19, 130.64, 129.81, 127.44, 124.21, 117.40, 111.46, 100.92, 96.42, 49.81, 39.27, 36.54, 33.90, 33.62, 32.17, 31.58, 26.14, 25.75, 12.72, 9.88, 9.82.

## **2.2 Examination of the Relationship Between BChE Levels and Depression in Human Serum**

### **2.2.1 Study Sample**

Human serum samples collected in study “Alterations in Levels of 8-Oxo-2'-Deoxyguanosine and 8-Oxoguanine DNA Glycosylase 1 during a Current Episode and after Remission in Unipolar and Bipolar Depression”<sup>123</sup> were used to explore the association of the BChE levels with depression. The reference study complied with the Declaration of Helsinki's requirements and was authorized by the Dokuz Eylul University Hospital's local ethics committee (protocol no: 307-SBKA EK). Written informed permission was acquired by each subject. The details of the methodological procedure could be found in the reference article<sup>123</sup>. It is summarized as below:

In the referenced study, a total of 145 participants were evaluated, comprising 72 patients and 73 healthy controls. Participants were aged between 18 and 45 years old. Patients diagnosed with either unipolar depression (UD) or bipolar disorder with a current

depressive episode (BD) were recruited from the general and bipolar outpatient units of the Department of Psychiatry, Dokuz Eylul University, located in Izmir, Turkey. Healthy control (HC) groups consisted of volunteers who responded to announcements posted around the hospital campus. The DSM-IV-TR Structural Clinical Interview (SCID-I)<sup>124</sup> was used to confirm and screen the diagnoses of both patients and healthy controls by a trained psychiatrist. The exclusion criteria for all individuals were risky alcohol intake, substance usage (apart from tobacco use), pregnancy or breastfeeding, and any supplements usage. Patients who received a score of less than 18 on the 17-item Hamilton Depression Rating Scale Score (HDRS)<sup>125</sup> or more than 7 on the Young Mania Rating Scale (YMRS)<sup>126</sup> were excluded. Acute infection, any significant abnormality in routine blood and urine tests, any medical comorbidity, and any history of psychiatric diagnosis were the other exclusion criteria for patients. The exclusion criteria for healthy controls were the presence of major neurodevelopmental disorders in first-degree family members, a history of psychiatric diagnosis, and having score more than 7 on HDRS or YMRS. Following the exclusion of 26 participants (16 patients and 10 healthy controls) because of matching at least one the above-mentioned criteria and a healthy control (n=1) declined to participate, the reference study included 61 healthy controls and 57 patients with depression (33 unipolar and 24 bipolar). The self-report Perceived Stress Scale (PSS)<sup>127</sup> were filled out by every participant. After diagnostic and clinical evaluations, baseline blood and urine samples from the participants were collected.

According to the literature, treatment response has often become evident after 4-6 week<sup>128,129</sup>; therefore, 8 weeks breaks were allowed for symptomatic remission and patients were reevaluated in the eighth week of treatment. Remission was assessed using two criteria: the lower score of 7 on the HDRS, and not fulfilling the diagnostic criteria for depression in the eighth week of the SCID-I Interview. After excluding the patients who dropped out the study (n=22), a patient who (n=1) has an active manic episode, and the patients (n=4) that could not match the remission criteria, the reference article included 30 patients (16 patients with unipolar depression, 14 patients with bipolar disorder with depressive episode) for follow up data.

In this study, all experiments were conducted with the approval of the local ethical committee of Koç University, under protocol number 2023.282.IRB2.062. Additionally, all experiments were carried out in accordance with the guidelines provided by the

Committee on Human Research at Koç University, Turkey. Because of the lack (no remaining) of serum samples ( $n=18$ ), 100 participants between 18-45 years old (48 healthy control, 32 patients with unipolar depression, 20 patients with bipolar disorder with depressive episode) were included for 0<sup>th</sup> week BChE level analysis. Because of lack of serum data and/or remission HDRS data ( $n=15$ ), only 15 patients (11 patients with unipolar depression, 4 patients with bipolar disorder with depressive episode) were included for subsequent 8<sup>th</sup> week BChE level analysis. Detailed flow chart for study sample was represented in Figure 2.1.

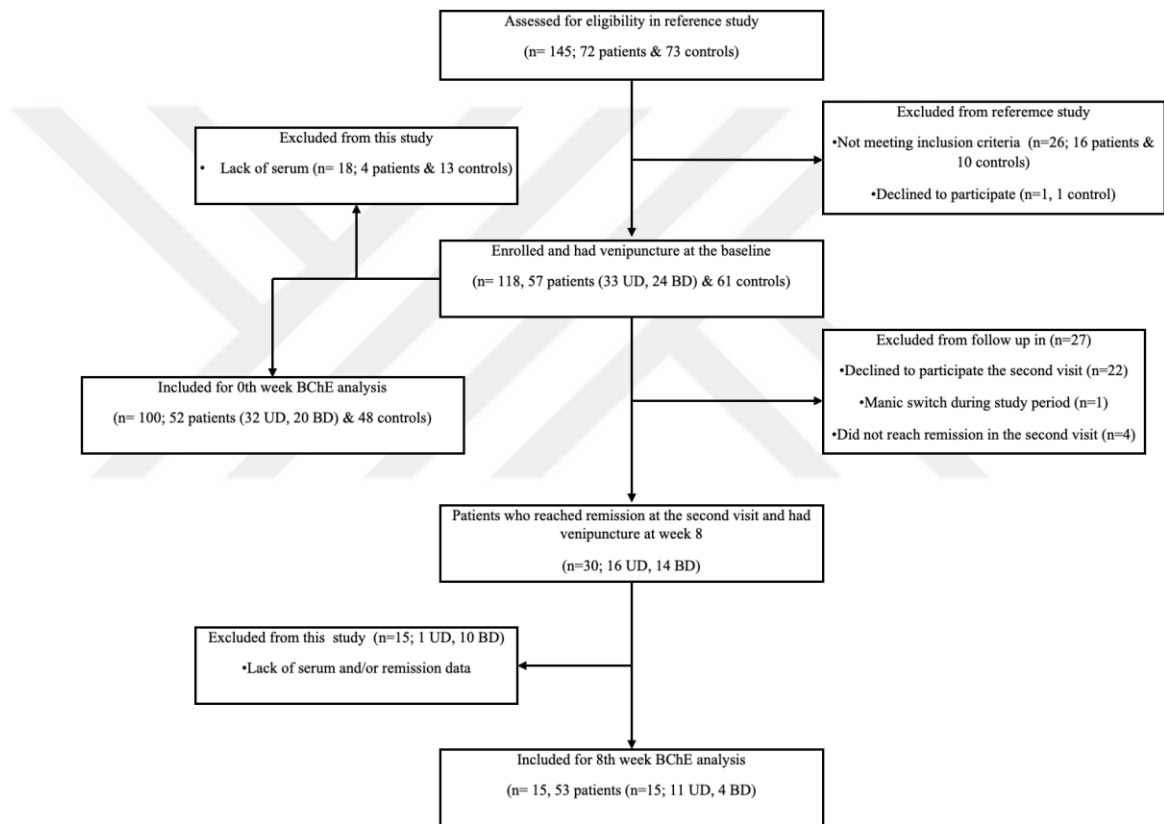


Figure 2.1: Detailed flow chart for human serum samples. 100 sample were used for the 0<sup>th</sup> week BChE activity measurement and 15 sample were used for the 8<sup>th</sup> week BChE activity measurement.

### 2.2.2 Collection and Storage of Human Serum Samples

In the referenced study <sup>123</sup>, the collection of human serum samples followed a standardized procedure: Participants underwent an 8-hour fasting period before a 10-milliliter blood sample was drawn between 09:00 and 10:00 am. After collection, the whole blood was left undisturbed at room temperature for 30 minutes to allow clot formation. Subsequently, the clot was separated through centrifugation at 1000g for 10

minutes in a refrigerated centrifuge, and the resulting serum was stored at -80°C for further analysis.

In this study, aliquoted 200µL human serum samples were taken with dry ice and stored at -80°C till analysis. Before the analysis, the samples were allowed to melt in the room temperature for 30 min and mixed with a vortex before % v/v dilution with DPBS.

### 2.2.3 Psychometric Scales

**Sociodemographic data:** Descriptive statistics of gender, age, smoking, working status, exercise, alcohol consumption, education by year, weight, and height were used for sociodemographic analysis. BMI was calculated as dividing a participant's weight in kilograms by their height in meters squared.

**Structural Clinical Interview (SCID-I) for DSM-IV-TR:** Structured Clinical Interview for DSM-IV-TR is a semi-structured interview guide to establish major DSM-4 diagnoses.<sup>124</sup> In the reference article, the Turkish version is applied by trained psychiatrists.<sup>123</sup>

**Hamilton Depression Rating Scale, 17-item version (HDRS-17):** A clinician-administered scale developed by Hamilton consists of 17 items investigating depressive symptoms.<sup>8</sup> In the scale items are marked between 0-4 or 0-2. Scores between 0 and 7 are regarded as normal, 8 and 16 as mild depression, 17 and 23 as moderate depression, and scores exceeding 24 as severe depression. Turkish version, whose reliability and validity study were conducted by Akdemir, was used in the reference article.<sup>123,125</sup>

**Young Mania Rating Scale (YMRS):** It was developed to measure the severity and change of the symptoms related to manic episode by Young et al.<sup>130</sup> YMRS is a scale consisting of 11 items, each containing five levels of severity. 7 items are five-point Likert type, and 4 items are 9-point Likert type. Symptoms are evaluated considering the last 1 week. Turkish version, whose validity and reliability study were conducted by Karadag et al., was used in the reference article.<sup>123,131</sup>

**Perceived Stress Scale (PSS):** The original scale was developed by Cohen et al.<sup>132</sup>, consists of 14 items designed to measure an individual's perception of the stressfulness of situations in their life. In addition to the original 14-item long form, the PSS also includes two shorter forms: a 10-item version and a 4-item version. Scores on the PSS-14 range from 0 to 56, scores on the PSS-10 range from 0 to 40, and scores on the PSS-4 range

from 0 to 16. Higher scores indicate higher perceived stress levels. In the referenced article, the Turkish version of the PSS, whose validity and reliability were established by Eskin et al., was utilized.<sup>123,133</sup>

#### 2.2.4 *Monitoring BChE Activity in Human Serum*

First, whether BCC can produce a luminescence signal in a human serum environment or not was checked. A human serum from a healthy control was diluted with DPBS in various concentrations (0-4%) and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. 100 $\mu$ M BCC (50 $\mu$ L, 2% DMSO) was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). Human serum had a final concentration in the interval of 0-2% (0.005%, 0.01%, 0.02%, 0.04%, 0.08%, 0.1%, 0.2%, 0.4%, 0.5%, 1%, 2%), while BCC had a final concentration of 50 $\mu$ M (1% DMSO in 100 $\mu$ L).

#### 2.2.5 *Inhibition of BChE Signal in Human Serum with Donepezil and Tacrine*

Second, whether the luminescence signal obtained from BCC in human serum medium was derived from BChE was checked by inhibition experiments. For donepezil inhibition study, the healthy control serum was diluted with DPBS to 0.2% and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. Then, various concentrations of donepezil (0-25 mM) were added to wells in 40 $\mu$ L total and the plated was incubated for 1h. Afterward, 10 $\mu$ L (100  $\mu$ M in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). Human serum had a final concentration of 0.1%, BCC had a final concentration of 10 $\mu$ M (0.5% DMSO in 100 $\mu$ L), and donepezil had a final concentration in the interval of 0-10 mM (0, 1, 2, 5, 10 mM).

For tacrine inhibition study, the healthy control serum was diluted with DPBS to 0.1% and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. Then, various concentrations of tacrine (0-250 mM) were added to wells in 40 $\mu$ L total and the plate was incubated for 1h. Afterward, 10 $\mu$ L (100  $\mu$ M in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently

monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). Human serum had a final concentration of 0.05%, BCC had a final concentration of 10 $\mu$ M (0.5% DMSO in 100 $\mu$ L), and tacrine had a final concentration in the interval of 0-10 mM (0, 2.5, 5, 10, 20, 50, 100 mM).

#### 2.2.6 Monitoring BChE Activity in Human Serum from Different Groups

All samples of human serum from three groups (healthy control, unipolar depression, and bipolar disorder with depressive episode) were diluted with DPBS to 1% concentration and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. Afterward, 100  $\mu$ M BCC (2% DMSO in 50 $\mu$ L DPBS) BCC was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). Human serum had a final concentration of 0.5%, while BCC had a final concentration of 50 $\mu$ M (1% DMSO in 100 $\mu$ L).

#### 2.2.7 Statistical Analysis

For all statistical analysis and visualization, the Statistical Package for Social Sciences (SPSS; Version 28.0.0), GraphPad Prism (Version 9.5) and Jamovi (Version 2.3.26.0) were used. Total luminescence score for BChE level at 0<sup>th</sup> week and 8<sup>th</sup> week were determined by employing BCC. All data were reviewed for quality control regarding missing or mistyped data.

Since BChE sample size is small (n=20 for BD) and assumptions of parameter tests, namely normality and homogeneity of variance, could not be achieved for most of the parameters, nonparametric tests were used where is applicable. To determine any potential differences between groups in terms of gender, smoking, working status, alcohol, and exercise, a chi-square test was used. Nonparametric Kruskal-Wallis with subsequent Bonferroni correction for multiple comparison was used for age, education, BMI, HDS, YMS, PSS, and BChE\_0W. Assumptions of multiple linear regression analysis (independence, linearity, normality, homoscedasticity, and absence of outliers) were checked. Being\_clinical (UD+BD), being\_UD, and being\_BD was introduced as new nominal variables. Age, BMI, and being\_clinical were assessed as predictors and multivariate linear regression model was used for analysis of predictors of the total

luminescence score for BChE level in 0<sup>th</sup> week. Same analysis was repeated by recoding group variable as being\_UD and being\_BD samples. A Pearson correlation was calculated for the association of BChE\_0W with the psychometric scales. A Pearson correlation was calculated for the association of BChE\_0W with the subitems of HDRS and with the subitems of PSS. Wilcoxon Signed Ranks Test was used for assessing the difference between total luminescence score for BChE level in 0<sup>th</sup> week and 8<sup>th</sup> in the patient group. HDRS and time were assessed as predictors and linear mixed model was used to determine predictors for the difference of total luminescence score for BChE level in 0<sup>th</sup> week and 8<sup>th</sup>. Same analysis was applied for HDRS#11 and HDRS#13. In all statistical analyses, threshold for p-value was taken as .05. Data were presented as mean  $\pm$  standard deviation.

### ***2.3 Examination of the Relationship Between BChE Levels with Chronic Stress and Animal Behavior***

#### ***2.3.1 Animal Characteristics and Chronic Unpredictable Mild Stress Model***

The data and samples used in this step were obtained from a preregistered study funded by TÜBİTAK-1001 (Project Number: 122Z771, Principal Investigator: Dr. Deniz Ceylan). The animal model experiments were conducted with approval from the local ethical committee of Koç University under protocol number 2022.HADYEK.014, and all procedures adhered to the guidelines set forth by the Committee on Animal Research at Koç University, Turkey.

Chronic Unpredictable Mild Stress (CUMS) is one of the frequently used depression models in animal which consists of days or weeks of systematic, repetitive exposure to erratic, unexpected, and unmanageable stressors.<sup>134,135</sup> It results in physical, physiological, neurological, biochemical, and behavioral symptoms due to the disruption of homeostasis.<sup>136–138</sup> One of the primary effects of CUMS is anhedonia, the inability to feel pleasure which is one of the core symptoms of the major depressive disorder.<sup>139–141</sup> In the original study, 35 days Chronic Unpredictable Mild Stress (CUMS) was used as a chronic stress model on animals based on literature<sup>142–146</sup>.

In brief, 6–12-week-old Wistar male rats (n=33, Weight range:124-425g) were used in the study. According to the procedure, animals were divided into three groups: 9

control (Control+Saline), 9 CUMS-exposed (CUMS+Saline), and 15 CUMS-exposed and subsequent fluoxetine-treated (CUMS+Fluoxetine). In all groups, rats were kept in groups of three in cages with *ad libitum* access to food and water and the cage were weekly cleaned.

In Control+Saline group rats were allowed to continue their normal cycle for 35 days without being exposed to any stress factors. Sterile saline (NaCl %0.9, 1.5 ml) injection was administered intraperitoneally each day starting from the 15<sup>th</sup> day. Rats in CUMS+Saline and CUMS+Fluoxetine groups were exposed to CUMS model for 35 days. The stressors were given at an unpredictable timing so that the animals could not get used to the stressors and adapt. The stressors used to create the CUMS model included cold exposure (+4 C°), overnight inclined cage, rotation in a shaker, food and water deprivation, interruptions in the day and night cycle, isolation in a single cage, exposure to aversive smell, flashlight application during the night cycle, floating, moist bedding, and group caging of all rats in the group. The detailed schedule for stressors during the procedure is given in Figure 2.2. Starting from day 15, the CUMS+Saline group was injected intraperitoneally with sterile saline (NaCl %0.9, 1.5 ml) daily whereas the CUMS+Fluoxetine group was injected intraperitoneally with fluoxetine (dissolved in saline) at a daily dose of 10 mg/kg (in total 1.5 ml) as used in similar studies.<sup>146</sup>

The procedure was terminated at the end of the 35<sup>th</sup> day and validation of the model was checked by sucrose preference test (SPT), forced swim test (FST), and weight% changes compared to baseline for each rat.

| Days | 08:00-12:00                  | 13:00- 17:00                 | 19:00 - 07:00                               |
|------|------------------------------|------------------------------|---------------------------------------------|
| 1    | 4 °C exposure for 1h         |                              | Keeping in an inclined (45°) cage overnight |
| 2    | Rotation in a shaker for 1h  |                              | Deprivation of water and food overnight     |
| 3    | Closing light for 3h         |                              | Overnight isolation                         |
| 4    |                              | 4 °C exposure for 1h         | Aversive smell exposure overnight           |
| 5    |                              |                              | Flashlight exposure overnight               |
| 6    | 4 °C exposure for 1h         |                              | Light exposure overnight                    |
| 7    |                              | Closing light for 3h         | Overnight isolation                         |
| 8    | Rotation in a shaker for 1h  | 4 °C exposure for 1h         |                                             |
| 9    | Closing light for 3h         |                              | Overnight isolation                         |
| 10   |                              |                              | Deprivation of water and food overnight     |
| 11   | Swimming (+18 °C) for 10 min |                              | Moist bedding overnight                     |
| 12   |                              | Closing light for 3h         | Aversive smell exposure overnight           |
| 13   | Rotation in a shaker for 1h  |                              | Group caging overnight                      |
| 14   | Closing light for 3h         |                              | Flashlight exposure overnight               |
| 15   |                              | Closing light for 3h         | Moist bedding overnight                     |
| 16   |                              | Swimming (+18 °C) for 10 min | Light exposure overnight                    |
| 17   |                              |                              | Deprivation of water and food overnight     |
| 18   |                              |                              | Overnight isolation                         |
| 19   |                              | Rotation in a shaker for 1h  | Aversive smell exposure overnight           |
| 20   | 4 °C exposure for 1h         |                              | Group caging overnight                      |
| 21   |                              |                              | Flashlight exposure overnight               |
| 22   |                              |                              | Light exposure overnight                    |
| 23   |                              |                              | Deprivation of water and food overnight     |
| 24   | Closing light for 3h         | 4 °C exposure for 1h         | Overnight isolation                         |
| 25   |                              |                              | Keeping in an inclined (45°) cage overnight |
| 26   |                              |                              | Moist bedding overnight                     |
| 27   |                              | Swimming (+18 °C) for 10 min | Overnight isolation                         |
| 28   | Rotation in a shaker for 1h  |                              | Aversive smell exposure overnight           |
| 29   |                              | Swimming (+18 °C) for 10 min | Inclined (45°) cage overnight               |
| 30   | 4 °C exposure for 1h         | Closing light for 3h         | Flashlight exposure overnight               |
| 31   |                              | Rotation in a shaker for 1h  | Deprivation of water and food overnight     |
| 32   |                              |                              | Overnight isolation                         |
| 33   | Closing light for 3h         |                              | Keeping in an inclined (45°) cage overnight |
| 34   |                              |                              | Moist bedding overnight                     |
| 35   | Closing light for 3h         | Swimming (+18 °C) for 10 min | Light exposure overnight                    |
| 36   |                              |                              | Overnight isolation                         |
| 37   |                              |                              |                                             |
| 38   |                              |                              |                                             |
| 39   |                              |                              |                                             |
| 40   |                              |                              |                                             |

Figure 2.2: Detailed 35 days CUMS Procedure. For each day, rats were exposed to different stressor in an unpredictable timing.

During the CUMS procedure, a rat (ID4) in the CUMS+Saline group died. As a result of the necropsy examination of KUTTAM's responsible veterinarian, it was reported that the cause of death may have been due to the stress-related complications during the swimming.

### 2.3.2 Behavioral Assessments

**Sucrose Preference Test:** The sucrose preference test is used to measure anhedonia, one of the main outcomes of the CUMS model, in animal depression model<sup>147–149</sup>. In the study, the reference procedure<sup>149</sup> was followed as summarized below:

Rats were given only water on the 1<sup>st</sup> and 2<sup>nd</sup> days of the 4-day adaptation period and two bottles containing 1% fresh sucrose solution on the 3<sup>rd</sup> day. Subsequently, the rats were deprived of food and water for 12 hours. On the 4th day, two different bottles were placed in the cages, one containing 1% fresh sucrose solution and the other containing only water for 12h. To exclude the side preference the place of the bottles was replaced

at 7<sup>th</sup> hour. Sucrose preference (%) was calculated as dividing sucrose intake (g) with total fluid intake (g) and then multiplying with 100.

**Forced Swim Test (FST):** The forced swim test or 'behavioral despair' test, developed in 1978 by Porsolt et al. is one of the common behavioral assessment models used in rodent for assessing antidepressant-like behavior.<sup>150</sup> FST was developed based on the fact that rats exposed to water cease their strong escape-directed behavior (e.g., swimming and climbing) and exhibit passive immobile behavior. The immobility is thought to indicate either behavioral despair, or the inability to continue escape-directed action in the face of stress, or the emergence of passive behavior that detaches the animal from proactive stress management techniques.<sup>151–153</sup> In the study, the reference procedure<sup>154</sup> was followed as summarized below:

Rats were placed in a cylinder (30 cm diameter, 80 cm height) containing 50 cm of clean water (22±1°C) for 5.5 min. Behaviors were recorded to be scored with a camera that can observe swimming behavior horizontally. The time they spent swimming and motionless was recorded by the observer. Swimming time is defined as the sum of periods of pedaling or making circular movements. Climbing is defined as the sum of vertical movements against the wall to escape. Immobility time is the sum of all periods of inactivity in which the rat maintains its ability to remain in the water with only minimal movements to keep its head above water. Rats were immediately placed in a dry and clean cage after each swimming test. After each animal, the water in the cylinder was changed and the cylinder was thoroughly cleaned. The scoring of the test was completed by three different observers watching the recordings and evaluating the swimming, climbing, and immobile times for each rat within the scope of the definitions stated above. For each scoring, the initial 30 second was accepted as adaptation process and excluded from the evaluation. Following 5 minutes were scored by each observant and mean values were calculated.

**Weight% Change:** Weight change is one of the significant outcome of chronic stress models in rat.<sup>155–158</sup> Thus, weight% change compared to the baseline was used to test the reliability of the model as similar to reported studies.<sup>136,138</sup> In the study, the weight of the rats was measured since the beginning. Then, by accepting baseline as 100%, weight% change per week compared to the weight at 0<sup>th</sup> were calculated.

### 2.3.3 Plasma Sample Preparation

Within the scope of the original project, 2-8 ml of blood had to be taken from each rat to obtain neuron-derived exosomes (NKE); therefore, blood samples were taken from the heart. In brief, rats were administered a ketamine/xylazine mixture (80 mg/kg; 10 mg/kg) via intraperitoneal injection (26-gauge needle and 1 mL syringe) as anesthesia. Depth of anesthesia was controlled by monitoring the loss of reflexes due to pinching of the tail or toes. Subsequently, rats were placed in a supine position and the abdominal area was cleaned with 70% ethanol. Lateral incisions were made using scissors starting from the level of the xiphoid protrusion of the abdominal cavity, and diaphragms were cut from the junction with the chest wall on both sides. The sternum was then lifted by reflecting it backwards and fixed with a sturdy clamp. The tissues connecting to the heart were carefully cut. Clamp the end of the sternum with the hemostat and place the hemostat over the head. Blood was collected via the left ventricle using a 21-gauge needle. After sufficient sample was collected, the needle was removed from the heart and blood was injected into the anticoagulant EDTA tube. Blood tubes were immediately stored on ice. 3 hours later, the tubes were centrifuged at 2800 rpm for 10 min. The plasma in the supernatant was transferred to sterile Eppendorf tubes. The tubes were stored at -80°C for further analysis.

### 2.3.4 Monitoring BChE Activity in Rat Plasma

Aliquoted rat plasma samples were taken with dry ice and stored at -80°C till analysis. Before the analysis, the samples were allowed to melt in the room temperature for 30 min and mixed with a vortex before diluting with DPBS.

First, whether BCC can produce a luminescence signal in a rat plasma environment or not was checked. A Control+Saline plasma was diluted with DPBS in various concentrations (0-4%) and 50µL of each was added in an opaque (black-sided) 96-well plate. 20µM BCC (50µL, 1% DMSO) was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). Rat plasma had a final concentration interval of 0-2% (0.005%, 0.01%, 0.02%, 0.04%, 0.08%, 0.1%,

0.2%, 0.4%, 0.5%, 1%, 2%), while BCC had a final concentration of 10 $\mu$ M (0.5% DMSO in 100 $\mu$ L).

### 2.3.5 *Inhibition of BChE Signal in Rat Plasma with Donepezil and Tacrine*

Second, whether the luminescence signal obtained from BCC in rat plasma medium was derived from BChE was checked by inhibition experiments. For donepezil inhibition study, the Control+Saline plasma was diluted with DPBS to 0.1% and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. Then, various concentrations of donepezil (0-25 mM) were added to wells in 40 $\mu$ L total and the plated was incubated for 1h. Afterward, 10 $\mu$ L (100  $\mu$ M in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently monitored for 102 min using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). The rat plasma had a final concentration of 0.05%, BCC had a final concentration of 10 $\mu$ M (0.5% DMSO in 100 $\mu$ L), and donepezil had a final concentration in the interval of 0-10 mM (0, 1, 2, 5, 10 mM).

For tacrine inhibition study, the Control+Saline plasma was diluted with DPBS to 0.1% and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. Then, various concentrations of tacrine (0-100 mM) were added to wells in 40 $\mu$ L total and the plate was incubated for 1h. Afterward, 10 $\mu$ L (100  $\mu$ M in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently monitored for 102 min using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). The rat plasma had a final concentration of 0.05%, BCC had a final concentration of 10 $\mu$ M (0.5% DMSO in 100 $\mu$ L), and tacrine had a final concentration in the interval of 0-10 mM (0, 2.5, 5, 10, 20, 50, 100 mM).

### 2.3.6 *Monitoring BChE Activity in Rat Plasma from Different Groups*

All samples of rat plasma from three groups (Control+Saline, CUMS+Saline, and CUMS+Fluoxetine) were diluted with DPBS to 2% concentration and 50 $\mu$ L of each was added in an opaque (black-sided) 96-well plate. Afterward, 40  $\mu$ M BCC (2% DMSO in 50 $\mu$ L DPBS) BCC was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=3). The rat plasma had a final

concentration of 1%, while BCC had a final concentration of 20 $\mu$ M (1% DMSO in 100 $\mu$ L).

### 2.3.7 Statistical Analysis

For all statistical analysis and visualization, the Statistical Package for Social Sciences (SPSS; Version 28.0.0) and GraphPad Prism (Version 9.5) were used. ID, group, starting age by week, age before termination by week, weight at 0<sup>th</sup> week, weight at 2<sup>nd</sup> week, weight at 3<sup>rd</sup> week, weight at 4<sup>th</sup> week, weight at 5<sup>th</sup> week, water intake (g), sucrose intake (g), total intake (g), sucrose preference%, immobile time (s), swimming time (s) and climbing time (s) were used for descriptive statistics. BChE level were determined as total luminescence score by employing BCC. To determine any potential differences in terms of parameters stated above between group, nonparametric Kruskal-Wallis test was used. Starting age by days and sucrose preference% were assessed as predictors and multivariate linear regression model was used for analysis of predictors of the total luminescence score for BChE level. Data were presented as the mean as mean  $\pm$  standard error mean.

## 2.4 *Validating the Link Between BChE Levels and Chronic Stress: Utilizing PC-12 Adh Cells and Modulating with Corticosterone, Noradrenaline, and Fluoxetine*

### 2.4.1 Cell Culture

PC-12 Adh (CRL-1721.1) cell line was kindly received from Hasirci Group (Acibadem University, Istanbul). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (D0547), 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (Tacrine – A79922), donepezil hydrochloride (D6821), corticosterone (27840) and dl-norepinephrine hydrochloride (A7256) were purchased from Sigma-Aldrich. Heat inactivated Fetal Bovine Serum (S181H) and Penicillin-Streptomycin 100X antibiotics (L0022) were purchased from BioWest. 0,05% Trypsin/EDTA (325-542 EL) were purchased from Multicell. All absorbance and luminescence measurements were done by employing Biotek Synergy H1 Synergy H1 Reader (Biotek, USA) microplate reader.

PC12 cells was maintained in 90% DMEM/F12, 10% Fetal Bovine Serum, and 1% Penicillin-Streptomycin (5,000 U/mL) at 37°C in an Eppendorf Galaxy 170S incubator

(humidified, 37 °C, 5% CO<sub>2</sub>). The cells were passaged with DPBS and 0.05% Trypsin/EDTA at ~70% confluency.

#### 2.4.2 Cell Viability Measurements

All cell viability measurements were done by using MTT assay, which assesses the cell's viability by determining cellular mitochondrial dehydrogenase activity. In brief,  $2 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Then, the medium was aspirated, washed with DPBS two times, and the cells were treated with different concentrations of BCC (0-50  $\mu$ M, 1% DMSO, 100 $\mu$ L total) for 1h. Subsequently, the medium was replaced with fresh media, and the cells were further incubated for 23h. Following incubation, the media was removed, washed with DPBS two times, and MTT solution (final concentration: 0.5 mg/mL, 100 $\mu$ L total) was exposed for 4 hours. Next, the media was aspirated and 100 $\mu$ L of DMSO was used to solubilize formazan crystals, which are directly correlated with the quantity of live cells. The plate was shaken on a microtiter plate shaker to ensure a homogeneous solution, and the absorbance, where optical density (OD) equals to the 570 nm, was read by using a Biotek Synergy H1 Synergy H1 Reader (Biotek, USA) microplate reader (number of technical replicates=6).

In tacrine treated cell viability,  $2 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Then, the medium was aspirated, washed with DPBS two times, and the cells were treated with different concentrations of tacrine (0, 2, 5, 10, 20, 50, 100, 200  $\mu$ M, 100 $\mu$ L total) for 1h. Subsequently, the medium was replaced with fresh media, and the cells were further incubated for 23h. Following incubation, same MTT procedure stated above was applied to assign cell viability (number of technical replicates=6).

In corticosterone (CORT) treated cell viability assay,  $2 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Then, the medium was aspirated, washed with DPBS two times, and the cells treated with various concentrations of corticosterone (0, 50, 100, 200, 400, 500  $\mu$ M, 1% DMSO, 100 $\mu$ L total) for 24h. Following incubation, the same MTT procedure stated above was applied to assign cell viability (number of technical replicates=6).

In norepinephrine (NE) treated cell viability assay,  $2 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Then, the medium was aspirated, washed with DPBS two times, and the cells treated with various concentrations of norepinephrine (0, 10, 20, 50, 100, 200, 400, 500, 1000  $\mu$ M, 100 $\mu$ L total) for 24h. Following incubation, the same MTT procedure stated above was applied to assign cell viability (number of technical replicates=6).

#### 2.4.3 Monitoring BChE Activity in PC-12 Adh Cells

For determination of BChE activity in PC-12 Adh cells,  $2 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Then, the medium was aspirated, washed with PBS two times, and the cells were treated with various concentrations (0, 1, 2, 5, 10, 20, 50  $\mu$ M, 1% DMSO in 100 $\mu$ L DPBS) of BCC. The kinetic luminescence measurement was started directly after the treatment and monitored 200 min using a Biotek Synergy H1 MF microplate reader (number of technical replicates=6). For the inhibition experiment, cells were treated with various concentrations (0, 50, 100, 200  $\mu$ M) of tacrine for 1h prior to 10 $\mu$ M BCC (1% DMSO in 100 $\mu$ L DPBS) treatment and its subsequent kinetic luminescent measurement (number of technical replicates=3).

#### 2.4.4 Monitoring BChE Activity in CORT treated PC-12 Adh Cells

$2 \times 10^4$  cells/well were seeded for CORT-treated group whereas  $1 \times 10^4$  cells/well were seeded for control group in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Following incubation, 200  $\mu$ M CORT (1% DMSO) was treated for the CORT group whilst medium (1% DMSO) replacement applied to the control group. Upon treatment, cells were incubated for 24h. Following incubation, all mediums were discarded, washed with DPBS twice, and the groups were treated with 10  $\mu$ M BCC (1% DMSO in 100 $\mu$ L DPBS). The kinetic luminescence measurement was started directly after the treatment and monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=6).

#### 2.4.5 *Monitoring BChE Activity in NE treated PC-12 Adh Cells*

$2 \times 10^4$  cells/well were seeded for the NE-treated group whereas  $1 \times 10^4$  cells/well were seeded for the control group in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Following incubation, 1 mM NE was treated for the NE group whilst only medium replacement applied to the control group. Upon treatment, cells were incubated for 24h. Following incubation, all mediums were discarded, washed with DPBS twice, and the groups were treated with 10  $\mu$ M BCC (1% DMSO in 100 $\mu$ L DPBS). The kinetic luminescence measurement was started directly after the treatment and monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=6).

#### 2.4.6 *Monitoring BChE Activity in CORT treated PC-12 Adh Cells with Subsequent Fluoxetine Treatment*

$1 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Following incubation, the psychological stress was created for all cells with 200  $\mu$ M CORT (1% DMSO) treatment for 24h. Upon incubation, all mediums were discarded, washed with DPBS twice, and the groups were treated with 0 or 5 $\mu$ M fluoxetine (dissolved in water) and incubated for an additional 24h. Following incubation, all mediums were discarded, washed with DPBS twice, and the groups were treated with 10  $\mu$ M BCC (1% DMSO in 100 $\mu$ L DPBS). The kinetic luminescence measurement was started directly after the treatment and monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=6).

#### 2.4.7 *Monitoring BChE Activity in NE treated PC-12 Adh Cells with Subsequent Fluoxetine Treatment*

$1 \times 10^4$  cells/well were seeded in an opaque (black-sided) 96-well plate and incubated overnight for cell attachment. Following incubation, the psychological stress was created for all cells with 1 mM NE treatment for 24h. Upon incubation, all mediums were discarded, washed with DPBS twice, and the groups were treated with 0 or 5 $\mu$ M fluoxetine (dissolved in water) and incubated for additional 24h. Following incubation, all mediums were discarded, washed with DPBS twice, and the groups were treated with

10  $\mu$ M BCC (1% DMSO in 100 $\mu$ L DPBS). The kinetic luminescence measurement was started directly after the treatment and monitored for 2h using a Biotek Synergy H1 MF microplate reader (number of technical replicates=6).

#### 2.4.8 *Statistical Analysis*

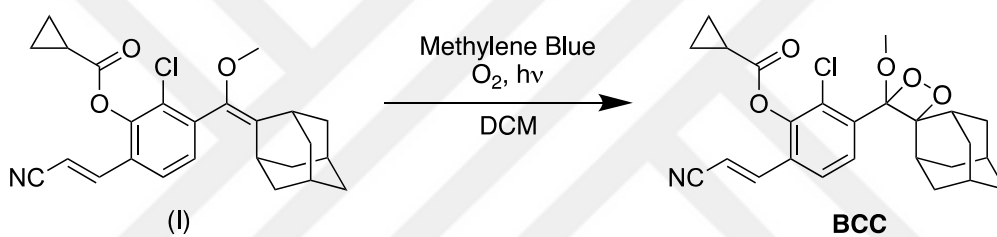
For all the kinetic luminescence experiments, the signal was reported as intensity with relative light units (RLU), and the total luminescence signal was declared as the area under the curve (AUC, min x intensity). For all statistical analysis and visualization, GraphPad Prism (Version 9.5) was used. Simple linear regression analysis was used for the calibration curve. Nonparametric Mann-Whitney test was applied for the comparison of two groups, nonparametric Kruskal-Wallis test was applied for multiple group comparisons. Data were presented as the mean  $\pm$  standard error mean.

## Chapter 3:

## RESULTS

### 3.1 Synthesis

The synthesis of BCC was successfully carried out by following the procedure in the recently reported article<sup>118</sup>. Briefly, as shown in the Scheme 3.1., 1,2-dioxatane bond was produced by reacting cyclopropyl bearing chemiluminescent precursor with singlet oxygen ( $^1\text{O}_2$ ) generated *in situ* by an irradiation of methylene blue with a constant 595 nm LED light. After column chromatography, the yield% was measured as 52.2% and the structural characterization of BCC was done with both  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy (Appendix A).



Scheme 3.1: Synthesis of BCC

### 3.2 Examination of the Relationship Between BChE Levels and Depression in Human Serum

#### 3.2.1 Optimization of BCC in Human Serum

The preliminary capability of BCC to monitor BChE level was determined in both *in vitro* and *in vivo* conditions by Kolen Lab in their recent report.<sup>118</sup> However, its competence was not determined in complex biological samples such as human serum. Therefore, first, whether BCC generates a luminescence signal in human serum or not (*proof of principle*) was checked. The time-dependent intensity of the relative luminescence unit of 0.005% human serum, 50  $\mu\text{M}$  BCC (1% DMSO), and 0.005% human serum with 50  $\mu\text{M}$  BCC (1% DMSO) was compared. As shown in Figure 3.1.a, while there is negligible luminescence signal observed from solely 0.005% human serum and 50  $\mu\text{M}$  BCC (1% DMSO) itself. The luminescence signal rapidly increased when BCC(50  $\mu\text{M}$ ) was added to 0.005% human serum and reached the peak point at 27 min

(20-fold difference). Furthermore, when their total luminescence was compared, 0.005% human serum + 50 $\mu$ M BCC was 17-fold greater than 0.005% human serum and 15-fold greater than 50 $\mu$ M BCC, respectively (Figure 3.1.b).

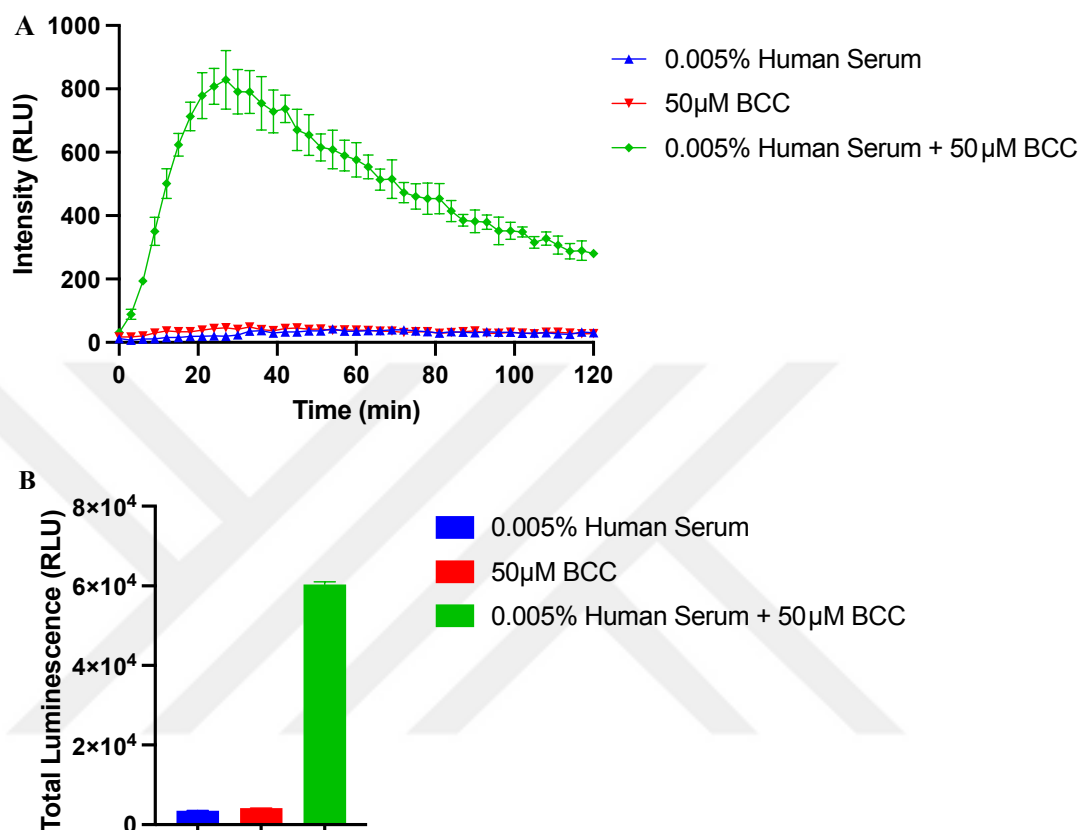


Figure 3.1: Proof of principle of BCC in human serum environment. Time dependent (a) and total Luminescence (b) comparison of 0.005% human Serum, 50 $\mu$ M BCC, and 0.005% human serum with 50 $\mu$ M BCC (number of technical replicates = 3). While there is negligible luminescence signal in serum only and probe only, there is a 20-fold increase 50 $\mu$ M BCC in 0.005% human serum.

After showing BCC can function in human serum environment, a dose-dependent titration of human serum (0.005-2%) was performed with 50 $\mu$ M BCC (1% DMSO in 100 $\mu$ L) for 120 min. As shown in Figure 3.2.a, as the concentration increased peak signal was obtained later as expected. On top of that, total luminescence of BCC in various human serum concentration and its subsequent simple linear regression was investigated (Figure 3.2.b). It was determined that BCC shows perfectly linear response between 0.005%-0.1% concentration ( $R^2=0.9906$ ), then becomes gradually saturated. (Figure 3.2.c)

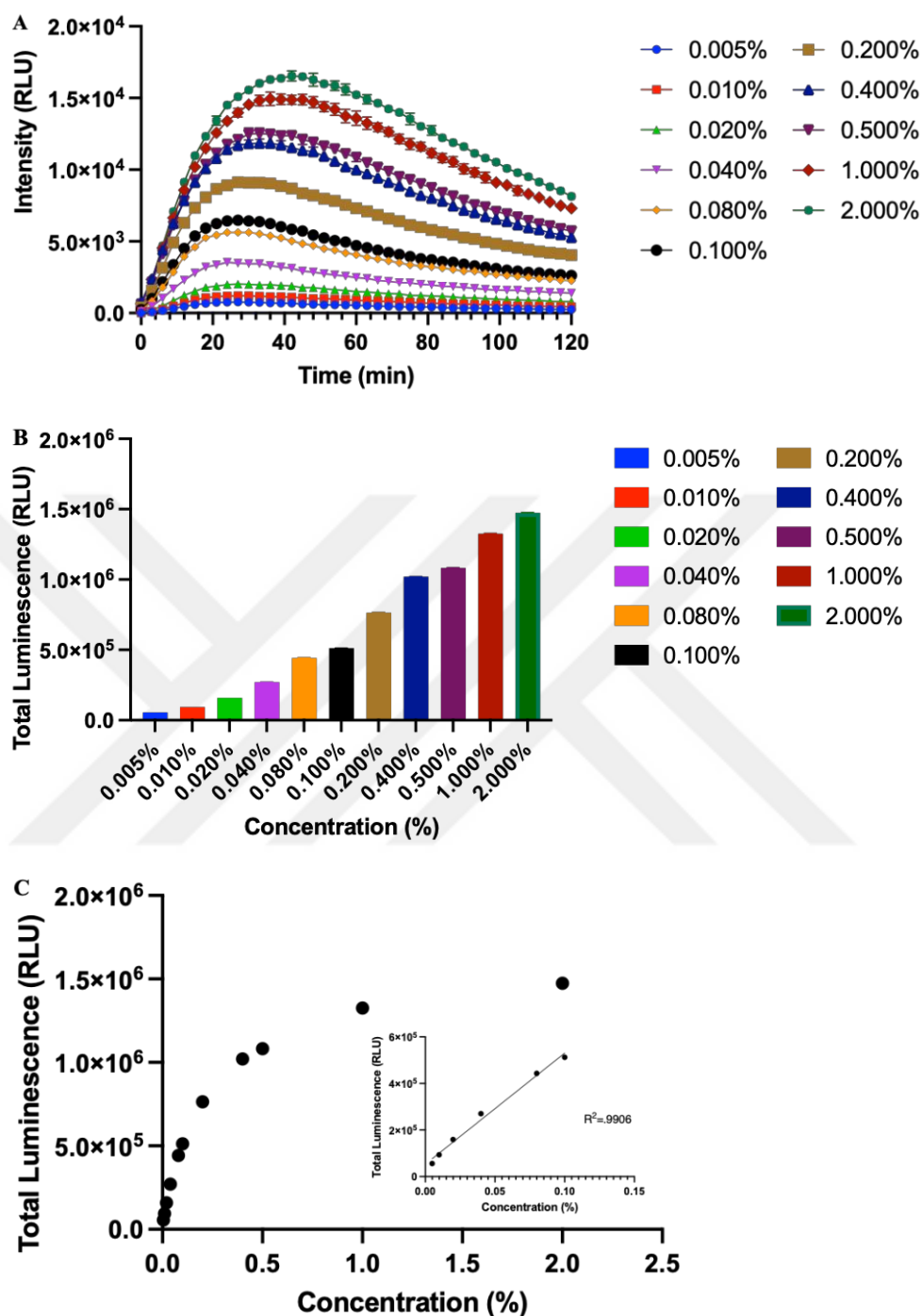


Figure 3.2: Dose-dependent titration in the range of 0.005%-2% human serum with 50 $\mu$ M BCC. Time-dependent luminescence (a), total luminescence score (b), and calibration curve (c) of BCC (number of technical replicates= 3). BCC can perfectly monitor the luminescence signal till 0.5% human serum, then it becomes saturated with increasing concentration.

To confirm that the source of the luminescence signal is only derived from BChE, the human serum was first treated with donepezil (0-10mM), a common cholinesterase inhibitor<sup>159</sup>, and then tacrine (0-100mM), a specific BChE inhibitor<sup>160</sup>, in various

concentrations. As shown in Figure 3.3 and Figure 3.4, the total luminescence of BCC gradually decreased as the concentration of inhibitor increased, which ensures that the luminescence signal is derived from BChE.

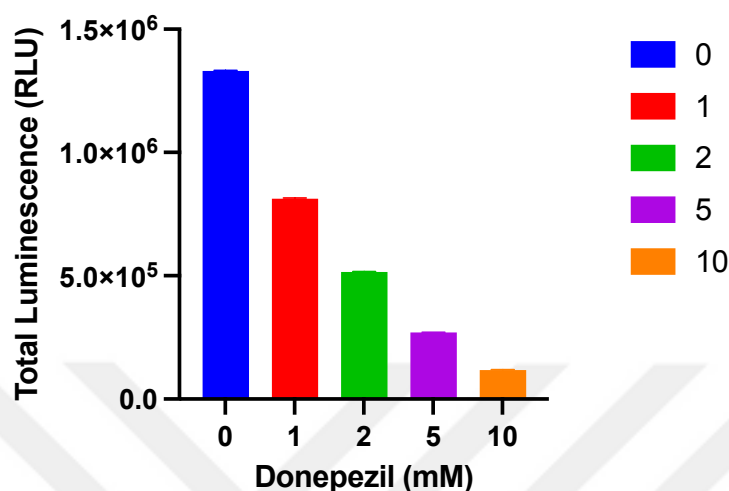


Figure 3.3: Total luminescence of 10 $\mu$ M BCC in 0.1% human serum treated with different concentrations (0-10 mM) of donepezil (number of technical replicates= 3). As concentration of donepezil increases, the luminescence signal decreases indicate cholinesterase-derived luminescence signal.

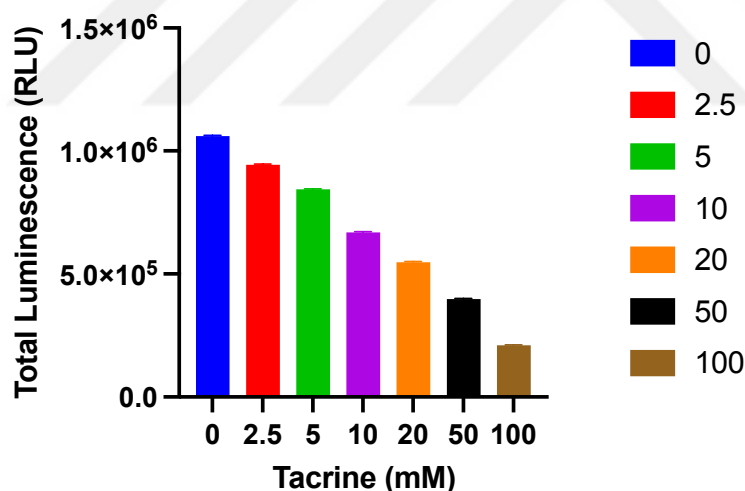


Figure 3.4: Total luminescence of 10 $\mu$ M BCC in 0.05% human serum treated with different concentrations (0-100 mM) of tacrine (number of technical replicates= 3). As concentration of tacrine increases, the luminescence signal decreases indicate BChE-derived luminescence signal.

### 3.2.2 Monitoring BChE Activity in Clinical and Non-Clinic Human Serums

After showing BCC's competency to monitor BChE activity in human serum environment, 0.5% human serums (diluted with DPBS) from clinical and non-clinical samples at 0<sup>th</sup> week and clinical samples at 8<sup>th</sup> week were analyzed with 50 $\mu$ M (1%

DMSO in 100 $\mu$ L) BCC for 2h. Total luminescence score at 0<sup>th</sup> week based on groups (HC, UD, and BD) was provided in Figure 3.5. Total luminescence score for each human serum sample at 0<sup>th</sup> week and 8<sup>th</sup> week were provided at Appendix B. Two new variable named as BChE\_0W and BChE\_8W was created and each total luminescence score added to the SPSS.

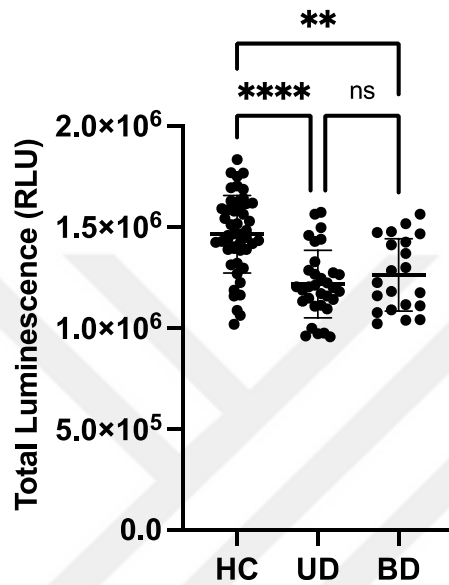


Figure 3.5: Total luminescence score comparison of HC, UD, and BD serum samples. There is a significant difference between HC with both UD ( $p < .001$ ) and BD ( $p = .003$ ), no significant difference between patient groups ( $p = 1.00$ ) (number of technical replicates = 3).

Of the 100 participants, 48 (48.0%) of those were the healthy controls (HC), 32 (32.0%) of the participants were unipolar depression (UD), and 20 (20.0%) of them were bipolar disorder with depressive episode (BD). 69 of the participants (69.0%) were female; 31 of the participants (31.0%) were male. 45 of the participants (45.0%) were smoker; 55 of the participants (55.0%) were non-smoker. 70 of the participants (70.0%) were single; 30 of the participants (30.0%) were married. 50 of the participants (50.0%) were working; 50 of the participants (50.0%) were not working. 50 of the participants (50.0%) do not consume alcohol; 50 of the participants (50.0%) consume alcohol. 32 of the participants (32.0%) do exercise; 68 of the participants (68.0%) do not exercising. The mean age of the participants was  $29.81 \pm 8.58$  years (min:18, max: 45). The mean year of the education was  $14.31 \pm 3.84$  (min:5, max: 23). The mean of body-mass index (BMI) was  $23.92 \pm 4.66$  (min:16, max: 41). The mean total score of Hamilton Depression Scale was  $11.72 \pm 11.47$  (min:0, max: 35). The mean total score of Young Mania Scale was  $0.81 \pm 1.38$  (min:0, max: 6). The mean total score of Perceived Stress Scale ( $n=83$ )

was  $27.17 \pm 8.06$  (min:11, max: 52). The mean BChE\_0W was  $1342516.30 \pm 213987.03$  (min:956929.00, max: 1832027.00). The detailed comparison of groups in terms of sociodemographic, total score psychometric scales, and total luminescence score of BCC at 0<sup>th</sup> week (BChE\_0W) is given in Table 3.1.

**Table 3.1:** Groups comparison of sociodemographic data, psychometric data and total BCC luminescence score

| Groups               | HC<br>(n=48)                | UD<br>(n=32)                | BD<br>(n=20)                | P                            |                                                               |
|----------------------|-----------------------------|-----------------------------|-----------------------------|------------------------------|---------------------------------------------------------------|
| Sex<br>(female%)     | 31 (64.6%)                  | 24 (75.0%)                  | 14 (70.0%)                  | $\chi^2$ : 0.986, $p = .611$ |                                                               |
| Smoking<br>(smoker%) | 18 (37.5%)                  | 20 (62.5%)                  | 7 (35.0%)                   | $\chi^2$ : 5.859, $p = .053$ |                                                               |
| WS<br>(Yes%)         | 22 (45.8%)                  | 15 (46.9%)                  | 13 (65.0%)                  | $\chi^2$ : 2.258, $p = .323$ |                                                               |
| Alcohol<br>(No%)     | 20 (41.7%)                  | 20 (62.5%)                  | 10 (50.0%)                  | $\chi^2$ : 5.697, $p = .223$ |                                                               |
| Exercise<br>(Yes%)   | 20 (41.7%)                  | 6 (18.8%)                   | 6 (30.0%)                   | $\chi^2$ : 4.615, $p = .099$ |                                                               |
|                      | Mean $\pm$ SD<br>(min-max)  | Mean $\pm$ SD<br>(min-max)  | Mean $\pm$ SD<br>(min-max)  | $p^*$                        | $p^{**}$                                                      |
| Age                  | 26.48 $\pm$ 7.50<br>(18-45) | 32.78 $\pm$ 8.85<br>(18-45) | 32.71 $\pm$ 7.86<br>(19-45) | <b>.001</b>                  | <b>.006 (HC- UD)</b><br><b>.011 (HC- BD)</b><br>1.00 (UD- BD) |
| Education<br>(year)  | 14.85 $\pm$ 2.67<br>(5-20)  | 12.63 $\pm$ 4.82<br>(5-20)  | 15.80 $\pm$ 3.58<br>(8-23)  | .086                         |                                                               |
| BMI                  | 22.42 $\pm$ 3.58<br>(16-32) | 25.24 $\pm$ 5.45<br>(17-41) | 25.31 $\pm$ 4.62<br>(17-34) | <b>.013</b>                  | <b>.047 (HC- UD)</b><br><b>.048 (HC- BD)</b><br>1.00 (UD- BD) |
| HDRS                 | 0.42 $\pm$ 1.13<br>(0-5)    | 21.69 $\pm$ 4.07<br>(18-32) | 22.00 $\pm$ 6.99<br>(4-35)  | <b>.000</b>                  | <b>.000 (HC- UD)</b><br><b>.000 (HC- BD)</b><br>1.00 (UD- BD) |
| YMRS                 | 0.17 $\pm$ 0.60<br>(0-3)    | 1.38 $\pm$ 1.43<br>(0-4)    | 1.38 $\pm$ 1.91<br>(0-6)    | <b>&lt; .001</b>             | <b>.000 (HC- UD)</b><br><b>.004 (HC- BD)</b><br>1.00 (UD- BD) |

|                  |                                                                                                     |                                                                                                     |                                                                                                     |        |                                                 |
|------------------|-----------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|--------|-------------------------------------------------|
| PSS*             | 22.30± 6.60<br>(11-35)                                                                              | 32.64± 6.73<br>(19-52)                                                                              | 31.50± 6.23<br>(20-42)                                                                              | < .001 | .000 (HC- UD)<br>.000 (HC- BD)<br>1.00 (UD- BD) |
| BChE_0W<br>(RLU) | 1.46x10 <sup>6</sup> ±<br>1.95x10 <sup>5</sup><br>(1.02x10 <sup>6</sup> -<br>1.83x10 <sup>6</sup> ) | 1.22x10 <sup>6</sup> ±<br>1.67x10 <sup>5</sup><br>(9.57x10 <sup>5</sup> -<br>1.57x10 <sup>6</sup> ) | 1.26x10 <sup>6</sup> ±<br>1.79x10 <sup>5</sup><br>(1.02x10 <sup>6</sup> -<br>1.56x10 <sup>6</sup> ) | < .001 | .000 (HC- UD)<br>.003 (HC- BD)<br>1.00 (UD- BD) |

Notes. HC: Healthy Controls; UD: Unipolar Depression; BD: Bipolar Disorder with depressive episode; WS: Working Status; BMI: Body-Mass-Index; HDRS: Total Hamilton Depression Rating Score; YMS: Total Young Mania Score; PSS: Total Perceived Stress Score; BChE\_0W: The Total Luminescence Score of BCC at 0th week. For perceived stress score, n=41 for healthy controls, n=25 for unipolar depression, n=17 for bipolar disorder with depressive episode. *p*\*: *Kruskal-Wallis test*; *p*\*\*: *Bonferroni correction*

The multiple linear regression model constitutes a statistical method employed to elucidate the association between a singular continuous variable and two or more predictor variables, which may themselves be either continuous or categorical. Regression models are based on five major assumptions: independence, linearity, normality, homoscedasticity, and absence of outliers.<sup>161</sup> Every observation should be independent, and there should be a linear relationship between the dependent and independent variables. The independent variables in multivariate linear regression shouldn't correlate too strongly. The dependent variable's distribution needs to be normal for every value of the independent variable. For any value of the independent variable, the variance of the dependent variable's distribution should remain constant. Lastly, there should be no outliers.

After observing that the distribution of age and BMI is not the same across of groups, each of the preliminary assumptions were checked and multiple linear regression analysis was used to test if age, BMI, and group significantly predicted BChE\_0W. First, the group was re-coded by nonclinical (health control) and clinical (unipolar depression + bipolar disorder with unipolar depression), the result of the regression indicated that model explained 37.6% of the variance and the model was a significant predictor of the total luminescence score at BChE\_0W ( $F(3, 96) = 19.25, p < .001$ ). The fitted model was:  $BChE\_0W = (1821474.34 \pm 98503.36) + (-154973.52 \pm 37972.07) * clinical\_group + (-6287.05 \pm 2230.84) * age + (-8820.57 \pm 3999.12) * BMI$ . All predictors were found as

statistically significant:  $p < .001$  for group,  $p = .006$  for age,  $p = .030$  for BMI. Standardized coefficients ( $\beta$ ) for each predictors as following: -.364, -.252, and -.192 respectively.

**Table 3.2:** *Multivariate linear regression model to assess predictors of BChE\_0W by dividing group with clinical and non-clinical samples*

| Model          | B          | Coefficients Std. Error | p     |
|----------------|------------|-------------------------|-------|
| (Constant)     | 1821474.34 | 98503.36                | <.001 |
| Clinical_group | -154973.52 | 37972.07                | <.001 |
| Age            | -6287.05   | 2230.84                 | .006  |
| BMI            | -8820.57   | 3999.12                 | .030  |

Second, because of group has three condition (health control, unipolar depression, and bipolar disorder with depressive episode), we recoded group variables by replacing “group” variable with new introduced 2 variables named as being\_UD, and being\_BD. The result of the regression indicated that model explained 38.3% of the variance and the model was a significant predictor of the total luminescence score at BChE\_0W ( $F(4, 95) = 14.74$ ,  $p < .001$ ). The fitted model was:  $BChE\_0W = (1822907.02 \pm 98437.91) + (-6310.00 \pm 2229.25) *age + (-8857.37 \pm 3896.24) *BMI + (-174856.23 \pm 42247.05) *being\_UD + (-122504.18 \pm 48580.50) *being\_BD$ . All predictors were found as statistically significant:  $p = .006$  for age,  $p = .029$  for BMI,  $p < .001$  for being\_UD, and  $p = .013$  for being\_BD. Standardized coefficients ( $\beta$ ) for each predictors as following: -.253, -.193, -.383, and -.230 respectively.

**Table 3.3:** *Multivariate linear regression model to assess predictors of BChE\_0W.*

| Model      | B          | Coefficients Std. Error | p     |
|------------|------------|-------------------------|-------|
| (Constant) | 1822907.02 | 98437.91                | <.001 |
| Age        | -6310.00   | 2229.25                 | .006  |
| BMI        | -8857.37   | 3896.24                 | .029  |
| Being_UD   | -174856.23 | 42247.05                | <.001 |
| Being_BD   | -122504.18 | 48580.50                | .013  |

A Pearson correlation coefficient was computed to determine the association between BChE\_0W with psychometric scales namely between Total Hamilton Depression Score and BChE\_0W, and between Total Perceived Stress Score and

BChE\_0W. The results indicate that there is a significant negative correlation between HDRS and BChE\_0W, [ $r(98) = -.496, p < .001$ ] and a significant positive correlation between HDRS and PSS, [ $r(81) = .618, p < .001$ ]. However, the negative correlation between PSS and BChE\_0W is not significant, [ $r(81) = -.041, p = .712$ ].

**Table 3.4:** Correlation table of BChE\_0W, HDRS, and PSS

|           | 1       | 2      |
|-----------|---------|--------|
| 1 BChE_0W |         |        |
| 2 HDRS    | -.496** |        |
| 3 PSS     | -.041   | .618** |

Notes. N=100 for BChE\_0W and HDRS, n= 83 for PSS; \*\*  $p < .01$

When the Pearson correlation coefficient was determined in the only clinical group, the correlations between Total Hamilton Depression Score, Total Perceived Stress Score, and BChE\_0W changed. The results indicate that a non-significant positive correlation between HDS and PSS, [ $r(40) = .190, p = .229$ ], and also there is a non-significant negative correlation between HDS and BChE\_0W, [ $r(50) = -.039, p = .782$ ]. However, the significant positive correlation between PSS and BChE\_0W was found, [ $r(40) = .359, p = .019$ ].

**Table 3.5:** Correlation table of BChE\_0W, HDRS, and PSS only for clinical group

|           | 1     | 2    |
|-----------|-------|------|
| 1 BChE_0W |       |      |
| 2 HDRS    | -.039 |      |
| 3 PSS     | .359* | .190 |

Notes. N=52 for BChE\_0W and HDRS, n= 42 for PSS; \*  $p < .05$

A Pearson correlation coefficient was computed to determine the association between BChE\_0W with subitems of HDRS. Results indicate a significant negative correlation between BChE\_0W and Hamilton#1, [ $r(92) = -.467, p < .001$ ], BChE\_0W and Hamilton#2, [ $r(92) = -.429, p < .001$ ], BChE\_0W and Hamilton#3, [ $r(92) = -.374, p < .001$ ], BChE\_0W and Hamilton#6, [ $r(92) = -.310, p = .002$ ], BChE\_0W and Hamilton#7, [ $r(92) = -.398, p < .001$ ], BChE\_0W and Hamilton#8, [ $r(92) = -.334, p = .001$ ], BChE\_0W and Hamilton#9, [ $r(92) = -.400, p < .001$ ], BChE\_0W and Hamilton#10, [ $r(92) = -.393, p < .001$ ], BChE\_0W and Hamilton#11, [ $r(92) = -.542, p < .001$ ], BChE\_0W and Hamilton#12, [ $r(92) = -.397, p < .001$ ], BChE\_0W and Hamilton#13, [ $r(92) = -.336, p < .001$ ], BChE\_0W and Hamilton#16, [ $r(92) = -.337, p < .001$ ]. Detailed table was provided in Appendix C.

When the correlation coefficient between BChE\_0W with subitems of HDRS was determined only for clinical group, the findings were changed. There was a significant negative correlation between BChE\_0W and HDRS suicide item (#11), [ $r(50) = -.364$ ,  $p=.008$ ] and a significant positive correlation between BChE\_0W and HDRS somatic anxiety item (#13), [ $r(50) = .277$ ,  $p=.047$ ]. Detailed table was provided in Appendix D.

A Pearson correlation coefficient was computed to determine the association between BChE\_0W with subitems of PSS. Results indicate a significant positive correlation between BChE\_0W and PSS#4, [ $r(85) = .333$ ,  $p=.002$ ], BChE\_0W and PSS#11, [ $r(86) = .235$ ,  $p=.030$ ], and a significant negative correlation between BChE\_0W and PSS#14, [ $r(85) = -.225$ ,  $p=.039$ ]. Detailed table was provided in Appendix E.

When the correlation coefficient between BChE\_0W with subitems of PSS was determined only for clinical group, the findings were changed. There was a significant negative correlation between BChE\_0W and PSS#7, [ $r(44) = -.369$ ,  $p=.014$ ] and a significant positive correlation between BChE\_0W and PSS#11, [ $r(44) = .430$ ,  $p=.004$ ]. Detailed table was provided in Appendix F.

On the 8 week follow up, HDRS total score was followed for 15 patients ( $n=15$ : 11 UD, 4 BD) and HDRS scores significantly decreased in the follow up ( $p<.001$ ). While the mean total score of Hamilton Depression Rating Scale was  $21.73 \pm 4.46$  (min:18, max:30) at the baseline, the mean was decreased to  $2.33 \pm 2.47$  (min:0, max:7) at 8<sup>th</sup> week.

When the change in the level of BChE in clinical group ( $n=15$ : 11 UD, 4 BD) between baseline and the 8<sup>th</sup> week was investigated, a significant increase was found ( $n=15$ ,  $p<.001$ ) as shown in Figure 3.7. The mean of the total luminescence BChE at baseline was  $1210869.42 \pm 150564.18$  (min: 974342.00, max:1496104.00). After the treatment, the following mean of total luminescence BChE at 8<sup>th</sup> week for those group was determined as  $1515727.29 \pm 1433771.98$  (min: 1254927.00, max: 1749392.00).

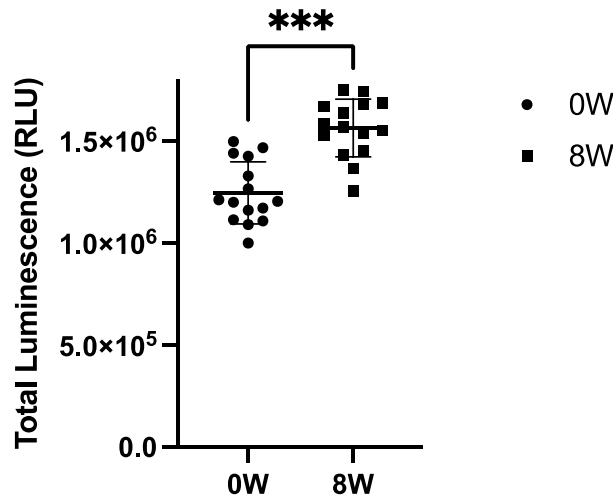


Figure 3.6: Total luminescence score was increased with remission, comparison of 0<sup>th</sup> Week and 8<sup>th</sup> week ( $n=15$ , number of replicates=3,  $p<.001$ ).

The linear mixed model was applied to investigate whether the change between BChE\_0W and BChE\_8W is significantly predicted by the change between HDRS and follow up HDRS or not. Therefore, total luminescence of BChE was included as dependent variable, time was added as factors, HDRS was added as covariates, and ID was added as cluster variables. In the model, time and HDRS was included as fixed effects, whereas intercept | id was included as random coefficients. Overall, model specification as follows:  $BChE \sim 1 + Time + HDRS + (1 | ID)$ . Fixed effect results revealed a significance in intercept ( $p<.001$ ) as expected because the baseline level of BChE luminescence that is not zero. Furthermore, there is no significance in time ( $p=.913$ ) underscores that the passage of time does not significantly influence BChE activity over the 8-week period. Also, a negative significance in HDRS ( $p=.024$ ) was found which means that as the depression severity of patients (HDRS) decreases, BChE luminescence score tends to increase. This finding supports the idea that improvements in depressive symptoms are associated with increases in BChE activity. Also, the small SD and variance for ID suggest minimal variability in BChE activity between individuals. The extremely low intraclass correlation coefficient (ICC) suggests negligible clustering effect, indicating that BChE activity is not significantly influenced by ID.

**Table 3.6:** Fixed effects parameters estimates for mixed linear analysis of BChE\_0W - BChE\_8W with HDRS.

| Estimates of Fixed Effects |                      |            |                         |                      |             |
|----------------------------|----------------------|------------|-------------------------|----------------------|-------------|
| Parameter                  | Estimate             | Std. Error | 95% Confidence Interval |                      | p           |
|                            |                      |            | Lower Bound             | Upper Bound          |             |
| Intercept                  | 1.41x10 <sup>6</sup> | 24501      | 1.36x10 <sup>6</sup>    | 1.45x10 <sup>6</sup> | <.001       |
| Time                       | 15753                | 144940     | -268325                 | 299830               | .913        |
| HDRS                       | -15818               | 7031       | -29599                  | -2037                | <b>.024</b> |

Notes. Random components: SD for ID is  $6.41 \times 10^{-5}$ , SD for residual is 134196. Variance for ID is  $4.11 \times 10^{-9}$ , variance for residual is  $1.80 \times 10^{10}$ . ICC is  $2.28 \times 10^{-19}$ . Number of Observation: 30, groups: ID 15.

The same analysis was computed only for subitem of HDRS suicide item (#11) where the model is BChE ~ 1 + Time + HDRS suicide item + (1 | ID). Initially, a significant effect of time on BChE activity ( $p=.031$ ). However, in the post-hoc comparison, the difference found between time points was not significant after Bonferroni correction ( $p = .051$ ). This indicates that while there may be a trend over time, the specific differences between time points may not be statistically significant. Also, a negative significance in HDRS suicide item ( $p=.004$ ) was found which means that as the severity of HDRS suicide item decreases, BChE luminescence score tends to increase. Small SD and variance for ID suggest minimal variability in BChE activity between individuals. The extremely low ICC suggests negligible clustering effect, indicating that BChE activity is not significantly influenced by ID.

**Table 3.7:** Fixed effects parameters estimates for mixed linear analysis of BChE\_0W - BChE\_8W with HDRS suicide item

| Estimates of Fixed Effects |                      |            |                         |                      |             |
|----------------------------|----------------------|------------|-------------------------|----------------------|-------------|
| Parameter                  | Estimate             | Std. Error | 95% Confidence Interval |                      | p           |
|                            |                      |            | Lower Bound             | Upper Bound          |             |
| Intercept                  | 1.41x10 <sup>6</sup> | 23319      | 1.36x10 <sup>6</sup>    | 1.45x10 <sup>6</sup> | <.001       |
| Time                       | 158146               | 73470      | 14148                   | 302144               | <b>.031</b> |
| HDRS suicide item          | -91376               | 31539      | -153191                 | -29562               | <b>.004</b> |

Notes. Random components: SD for ID is  $1.72 \times 10^{-4}$ , SD for residual is 127721. Variance for ID is  $2.96 \times 10^{-8}$ , variance for residual is  $1.63 \times 10^{10}$ . ICC is  $1.81 \times 10^{-18}$ . Number of Observation: 30, groups: ID 15.

**Table 3.8:** Post Hoc comparison for mixed linear analysis of BChE\_0W - BChE\_8W with HDRS suicide item

| Post Hoc Comparisons |         |            |       |                         |
|----------------------|---------|------------|-------|-------------------------|
| Comparison           |         | Difference | SE    | $P_{\text{bonferroni}}$ |
| Time                 | Time    |            |       |                         |
| BChE_0W              | BChE_8W | -158146    | 76770 | .051                    |

Lastly, the mixed linear analysis was computed only for subitem of HDRS somatic anxiety item (#13) where the model is  $BChE \sim 1 + \text{Time} + \text{HDRS somatic anxiety item} + (1 | \text{ID})$ . A significant effect of time on BChE activity ( $p < .001$ ) and the difference found between time points was also significant after Bonferroni correction ( $p < .001$ ). This indicates that there are significant differences in BChE activity over time. A positive effect of change in HDRS somatic anxiety item was not found significant ( $p = .389$ ) means alterations in HDRS somatic anxiety item are not strongly associated with changes in BChE activity. Besides minimal changes, same results were valid for random variables.

**Table 3.9:** Fixed effects parameters estimates for mixed linear analysis of BChE\_0W - BChE\_8W with HDRS somatic anxiety item

| Estimates of Fixed Effects |                    |            |                         |                    |       |
|----------------------------|--------------------|------------|-------------------------|--------------------|-------|
| Parameter                  | Estimate           | Std. Error | 95% Confidence Interval |                    | $p$   |
|                            |                    |            | Lower Bound             | Upper Bound        |       |
| Intercept                  | $1.41 \times 10^6$ | 26339      | $1.35 \times 10^6$      | $1.46 \times 10^6$ | <.001 |
| Time                       | 374360             | 79890      | 217778                  | 530943             | <.001 |
| HDRS somatic anxiety item  | 29848              | 34651      | -38067                  | 97764              | .389  |

Notes. Random components: SD for ID is  $6.63 \times 10^{-3}$ , SD for residual is 144264. Variance for ID is  $4.39 \times 10^{-5}$ , variance for residual is  $2.08 \times 10^{10}$ . ICC is  $2.11 \times 10^{-15}$ . Number of Observation: 30, groups: ID 15.

**Table 3.10:** Post Hoc comparison for mixed linear analysis of BChE\_0W - BChE\_8W with HDRS somatic anxiety item

| Post Hoc Comparisons |         |            |       |                         |
|----------------------|---------|------------|-------|-------------------------|
| Comparison           |         | Difference | SE    | $P_{\text{bonferroni}}$ |
| Time                 | Time    |            |       |                         |
| BChE_0W              | BChE_8W | -374360    | 83069 | <.001                   |

### 3.3 Examination of the Relationship Between BChE Levels with Chronic Stress and Animal Behavior

#### 3.3.1 Optimization of BCC in Rat Plasma

Capacity of BCC to monitor BChE activity in the rat plasma environment was also investigated. First, whether BCC generates a luminescence signal in rat plasma or not (*proof of principle*) was checked. The time-dependent intensity of the relative luminescence unit of 0.005% rat plasma, 10 $\mu$ M BCC (0.5% DMSO), and 0.005% rat plasma with 10 $\mu$ M BCC (0.5% DMSO) was compared. Like human serum experiments, while there is negligible luminescence signal detected from solely 0.005% rat plasma and solely 10 $\mu$ M BCC (0.5% DMSO), the luminescence signal was quickly observed in their mixture and reached the peak at 30 min (60-fold difference) as shown in Figure 3.7.a. Additionally, when their total luminescence was compared, 0.005% rat plasma + 10 $\mu$ M BCC was 60-fold greater than 0.005% rat plasma and 50-fold greater than 10 $\mu$ M BCC, respectively (Figure 3.7.b).

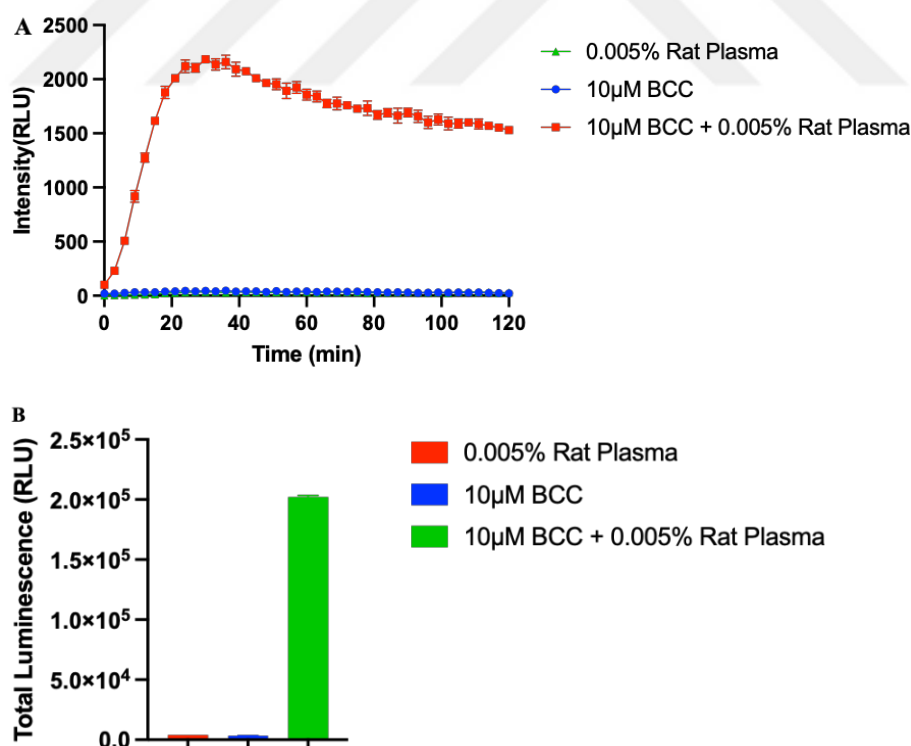


Figure 3.7: Proof of principle of BCC in rat plasma environment. Time dependent (a) and total luminescence (b) comparison of 0.005% rat plasma, 10 $\mu$ M BCC, and 0.005% rat plasma with 10 $\mu$ M BCC (number of technical replicates = 3). While there is negligible luminescence in control groups, there is more than 50-fold increase in their mixture

Upon promising response of BCC activity in rat plasma, a dose-dependent titration of rat plasma (0.005-2%) was done with 10 $\mu$ M BCC (0.5% DMSO in 100 $\mu$ L) for 120 min. As shown in Figure 3.8.a, the peak signal was observed at 30 min in the case of lower concentration. However, it shifted to the 36 min as the concentration increases.

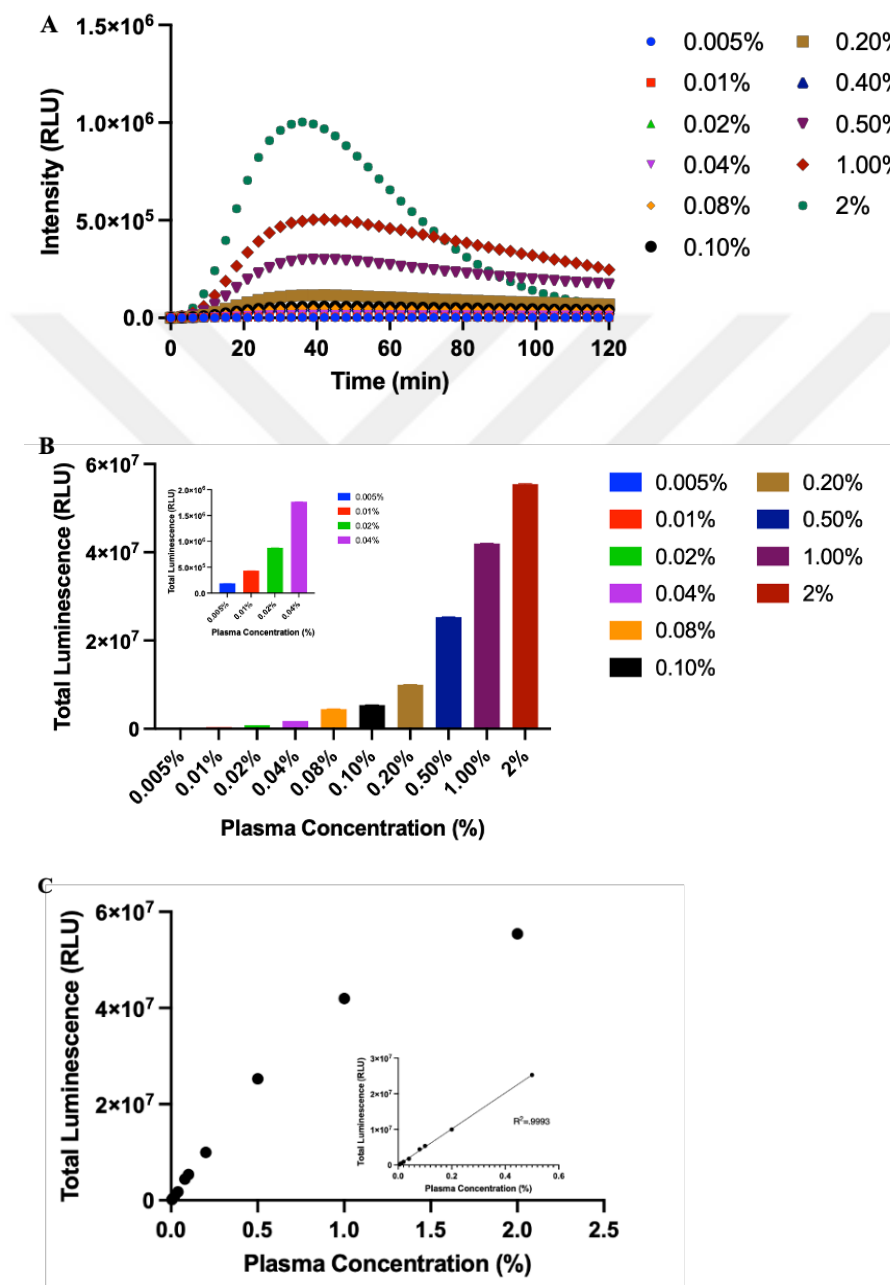


Figure 3.8: Dose-dependent titration in the range of 0.005%-2% rat plasma with 10 $\mu$ M BCC. Time-dependent luminescence (a), total luminescence score (b), and calibration curve (c) of BCC (number of technical replicates= 3). BCC is responsive to the rat plasma concentration with high sensitivity.

In addition, the total luminescence of BCC in different concentrations of rat plasma as well as its corresponding simple linear regression were examined (Figure 3.8.b and Figure 3.8.c). It was determined that BCC shows perfect linearity between 0.005%-0.5% concentration ( $R^2=0.9963$ ), then becomes gradually saturated.

The rat plasma was first treated with donepezil (0-10mM) and subsequently tacrine (0-100mM) at different concentrations to verify that the luminescence signal's source is BChE. As shown in Figure 3.9 and Figure 3.10, the progressive decrease in BCC's total luminescence with increasing inhibitor concentration confirms that the luminescence signal originated from BChE.

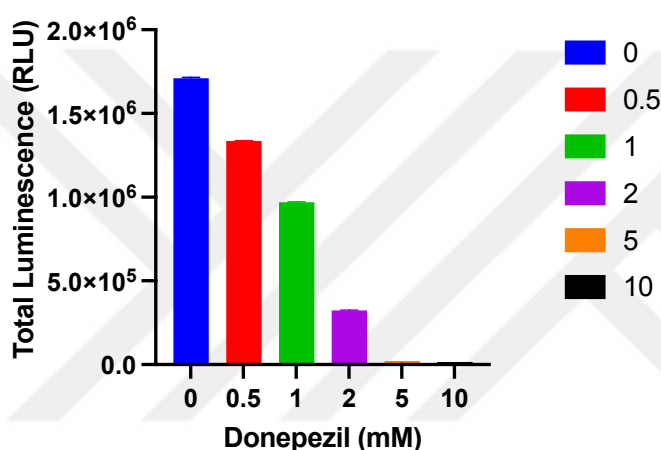


Figure 3.9: Total luminescence of 10 $\mu$ M BCC in 0.05% rat plasma treated with different concentrations (0-10 mM) of donepezil (number of technical replicates= 3). The fact that decreasing of luminescence signal with increasing of donepezil concentration indicates cholinesterase-derived luminescence signal.

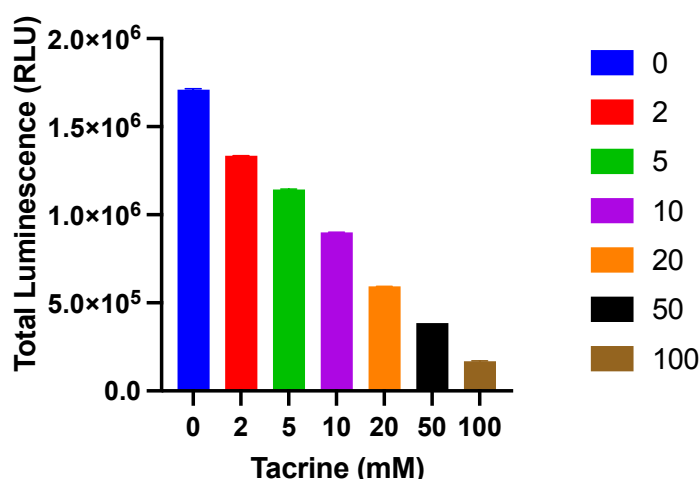


Figure 3.10: Total luminescence of 10 $\mu$ M BCC in 0.05% rat plasma treated with different concentrations (0-100 mM) of tacrine (number of technical replicates= 3). The fact that decreasing of luminescence signal with increasing of tacrine concentration indicates BChE-derived luminescence signal.

### 3.3.2 Monitoring BChE Activity in CUMS exposed Rat Plasma

Upon showing BCC's competency to monitor BChE activity in rat plasma environment, 1% rat plasma (diluted with DPBS) from CUMS study was analyzed with 20 $\mu$ M (1% DMSO in 100 $\mu$ L) BCC for 2h. Total luminescence score based on groups (HC, UD, and BD) was provided in Figure 3.5. Total luminescence score for each rat plasma sample was provided at Appendix G. A new variable named as BChE was created and each total luminescence score added to the SPSS.

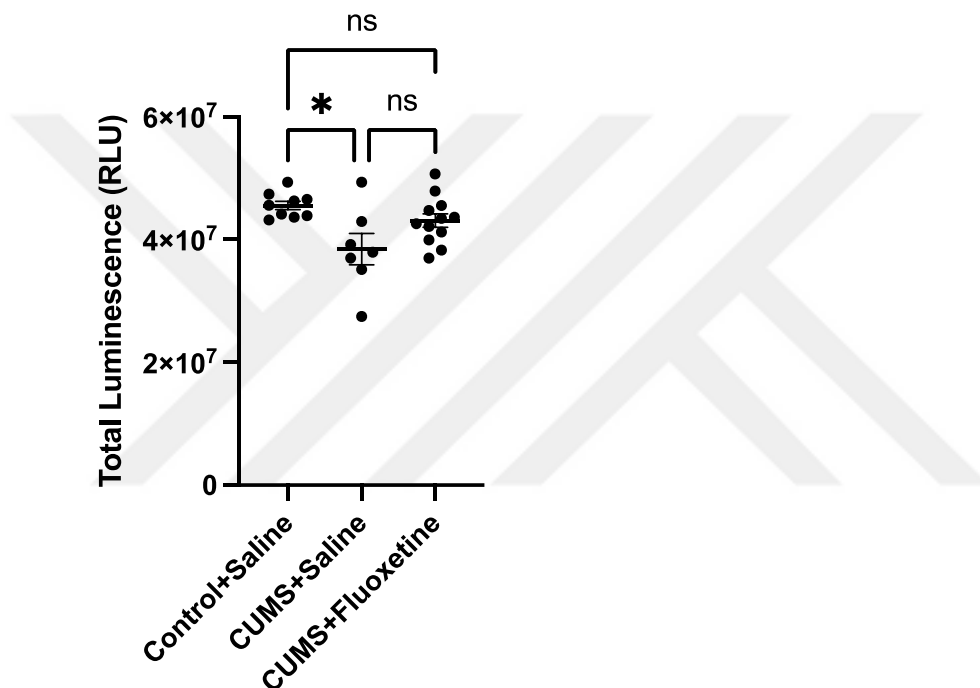


Figure 3.11: Total luminescence score comparison of Control+Saline, CUMS+Saline, and CUMS+Fluoxetine plasma samples. There is a significant difference between Control+Saline and CUMS+Saline ( $p=.011$ ). There is no significant difference between Control+Saline and CUMS+Fluoxetine ( $p=.323$ ) and CUMS+Saline and CUMS+Fluoxetine ( $p=.345$ ) ( $n=28$ , number of technical replicates= 3)

### 3.3.3 Sucrose Preference Test (SPT)

4 rats were excluded from the study by the researchers. According to the report, ID5 from CUMS+Saline group was excluded from the study because of resilience to the chronic stress. ID19 from CUMS+Fluoxetine group was excluded from low total intake, ID25 and ID26 from CUMS+Fluoxetine group was excluded from resistance to the fluoxetine treatment. After exclusion of 4 rats, new sample composition was 9 Control+Saline, 7 CUMS+Saline, and 12 CUMS+Fluoxetine groups.

The comparison of the groups for the Sucrose Preference Test was done by Kruskal-Wallis Test. As shown in Figure 3.12, there was a significant difference between Control+Saline ( $91.41 \pm 2.87$ ) and CUMS+Saline ( $83.25 \pm 3.90$ ),  $p=.011$ . However, CUMS+Fluoxetine ( $86.57 \pm 6.92$ ) did not show any significance compared to Control+Saline ( $p=.437$ ) or CUMS+Fluoxetine ( $p=.249$ ).

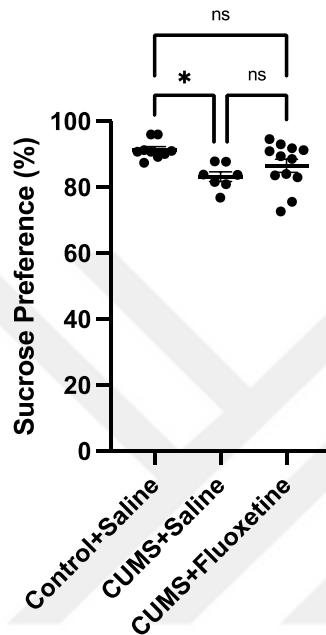


Figure 3.12: Sucrose preference % comparison by group (n=28). Rats exposed to CUMS have significantly lower sucrose preference% compared to Control+Saline ( $p=.011$ ).

### 3.3.4 Forced Swim Test (FST)

The Forced Swim Test scores for groups were compared for immobile, swimming, and climbing time. When immobility time was compared between groups, as shown in Figure 3.13, Control+Saline ( $71.2 \pm 8.48$ ) showed lower immobility compared to CUMS+Saline ( $95.5 \pm 13.7$ ) and CUMS+Fluoxetine ( $84.5 \pm 10.9$ ) but no significant difference between groups was found ( $p=.139$ ).

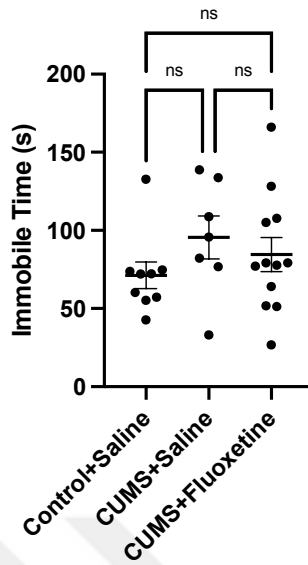


Figure 3.13: Comparison of immobility time by groups. No significance difference between groups was found ( $n=28$ ,  $p=.139$ ).

When swimming time was compared between groups, as shown in Figure 3.14, a significant difference between groups was found ( $p=.001$ ). According to the post hoc comparison, there is a significant difference between Control+Saline ( $145.0 \pm 10.4$ ) and CUMS+Fluoxetine ( $71.5 \pm 8.87$ ) was found ( $p=.001$ ). On the other hand, there is no significant difference between CUMS+Saline ( $123.0 \pm 11.3$ ) with Control+Saline ( $145.0 \pm 10.4$ ) ( $p=.936$ ) or CUMS+Fluoxetine ( $71.5 \pm 8.87$ ) ( $p=.052$ ).

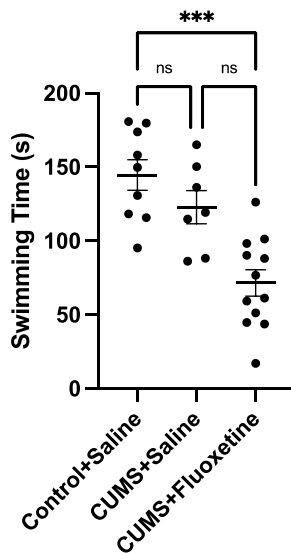


Figure 3.14: Swimming time comparison by group ( $n=28$ ). Control+Saline group has significant higher swimming time compared to CUMS+Fluoxetine group ( $p=.001$ ).

When climbing time was compared between groups, as shown in Figure 3.15, a significant difference between groups was found ( $p=.007$ ). According to the post hoc comparison, there is a significant difference between a significant difference between Control+Saline ( $84.15 \pm 10.91$ ) and CUMS+Fluoxetine ( $144.0 \pm 14.66$ ) ( $p=.028$ ). Also, there is a significant difference between CUMS+Saline ( $81.57 \pm 9.42$ ) and CUMS+Fluoxetine ( $144.0 \pm 14.66$ ) ( $p=.021$ ). However, there is no significant difference between Control+Saline ( $84.15 \pm 10.91$ ) and CUMS+Saline ( $81.57 \pm 9.42$ ) ( $p>.99$ ).

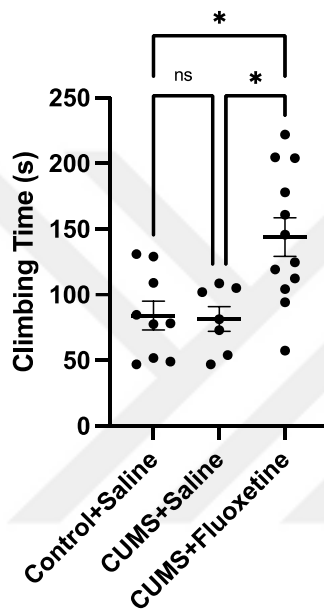


Figure 3.15: Climbing Time Comparison by Group (n=28). CUMS+Fluoxetine group has significantly higher climbing time compared to Control+Saline ( $p=.028$ ) and CUMS+Saline ( $p=.021$ ).

### 3.3.5 Weight% Change

By accepting baseline as 100, weight% change per week compared to the weight at 0<sup>th</sup> were calculated for between groups.

When weight% changes of the groups compared to the 2nd week are compared, a significant difference between groups was found ( $p<.001$ ). According to the post hoc comparison, there is a significant difference between Control+Saline ( $156.2 \pm 7.85$ ) and CUMS+Fluoxetine ( $103.2 \pm 0.79$ ) ( $p<.001$ ). However, there is no significance between Control+Saline and CUMS+Saline ( $106.5 \pm 1.64$ ) ( $p=.0272$ ) and between CUMS+Saline and CUMS+Fluoxetine ( $p=.173$ ).

When weight% changes of the groups compared to the 3rd week are compared, a significant difference between groups was found ( $p<.001$ ). According to the post hoc

comparison, Control+Saline ( $186.1 \pm 6.39$ ) significantly gained more weight than CUMS+Fluoxetine ( $96.80 \pm 0.89$ ) ( $p < .001$ ). Also, there is a significant difference between CUMS+Saline ( $106.9 \pm 0.78$ ) and CUMS+Fluoxetine ( $96.80 \pm 0.89$ ) ( $p = .046$ ). No significant between Control+Saline and CUMS+Saline was found ( $p = .161$ ).

When weight% changes of the groups compared to the 4th week are compared, a significant difference between groups was found ( $p < .001$ ). According to the post hoc comparison, Control+Saline ( $205.1 \pm 8.12$ ) significantly gained more weight than CUMS+Fluoxetine ( $99.78 \pm 2.59$ ) ( $p < .001$ ). However, there is no significance between Control+Saline and CUMS+Saline ( $112.3 \pm 1.38$ ) ( $p = .090$ ) and between CUMS+Saline and CUMS+Fluoxetine ( $p = .129$ ).

When weight% changes of the groups compared to the 5th week are compared, a significant difference between groups was found ( $p < .001$ ). According to the post hoc comparison, Control+Saline ( $222.8 \pm 9.78$ ) significantly gained more weight than CUMS+Fluoxetine ( $96.88 \pm 1.39$ ) ( $p < .001$ ). Moreover, there is a significant difference between and CUMS+Saline ( $115.2 \pm 1.15$ ) and CUMS+Fluoxetine ( $p = .046$ ). No significant between Control+Saline and CUMS+Saline was found ( $p = .161$ ).

Overall weight% changes of the groups compared to the baseline between 2<sup>nd</sup> week to 5<sup>th</sup> week was given at Figure 3.16.

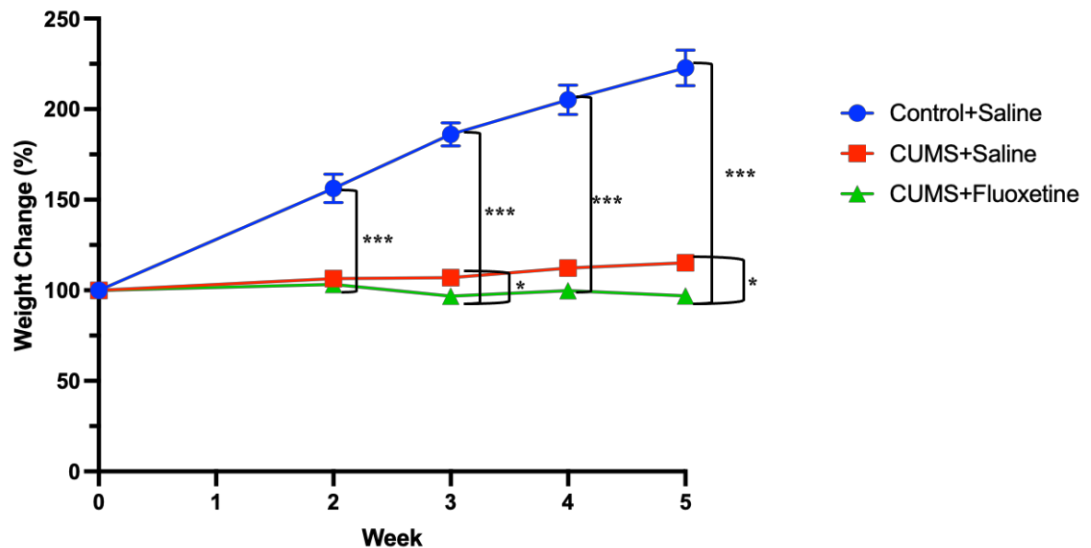


Figure 3.16: Overall weight% changes of the groups compared to baseline between 2<sup>nd</sup> week to 5<sup>th</sup> week. Control+Saline significantly gained more weight than CUMS+Fluoxetine group for each week comparison. CUMS+Saline group significantly gained more weight than CUMS+Fluoxetine group for 2<sup>nd</sup> and 5<sup>th</sup> week. For \*  $p < .05$ ; for \*\*\*  $p < .001$ .

A Pearson correlation coefficient was computed to determine the association between total luminescence score of BChE in rat plasma for each rat with behavioral test parameters namely sucrose preference%, immobility time, swimming time, and climbing time. The results indicate that there is a significant positive correlation between BChE score and sucrose preference%, [ $r(28) = .544, p = .003$ ]. However, there is no significant correlation was found BChE score with immobility ( $p = .184$ ), climbing ( $p = .448$ ), and swimming ( $p = .875$ ).

**Table 3.11:** Correlation table of BChE total luminescence score with behavioral test parameters

|                          | 1      | 2     | 3       | 4       |
|--------------------------|--------|-------|---------|---------|
| 1 BChE total lum. score  |        |       |         |         |
| 2 Sucrose preference (%) | .544** |       |         |         |
| 3 FST immobile time      | .259   | -.013 |         |         |
| 4 FST climbing time      | -.150  | -.078 | -.487** |         |
| 5 FST swimming time      | -.031  | .097  | -.221   | -.744** |

Note: N=28; \*\*  $p < .01$

### 3.3.6 Age-Behavioral Measurements of Animal Characteristics

| Groups                    | Control + SF<br>(n=9)            | CUMS + SF<br>(n=7)               | CUMS + FLX<br>(n=12)             | $p^*$ | $p^{**}$             |
|---------------------------|----------------------------------|----------------------------------|----------------------------------|-------|----------------------|
| Age (day)                 | 47.44 ± 10.3<br>(42.0-49.0)      | 78.00 ± 2.12<br>(73.5-84.0)      | 79.63 ± 1.07<br>(73.5-84.0)      | <.001 | .004<br>.000<br>1.00 |
| Sucrose<br>Preference (%) | 91.41 ± 0.96<br>(87.43-96.08)    | 83.25 ± 1.48<br>(76.84-87.84)    | 86.57 ± 2.00<br>(72.73-94.59)    | .014  | .011<br>.437<br>.248 |
| Immobility (s)            | 71.22 ± 8.48<br>(42.67-132.67)   | 95.52 ± 13.74<br>(33.00-138.67)  | 84.50 ± 10.88<br>(26.67-166.00)  | .139  | -                    |
| Swimming (s)              | 144.63 ± 10.36<br>(95.33-180.67) | 122.90 ± 11.25<br>(86.33-165.00) | 71.53 ± 8.87<br>(17.00-126.33)   | <.001 | .936<br>.001<br>.052 |
| Climbing (s)              | 84.15 ± 10.91<br>(47.00-131.00)  | 81.57 ± 9.42<br>(47.00-108.67)   | 143.97 ± 14.65<br>(57.33-222.00) | .007  | 1.00<br>.028         |

|            |                                  |                                  |                                 |                 |             |
|------------|----------------------------------|----------------------------------|---------------------------------|-----------------|-------------|
|            |                                  |                                  |                                 |                 | <b>.021</b> |
|            |                                  |                                  |                                 |                 | .272        |
| Week2 (%)  | 156.23 ± 7.85<br>(103.03-189.52) | 106.52 ± 0.62<br>(105.49-110.00) | 103.24 ± 0.79<br>(98.82-107.41) | <b>&lt;.001</b> | <b>.000</b> |
|            |                                  |                                  |                                 |                 | .173        |
|            |                                  |                                  |                                 |                 | .161        |
| Week3 (%)  | 186.10 ± 6.39<br>(173.06-227.42) | 106.92 ± 0.78<br>(103.61-110.29) | 96.80 ± 0.89<br>(92.47-102.53)  | <b>&lt;.001</b> | <b>.000</b> |
|            |                                  |                                  |                                 |                 | <b>.046</b> |
|            |                                  |                                  |                                 |                 | .090        |
| Week4 (%)  | 205.14 ± 8.12<br>(188.08-254.84) | 112.28 ± 1.38<br>(109.72-118.86) | 99.78 ± 2.59<br>(91.53-126.13)  | <b>&lt;.001</b> | <b>.000</b> |
|            |                                  |                                  |                                 |                 | .129        |
|            |                                  |                                  |                                 |                 | .161        |
| Week5 (%)  | 222.78 ± 7.78<br>(201.55-282.26) | 115.24 ± 1.15<br>(112.58-121.43) | 96.88 ± 1.39<br>(88.12-102.17)  | <b>&lt;.001</b> | <b>.000</b> |
|            |                                  |                                  |                                 |                 | <b>.046</b> |
|            | 4.55x10 <sup>9</sup> ±           | 3.84x10 <sup>9</sup> ±           | 4.55x10 <sup>9</sup> ±          |                 | <b>.011</b> |
| BChE (RLU) | 6.80x10 <sup>7</sup>             | 2.54x10 <sup>8</sup>             | 6.80 x10 <sup>7</sup>           | <b>.015</b>     | .323        |
|            | (4.32– 4.93)x10 <sup>9</sup>     | (2.75–4.93)x10 <sup>9</sup>      | (4.32– 4.93)x10 <sup>9</sup>    |                 | .345        |

Notes. Control+SF: Control+Saline; CUMS+SF: CUMS+Saline; CUMS+FLX: CUMS+Fluoxetine. For each comparison non-parametric Kruskal-Wallis test was used. For each significant analysis, Bonferroni multiple correction was applied. *P*\*: Kruskal-Wallis; *p*\*\*: Bonferroni correction. *P*\*\* value orders are Control+SF - CUMS+SF, Control+SF – CUMS+FLX, and CUMS+SF – CUMS+FLX. Data were presented as mean±SEM (min-max).

After observing that the distribution of starting age by day is not the same across of groups, each of the preliminary assumptions were checked and multiple linear regression analysis was used to test if age and sucrose preference% significantly predicted BChE\_score. When predictors were assessed as starting age by day and sucrose preference%, the regression indicated that model explained 32.9% of the variance and the model was a significant predictor of the total luminescence score at BChE\_0W ( $F(2, 25) = 6.14, p = .007$ ). The fitted model was:  $BChE\_score = (1.618 \times 10^9 \pm 1.686 \times 10^9) + (-6.961 \times 10^6 \pm 6.216 \times 10^6) * \text{starting age} + (3.589 \times 10^7 \pm 1.622 \times 10^7) * \text{sucrose preference\%}$ . Only sucrose preference% was found as statistically significant,  $p = .036$ . Standardized coefficients ( $\beta$ ) for each predictors as following: -.217, and .428 respectively.

**Table 3.12:** Multivariate linear regression model to assess predictors of BChE score in rat plasma

| Model               | B                      | Coefficients Std. Error | p           |
|---------------------|------------------------|-------------------------|-------------|
| (Constant)          | 1.618x10 <sup>9</sup>  | 1.686x10 <sup>9</sup>   | .347        |
| Starting Age        | -6.961x10 <sup>6</sup> | 6.216x10 <sup>6</sup>   | .273        |
| Sucrose preference% | 3.589x10 <sup>7</sup>  | 1.622x10 <sup>7</sup>   | <b>.036</b> |

### 3.4 Validating the Link Between BChE Levels and Chronic Stress: Utilizing PC-12 Adh Cells and Modulating with Corticosterone, Noradrenaline and Fluoxetine

After receiving promising results in human and rat studies, their validity was determined *in vitro* experiments.

First, the toxicity of BCC in various concentration was checked with MTT cell viability assay. As shown in Figure 3.17, BCC shows no toxicity between 0-50  $\mu$ M; therefore, it is suitable for *in vitro* imaging.

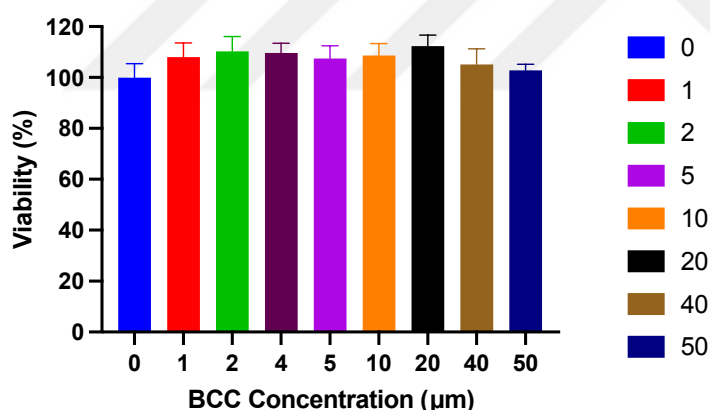


Figure 3.17: MTT Viability Assay of BCC treated PC-12 Adh (number of technical replicates=6)

Upon observing no noticeable cell toxicity between 0-50  $\mu$ M, its time-dependent and dose-dependent capacity were checked by determining relative luminescence unit response of different concentrations of BCC treated PC-12 Adh cells for 200 min. As shown in Figure 3.18.a, BCC responds quickly to presence of BChE in cell environment and shows an increasing trend about 60 min (Figure 3.18.a). Eventually, the signal gradually decreased after a plateau due to BCC consumption.

When the total luminescence signal was calculated with a function of the area under the curve, the dose-dependent increase was determined with great linearity (Figure 3.18.b and Figure 3.18.c).

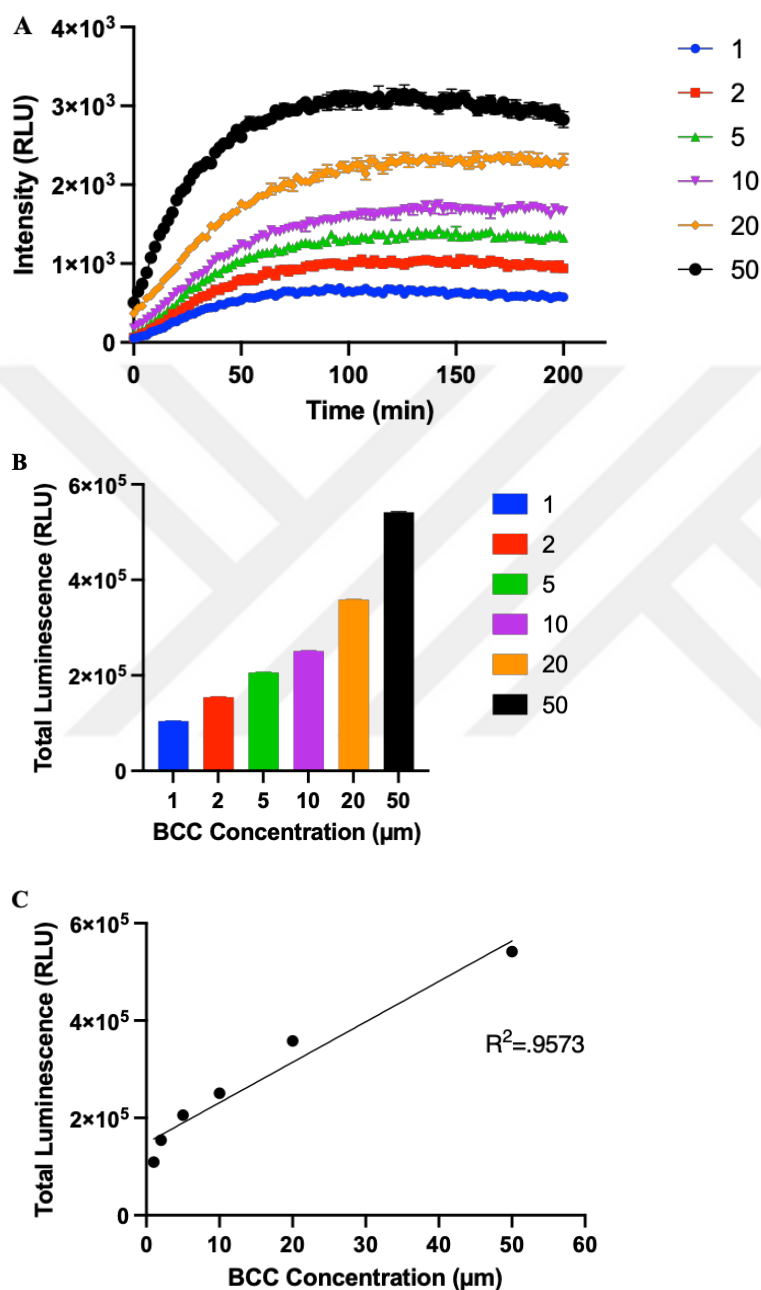


Figure 3.18: Dose-dependent titration of BCC in PC-12 Adh cells. Time-dependent luminescence (a), total luminescence score (b), and calibration curve (c) of BCC (number of technical replicates= 6). Total luminescence increases with the increasing concentration of BCC, demonstrates great linearity.

To confirm that the resource of the luminescence signal is only derived from endogenous BChE, the cell is treated with various concentration of tacrine. As shown in

Figure 3.19, the total luminescence was gradually decreased with increasing concentration of tacrine. Furthermore, it was determined that this decrease is not related with cell toxicity of tacrine (Figure 3.20).

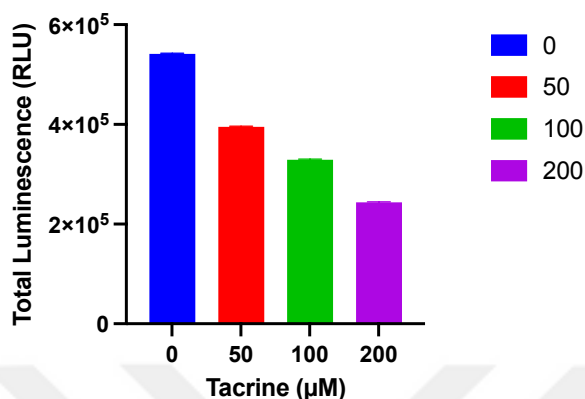


Figure 3.19: Total luminescence of PC-12 Adh cells treated with tacrine (number of technical replicates=3). The fact that decreasing of luminescence signal with increasing of tacrine concentration indicates BCHE-derived luminescence signal.

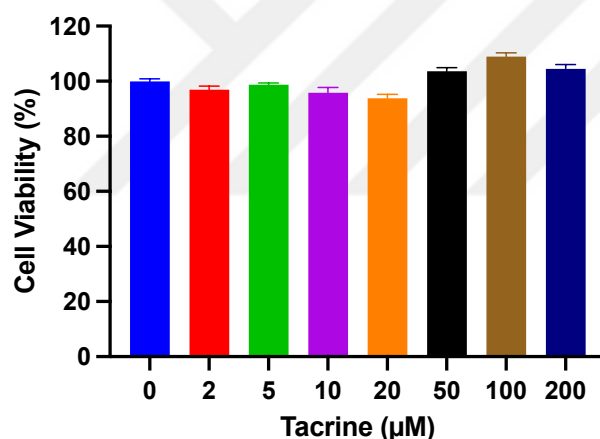


Figure 3.20: MTT Cell Viability Assay of Tacrine (number of technical replicates=6). The fact that there is no tacrine related cell toxicity in the range of 0-200µM supports BCC's BChE specificity.

After replicating the capacity of BCC *in vitro*, the corticosterone based psychological stress model by mimicking the outcome of HPA-axis was established. First, PC-12 Adh cells were treated with various concentration of CORT for 24h and subsequently MTT cell viability was measured. As reported, cell viability decreased with increasing concentration of CORT (Figure 3.21).

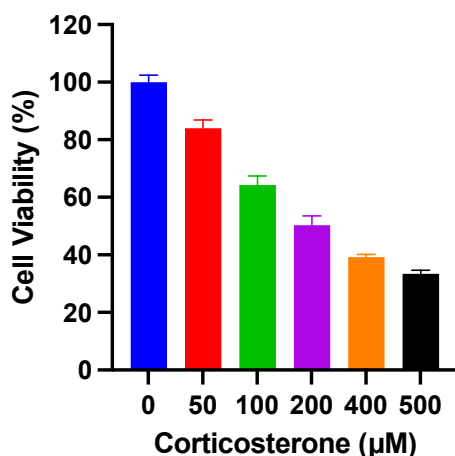


Figure 3.21: MTT cell viability of 24h CORT treated PC-12 Adh Cells. Increasing concentration of CORT induce cell apoptosis as mimicking the outcome of HPA-axis dysfunction (number of technical replicates=6).

For the model, the CORT concentration was chosen as 200μM, which is also considered as a reference in other articles and where the toxicity is equal to 50%.<sup>162,163</sup> Afterward, BChE activity change was compared with the control. As shown in Figure 3.22, BChE activity was significantly decreased in cells exposed to 200μM CORT for 24h compared to control ( $p=.0022$ ).

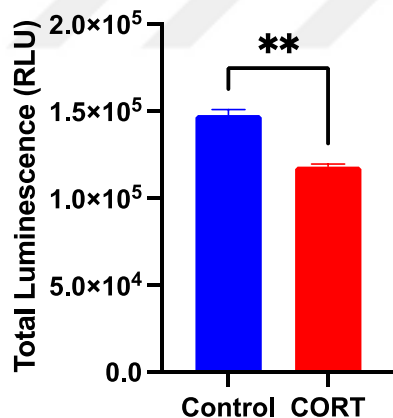


Figure 3.22: Comparison of BChE activity control vs 200μM CORT-treated PC-12 Adh cells. BChE activity significantly decreased in CORT-treated PC-12 Adh cells (number of technical replicates=6,  $p=.0022$ ).

After observing BChE activity decrease upon mimicking HPA-axis outcome, the norepinephrine based psychological stress model by imitating the outcome of SMA-axis was established. As similar methodology, first, PC-12 Adh cells were treated with various concentration of norepinephrine for 24h and subsequently MTT cell viability was measured. As shown in Figure 3.23, cell viability decreased with increasing concentration of NE.

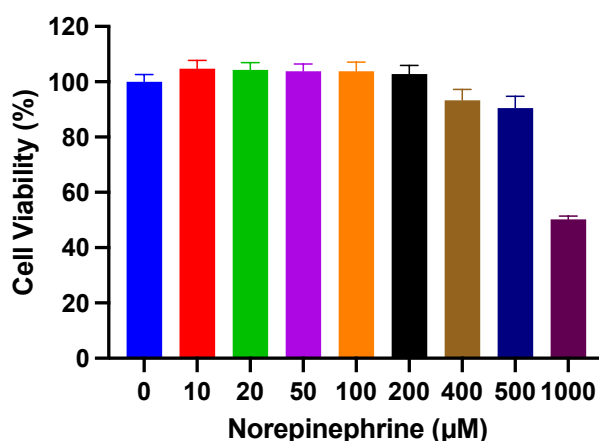


Figure 3.23: MTT Cell Viability of 24h NE Treated PC-12 Adh cells. Increasing concentration of NE induce cell apoptosis as mimicking the outcome of SAM-axis activation (number of technical replicates=6).

The concentration of NE for the model was selected as 1mM, where the toxicity equals to 50%. Then, BChE activity change was compared with control. As shown Figure 3.24, BChE activity was significantly increased in cells exposed to 1mM NE for 24h compared to control ( $p=.0022$ ).

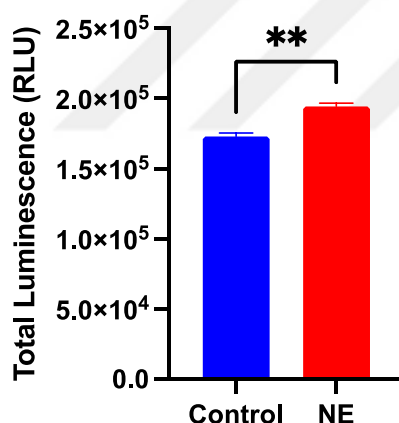


Figure 3.24: Comparison of BChE Activity Control vs 1mM NE-treated PC-12 Adh cells. BChE activity significantly increased in NE-treated PC-12 Adh cells (number of technical replicates=6,  $p=.0022$ ).

The effect on fluoxetine (common SSRI antidepressant) established two psychological stress models was investigated. First, CORT treated psychological stress model was created with 200μ CORT for 24h. Then, while one group was treated with 5μM fluoxetine for 24h, the other was replaced by only medium. As shown Figure 3.25, BChE activity was significantly increased in cells exposed to 5μM fluoxetine for 24h compared to control ( $p=.004$ ).

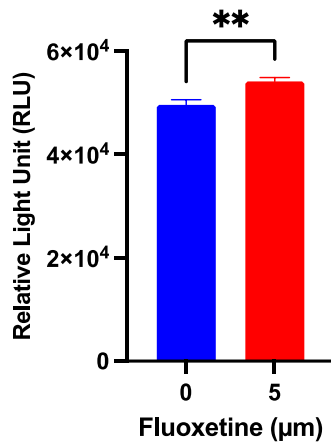


Figure 3.25: Comparison of BChE activity control vs 5μM fluoxetine-treated PC-12 Adh cells after CORT established psychological stress. Fluoxetine significantly increased BChE activity in CORT-established psychological stress model (number of technical replicates=6,  $p=.004$ ).

Lastly, NE treated psychological stress model was created with 1M NE for 24h. Then, while one group was treated with 5μM fluoxetine for 24h, the other was replaced by only medium. As shown Figure 3.26, BChE activity was significantly increased in cells exposed to 5μM fluoxetine for 24h compared to control ( $p=.010$ ).

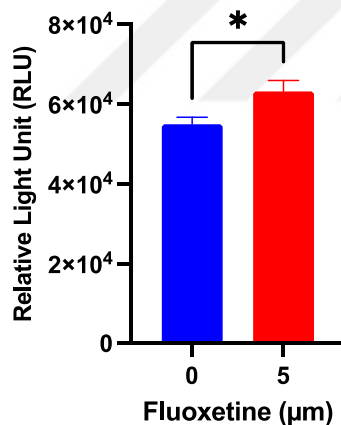


Figure 3.26: Comparison of BChE activity control vs 5μM fluoxetine-treated PC-12 Adh cells after NE established psychological stress. Fluoxetine significantly increased BChE activity in NE-established psychological stress model (number of technical replicates=6,  $p=.010$ ).

## Chapter 4:

### DISCUSSION

#### 4.1 *Synthesis*

The synthesis of the butyrylcholinesterase-responsive chemiluminescent probe (BCC) was conducted according to the protocol detailed in a previously published article.<sup>118</sup> Specifically, BCC was synthesized *via* the formation of a dioxetane bond on the precursor compound. The shift in the NMR spectrum due to dioxetane formation is consistent with that reported in the reference paper<sup>118</sup>; therefore, it confirmed that BCC synthesis was successful. The obtained yield was 52.2%, which, although slightly lower than the reported yield of 57.0%, was comparable. This discrepancy may be attributed to variations in reaction conditions or the efficiency of column chromatography. To enhance the yield, employing a purification method based on high-performance liquid chromatography (HPLC) as used in many studies could be beneficial.<sup>164–167</sup> Nevertheless, it can be concluded that the synthesis of BCC was successfully achieved.

#### 4.2 *Examination of the relationship between BChE levels and depression in human serum*

Although BCC's efficacy for monitoring BChE activity in various cell lines and tumor-bearing mice was previously demonstrated, its capability in human serum has not been tested yet. To address this, we compared luminescence signals from 0.005% human serum, 50  $\mu$ M BCC, and their mixture over 2 hours. A 20-fold increase in peak luminescence at 27 minutes and a 15-fold increase in total luminescence compared to controls indicate effective BCC response in human serum. A dose-dependent titration of human serum (0.005-0.10%) yielded an excellent  $R^2$  score (.9906), demonstrating BCC's sensitivity to BChE activity at low serum concentrations. The plateau observed after 0.5%, particularly in the 1-2% range, suggests the need for higher BCC concentrations for serum levels above 1%. Inhibition experiments with donepezil and tacrine confirmed that the luminescence signal originates from BChE activity, consistent with previous findings showing BCC's selectivity for BChE over other enzymes and components.<sup>118,168–</sup>

<sup>170</sup> Overall, these results confirm BCC's efficacy to real-time measure BChE activity in a human serum environment.

After confirming that BCC can sensitively and dose-dependently detect BChE activity in human serum, we screened BChE activity in serum samples from healthy controls, unipolar depression patients, and bipolar disorder patients with depressive episodes. Healthy controls showed significantly higher BChE activity than both unipolar and bipolar patients, with no significant difference between the patient groups. To explore these differences, we compared groups based on sociodemographic factors (sex, smoking, working status, alcohol use, exercise, age, education, and BMI) and psychometric data (HDRS, YMRS, and PSS). Significant differences were found only for age and BMI, with healthy controls being younger and having slightly lower BMI compared to other groups. The sample recruitment's strength is highlighted by the absence of notable distinctions between groups concerning factors like sex, smoking, alcohol consumption, and exercise, which are recognized to potentially impact BChE levels in both rat and human.<sup>171–183</sup> Two multiple linear regression analyses examined the effects of age, BMI, and group status on BChE activity. Both models were significant, explaining 37.6% and 38.3% of the variance, respectively indicating that differentiating the clinical group into UD and BD slightly enhanced the model's explanatory power for total BChE luminescence score.

In both models, age negatively impacted the BChE luminescence score with standardized coefficients of -.252 and -.253. In the literature, the effect of age on BChE levels is controversial. Some studies report no association between BChE levels and age<sup>180,184,185</sup>, while others have found a positive<sup>186</sup> or negative<sup>177,187,188</sup> correlation. Our findings suggest that BChE activity declines with increasing age. BMI also had a negative impact in both model with standardized coefficients of -.192 and -.193. Given that BChE is a serine hydrolase with lipolytic activity, mediating fatty acid use and storage, a decrease in BChE activity could result in increased lipid concentrations.<sup>189–192</sup> This aligns with existing research linking BChE to lipid metabolism, where increased BChE activity is associated with higher lipoprotein levels and hyperlipidemia.<sup>193–195</sup> In normal-weight individuals, higher BChE activity tends to be associated with a lower BMI.<sup>196,197</sup> However, BMI and BChE activity are positively correlated<sup>198</sup> in individuals with obesity, meaning that they generally have higher BChE activity than those with normal weight<sup>199–202</sup>. The mean BMI of the sample was  $23.92 \pm 4.66$  (range: 16–41). Consequently, it was

anticipated that BMI would increase as BChE total luminescence decreased. Our analysis confirmed this assumption, demonstrating a negative association between BMI and total luminescence score. As per the World Health Organization (WHO), obesity in adults is defined as having a BMI greater than or equal to 30.<sup>203</sup> In our study population, 12 participants had a BMI of 30 or higher, with a mean BMI of 33.09 (range: 30-41). Among them, 10 were classified as having class I obesity, 1 as class II obesity, and 1 as class III obesity. Notably, despite 10 participants in the clinical group (5 with UD, 5 with BD) being classified as obese, their obese condition did not significantly affect the total luminescence score as having lower BChE score than healthy controls. This finding is further supported by the standardized coefficient for BMI being lower than that for the group variable in both regression analyses. Therefore, even if there is a positive association in BChE activity and BMI in people with obesity, having depression has more substantial effect on BChE activity.

The first model, being in the clinical group negatively affected the score with -.364 standardized coefficient. In the second model, being in the UD group had a significant negative impact with -.383 standardized coefficient, and being in the BD group also had a significant negative impact but with -.230 standardized coefficient. Given that there are neurochemical differences between UD and BD<sup>204</sup>, this result indicates that BChE activity might also differentiate people from UD and BD at some extent. Overall, these results indicate that while age and BMI affect BChE activity, the most significant factor is having a depressive episode. In other words, depression's impact on BChE activity is more substantial than other factors like age and BMI. Notably, individuals in the bipolar disorder with depressive episode also exhibited significant reduced BChE activity, albeit to a lesser extent. Although there are a few articles directly investigate the association between BChE activity and depression, our findings align with existing literature. Joep et al. were among the first to report reduced plasma cholinesterase levels in patients with mania, depression, and schizophrenia compared to controls.<sup>205</sup> A recent study conducted by Awan et al. also stated that people with unipolar depression had a significant decrease in serum butyrylcholinesterase (BChE) activity when compared to healthy controls.<sup>206</sup> Also, that reported decrease was not influenced by demographic factors like gender or age as we found. Furthermore, a study that examined the effects of genetic variants in the apolipoprotein (APOE) and butyrylcholinesterase (BCHE) genes

on glucocorticoid (GC) secretion, personality traits, and cognitive functioning found that those who carried the E4 allele in the APOE gene and the K allele in the BCHE gene (A539T variant) had higher basal levels of GCs, lower levels of self-esteem, and more depressive symptoms than non-carriers of these variants.<sup>207</sup> Given that having K-variant of BCHE reduces its level<sup>208</sup>, this also supports our findings. Subsequently, the potential utility of butyrylcholinesterase (BChE) as a pharmacological agent capable of directly modulating stress-induced physiological and behavioral responses was underlined<sup>209</sup> Lastly, Zheng et al. observed a notable reduction in butyrylcholinesterase (BChE) levels among farmers exposed to pesticides, which corresponded to a markedly heightened susceptibility to experiencing moderate to severe depressive episodes and engaging in suicidal behaviors in comparison to individuals not engaged in farming activities.<sup>210</sup> There are a few studies reporting no significant change in BChE levels; however, in the relevant studies, employing old techniques to evaluate BChE levels, or small sample size, or recruiting patients on active medications, or not examining parameters affecting BChE levels such as BMI, smoking, and exercise has been noted.<sup>186,211,212</sup>

After establishing the significant impact of depression phenotype on BChE luminescence scores, we examined the key factors within the complexity of depression contributing to this association. Specifically, we investigated whether the observed reduction in BChE activity in depression extends to psychometric scales by correlating total scores on the HDRS and PSS. A significant negative correlation between BChE score and HDRS indicates that participants who have more score on HDRS have significantly reduced total BChE luminescence score. However, the correlation between BChE score and PSS is not significant. The reason for this could be small sample size of PSS compared to HDRS (n=100 for HDRS while n=83 for PSS) or the effect of change in BChE activity on people's perceived stress levels may not be reflected at a conscious level.

Second, same correlation analysis was determined for only clinical group. This time, the correlation between BChE score and HDRS became nonsignificant while the correlation between BChE score and PSS became significant. These findings means that the low BChE activity may be an important biomarker to determining a depressive episode within a population but BChE score does not assess depression's severity in the clinical sample. The weak correlation between BChE level and HDRS may be also due

to the multifaceted nature of depression as assessed by psychometric scales. Due to the multifaceted nature of depression such as molecular, cognitive, emotional, and physiological<sup>7</sup>, BChE score might be more closely associated with specific symptoms of depression rather than the broad spectrum of items covered by the HDRS. Furthermore, it may give an opportunity to elaborate the heterogeneous patient profile in depression by phenotyping of each patient. On the other hand, while BChE does not assess the perceived stress severity in a population, patients with depression reflect higher BChE scores with increasing levels of their perceived stress. This finding suggests that BChE may be elevated in response to the increased cholinergic activity linked to stress because it was reported that cholinergic transmission is altered in depression pathophysiology.<sup>213–215</sup> BChE, in conjunction with acetylcholinesterase (AChE), hydrolyzes acetylcholine, avoiding its excessive accumulation, and helps regulate acetylcholine levels during heightened cholinergic activity.<sup>216–219</sup> It is possible that this increase regulates acetylcholine levels and modifies stress responses as a compensatory mechanism.

To identify depression symptoms associated with BChE score, we examined the correlation and subsequent regression analysis between BChE score and HDRS subitems in the clinical group. According to correlation analysis, BChE score was found significantly correlated with HDRS suicide item (#11), and HDRS somatic anxiety item (#13). HDRS item 11, which assesses suicidal ideation and behavior, is scored on a scale from 0 to 4. A score of 0 indicates the absence of suicidal ideation, 1 reflects feelings that life is not worth living, 2 signifies a wish to be dead or thoughts of possible self-harm, 3 denotes suicidal ideas or gestures, and 4 indicates a suicide attempt.<sup>125,220</sup> In the study, depression patients who have higher suicidal ideation and behavior have significantly lower BChE activity compared to other patients. Although there is no explicit association between cholinergic transmission and neurobiology of suicide was reported<sup>221</sup>, this finding aligns with the recent report by Zheng et al., which demonstrated that farmers exposed to pesticides—substances that inhibit BChE activity—exhibit a significantly higher risk of moderate to severe depression and double the rate of suicide attempts compared to non-farmers.<sup>210</sup> Furthermore, this is also consistent with prior studies suggesting that pesticide exposure induces depression and suicidal ideation/attempt.<sup>222–226</sup> HDRS item 13 assesses somatic anxiety, including physiological symptoms such as gastrointestinal issues (e.g., dry mouth, wind, indigestion, diarrhea, cramps, belching),

cardiovascular symptoms (e.g., palpitations, headaches), respiratory symptoms (e.g., hyperventilation, sighing), urinary frequency, and sweating. This item is scored from 0 to 4, where 0 indicates the absence of symptoms, 1 indicates mild symptoms, 2 indicates moderate symptoms, 3 indicates severe symptoms, and 4 indicates incapacitating symptoms.<sup>125,220</sup> In the study, depression patients who have higher somatic anxiety has significantly higher BChE activity compared to other patients. Given that cholinergic transmission might promotes anxiety related behaviors<sup>227–229</sup> and BChE's reported associations with cardiac health<sup>230–233</sup>, stroke<sup>234</sup>, myocardial infarction<sup>235,236</sup> could explain this significant correlation. However, given that this item covers the symptom from a general perspective, a detailed scale that differentiates somatic anxiety symptoms such as Hamilton Anxiety Rating Scale as an addition could be used in further studies to elaborate on this association.<sup>237</sup>

To identify perceived stress symptoms associated with BChE score, we examined the correlation analysis between BChE score and PSS subitems in the clinical group. According to correlation analysis, BChE score was found significantly correlated with PSS#7, and PSS#11. PSS item 7 is part of the poor self-efficacy perception factor in the Perceived Stress Scale (PSS).<sup>127</sup> It measures the participant's ability to cope by asking, "In the last month, how often have you felt that things were going your way?". Participant rates him/herself up to 4 in an increasing order (0-never; 4- very frequently). In the study, depression patients who has lower score (thinks could not cope) have significantly higher BChE activity compared to other patients. Given that coping in specific anxious episodes could be impaired in depression, this question can be relatable with anxiety related stress perception.<sup>238,239</sup> Therefore, as cholinergic transmission has a modulatory effect on anxiety related behavioral states above-mentioned, it may be expected to have higher score of BChE.<sup>240,241</sup> PSS item 11 is part of the perception of stress/discomfort factor in the Perceived Stress Scale (PSS).<sup>127</sup> It measures the participant's anger level for could not change by asking, "In the last month, how often have you been angered because of things that happened that were outside of your control?". Participant rates him/herself up to 4 in an increasing order (0-never; 4- very frequently). In the study, depression patients who has higher score (thinks angered) have significantly higher BChE activity compared to other patients. As this question measures significantly related with anger driven by anxiety, it may be expected to have higher score of BChE's compensatory role in elevated

cholinergic transmission in anxiety before mentioned. However, a recent study conducted by Chen et al. demonstrated the potential role of BChE in attenuating aggression through modulation of the ghrelin axis in mice. Specifically, male mice housed in groups and overexpressing BChE via viral gene transfer exhibited a reduction in instances of aggression, contrasting with heightened aggression observed in BChE knockout mice.<sup>242</sup> While the aggression reported in the article is not anxiety-driven, further research is warranted to elucidate the broader role of BChE in aggression especially differentiate its role in cholinergic transmission and ghrelin axis.

After the 8-week treatment period, remission was assessed using an HDRS score of 7 or lower and the absence of depression criteria in the SCID-I Interview. Comparing BChE activity from serum samples at the 8th week with baseline total luminescence scores revealed a significant increase in patients in remission. The mixed linear analyses conducted on BChE activity with both total score and different subitems of the HDRS provide valuable insights into the factors influencing BChE activity in the context of depression. In the analysis involving the total HDRS score, while there is no significant effect of time in BChE activity, significant association was observed between BChE activity and remission. Specifically, decreases in HDRS scores were associated with increases in BChE activity, suggesting that improvements in depression severity are accompanied by increased BChE levels. These results highlight the potential value of BChE activity as a marker for tracking the intensity of depression and the effectiveness of treatment. Different trends appeared when the study was narrowed down to particular HDRS subitems. Changes in BChE activity were found to have a strong correlation with HDRS suicide item (#11) scores, which measure suicidal ideation and behavior. This suggests that fluctuations in suicidal ideation and behavior are linked to changes in BChE levels, highlighting the potential role of BChE activity as a marker for assessing suicide risk in individuals with depression. In contrast, no significant correlation was found between changes in BChE activity and HDRS somatic anxiety item scores, which measure somatic anxiety symptoms. This suggests that changes in somatic anxiety sensations could not have a significant impact on BChE activity in this context. Since HDRS somatic anxiety item includes many different somatic symptoms, such as gastrointestinal, cardiovascular, and respiratory, it could be reasonable to not find a

significance with remission. Given that BChE's association with some somatic diseases, it is better to evaluate BChE activity in specific somatic symptoms for further studies.

Overall, this section presents a comprehensive investigation into the utility of serum BChE activity as a potential marker for monitoring depression severity and treatment response. Firstly, it demonstrates the effectiveness of BChE in monitoring real-time activity under human serum conditions. Furthermore, it reveals a significant decrease in BChE activity among patients with unipolar depression and bipolar disorder with depressive episodes. Analysis of sociodemographic and psychometric factors identifies age and BMI as significant influencers of BChE luminescence scores, with both showing a negative impact. However, depressive episodes exert the most substantial effect on BChE activity. BChE activity is found to be negatively correlated with overall HDRS scores, while in the clinical group, it shows a significant positive correlation with perceived stress scores (PSS). Additionally, within the clinical group, BChE activity correlates negatively with HDRS suicide item (#11) and positively with HDRS somatic anxiety item, PSS#7 (poor self-efficacy perception factor), and PSS#11 (perception of stress/discomfort factor). Moreover, comparing BChE activity between baseline and 8th-week serum samples reveals a significant increase among patients in remission, indicating a potential association between BChE activity and treatment response. The mixed linear analyses conducted on different subitems of the Hamilton Depression Rating Scale (HDRS) elucidate further insights. While a significant association is observed between BChE activity and the total HDRS score, indicating that improvements in depression severity coincide with raised BChE levels, specific subitems yield distinct patterns. Notably, HDRS suicide item score shows a significant association with changes in BChE activity, suggesting a link between suicidal ideation and behavior and BChE levels. Conversely, HDRS somatic anxiety item scores do not significantly influence BChE activity probably because of broad screening of HDRS somatic anxiety item in somatic anxiety. Overall, these findings underscore the potential of BChE activity as a biomarker for monitoring depression severity, treatment response, and suicide risk. However, further research is warranted to elucidate underlying mechanisms and explore clinical implications comprehensively.

### 4.3 *Examination of the Relationship Between BChE Activity with Chronic Stress and Animal Behavior*

Similar approach used in human serum analyses were used to investigate the relationship between BChE activity with chronic stress and animal behavior. First, *proof of principle* of BCC in rat plasma environment was determined with comparing time-dependent luminescence signals from 0.005% rat plasma, 10 $\mu$ M BCC, and their mixture for 2h. A 60-fold increase in peak luminescence at 30 minutes and more than 50-fold increase in total luminescence compared to controls indicate effective BCC response in rat plasma. A dose-dependent titration of rat plasma (0.005-0.50%) yielded an excellent  $R^2$  score (.9963), demonstrating BCC's sensitivity to BChE activity at low rat plasma concentrations. The plateau observed after 1% suggests the need for higher BCC concentrations for serum levels above 1%. Inhibition experiments with donepezil and tacrine confirmed that the luminescence signal originates from BChE activity, consistent with literature. Overall, these results confirm BCC's efficacy to real-time measure BChE activity in a rat plasma environment.

Characterization of 35 days Chronic Unexpected Mild Stress (CUMS) were done with sucrose preference test, forced swim test, and weight% change. First, the sucrose preference test revealed a significant reduction in sucrose preference in the CUMS+Saline group compared to the Control+Saline group, indicating successful induction of anhedonia, a primary symptom of depression and the main outcome of the CUMS procedure. This finding aligns with existing literature, which indicates that CUMS induces depressive-like behaviors in rodents, including reduced sucrose preference, signifying a diminished capacity for experiencing pleasure.<sup>243-245</sup> However, although the sucrose preference in the CUMS+Fluoxetine group was higher on average than in the CUMS+Saline group, the difference did not reach a statistical significance. This suggests that fluoxetine administration may partially mitigate the anhedonic effects induced by CUMS, despite the lack of statistical significance. Studies indicate that antidepressants can normalize sucrose intake in animals exposed to CUMS<sup>246,247</sup>; however, the sensitivity of the test to minor design changes often complicates protocol reproducibility across different laboratories.<sup>248,249</sup> The lack of statistical significance in the model could be attributed to the small sample size in the CUMS+Saline group, the possibility that the

fluoxetine treatment was not sufficiently robust, or that a longer duration or higher dose of fluoxetine might be necessary for a more pronounced effect. The possible sucrose solution consumption suppressing effect of fluoxetine in rats<sup>250</sup> could explain the lack of significance.

Second, forced swimming test revealed that although the average immobility time of the CUMS+Saline group was higher than that of the other groups, no significant difference was observed between the groups. In terms of swimming time, a significant difference was found only between the Control+Saline and CUMS+Fluoxetine groups, with the Control+Saline group spending significantly more time swimming. Regarding climbing time, the CUMS+Fluoxetine group spent significantly more time climbing compared to both the Control+Saline and CUMS+Saline groups. These results indicate that the CUMS+Fluoxetine group had significantly higher climbing scores, as expected. Research conducted by Detke et al. (1995) has demonstrated that antidepressants that target serotonin, such as fluoxetine, improve swimming behavior, and those that target norepinephrine increase climbing.<sup>251–253</sup> Given that fluoxetine improved active behaviors in spite of the chronic stress setting, the considerable increase in climbing time observed in the CUMS+Fluoxetine group in the study implies that fluoxetine's impact may include both serotonergic and noradrenergic processes. Some studies have reported significant increases in norepinephrine in addition to increases in serotonin as a result of fluoxetine treatment in rats.<sup>254,255</sup> Based on findings, there could be some potential confounding variables to affect the outcome of forced swim test. First, the influence of strain and individual differences on FST outcomes has been emphasized in the literature.<sup>256–258</sup> Even, in same strain, difference from the supplier might yield inconsistent findings.<sup>259</sup> There are variations in the effectiveness of antidepressants in strains as well.<sup>260,261</sup> In the model, it was stated that only male Wistar rats supplied from Koç University Animal Center was used to eliminate those factors; however, the absence of significant differences in immobility time across groups still may be attributed to individual variability (genetic heterogeneity) within the strain. Additionally, although outlier analysis was conducted, some rats still displayed aberrant distribution compared to others supports the idea of an individual differences between them. Second, even though the study used 6- to 12-week-old rats to control for age, small age differences within this range may still have an impact on behavior such as physical activities or metabolism.<sup>262–</sup>

<sup>264</sup> While older rats may show more overt signs of depression, younger rats may be more energetic and stress-tolerant.<sup>258</sup> The way that medications like fluoxetine are metabolized by rats as they age can also have an impact on the effectiveness of these treatments as research showing that sensitivity to some antidepressants is strongly altered by age.<sup>265,266</sup> In the study, although there was no significant difference between CUMS+Saline and CUMS+Fluoxetine by age but Control+Saline group was significantly younger from other groups. Therefore, age difference could be an important factor for inconsistency in FST outcome and hypothesis especially in Control+Saline comparisons with other groups. Third, in parallel to age, the weight range of 124–425g is rather wide, and variations in weight could have an impact on the rats' capacity for climbing and swimming, which could skew the results. Physical performance in the FST may be impacted by the wide weight range (124–425g).<sup>258,267</sup> Along with to being more difficult to swim and climb, heavier rats may spend longer periods of time immobile, even when they are not depressed. On the other hand, lighter rats may be more agile, which could distort the findings. Fourth, the effects of the CUMS model and the antidepressant medication may be complicated by daily intraperitoneal injections of fluoxetine or saline. Stress from the injection process itself may have an impact on the behavioral assessments as shown in the literature.<sup>268–270</sup> In the model, rats in Control+Saline group also received intraperitoneal injections daily for 20 days. This may induce experiencing stress, even in control group which could affect how the CUMS model and fluoxetine treatment work. It can be challenging to separate the effects of the medication from the stress of the process itself because the stress related to injections may have an impact on the behavior of the rats in the FST. To overcome this, a control group without any injection procedure could be added for further studies. Fifth, even if the FST actions were rated by three observers, observer bias may still have an impact on the outcomes. Human observers may induce bias in the interpretation of some activities, like swimming, climbing, and immobility, even with efforts to standardize scoring. Variability in the data may result from variations in how observers see and document these actions. Using automated scoring and tracking systems<sup>271–275</sup> can improve the precision and dependability of behavioral data while lowering observer bias. By limiting human error, these devices can give objective measurements of behavior and movement. Lastly, another potential confounding variable in the forced swim test (FST) procedure could be the absence of a

pre-test. Traditionally, the rat FST includes a 15-minute pre-test session followed by a 5-minute test session 24 hours later.<sup>276</sup> The pre-test induces a state of behavioral despair, making it easier to observe the effects of antidepressant treatments in the subsequent test.<sup>277,278</sup> This session also involves elements of learning and memory, which may reflect cognitive aspects of major depression. However, pre-conditioning procedures that affect memory processing could yield false results.<sup>258,277</sup> To avoid such confounding effects, some studies using chronic antidepressant treatment employ a single 15-minute test session as we did in this study.<sup>279</sup> However, addition of a pre-test session could be taken into account to enhance the sensitivity and reliability of the FST in detecting depressive-like behaviors and the efficacy of antidepressant treatments for further studies. Overall, having significant difference between groups in terms of age and weight, not having group without any injection, absence of pre-test, and using human based scoring could be the main challenges in the forced swim test. More limited age range (therefore weight), adding a new group without any injection procedure, adapting automated scoring, having more sample size (for resilience possibility), and including a pre-test could be taken into consideration for more reliable and consistent data for further studies.

Third, weight% change every week compared to the weight at 0th was computed for each group by using a baseline of 100. For all week-based comparison (2<sup>nd</sup> week to 5<sup>th</sup> week), Control+Saline group has gained more weight than other groups which is expected because of their younger age and not receiving any stressor (except saline injection). Also, the significant weight loss in CUMS+Fluoxetine compared to CUMS+Saline group was observed in 3<sup>rd</sup> week and 5<sup>th</sup> week. This finding is consistent with the literature, which reports weight loss associated with fluoxetine treatment in both rats and humans, due to its possible role in increasing energy expenditure<sup>280–285</sup> or common side effect of reducing appetite.<sup>250,286,287</sup>

After behavioral assessments, plasma samples from all rat groups were screened with BCC and BChE activity were compared by groups. Rats exposed to CUMS and received no fluoxetine treatment show significantly lower total luminescence score from Control+Saline group. This is consistent with our human serum results because main anhedonia, main outcome of CUMS, is a core symptom of major depressive disorder as above-mentioned. Furthermore, this finding is consistent with previous research by Tagliari et al., which demonstrated that a 40-day Chronic Variable Stress model in Wistar

male rats significantly inhibited BChE activity.<sup>288</sup> However, in that study, Ellman's method was used to measure BChE activity,<sup>288</sup> which is problematic due to factors such as indirect measurement, instability, and low sensitivity.<sup>100,101</sup> Lastly, the increased effect of antidepressant treatment on BChE observed in human serum was not replicated in this model. This discrepancy could be attributed to factors mentioned in the behavioral assessment analyses, small sample size, particularly in the CUMS+Saline group, individual variance, and potential confounding parameters during the CUMS procedure.

Following BChE activity screening, a Pearson correlation coefficient assessed the relationship between BChE luminescence in rat plasma and behavioral parameters: sucrose preference percentage, immobility time, swimming time, and climbing time. A significant positive correlation emerged between BChE activity and sucrose preference, indicating that decreased BChE activity relate to decreased sucrose preference (anhedonia phenotype), mirroring findings in human serum where healthy controls exhibit notably higher BChE activity compared to depression patients. However, no significant correlations were detected between BChE activity and immobility, climbing, or swimming times, suggesting presence of potential confounding factors in the forced swim test procedure or minimal influence of BChE on these behavioral parameters.

Finally, considering the significant differences in the distribution of age between groups and sucrose preference% as a representative depression phenotype variable, whether age and percent sucrose preference significantly predicted BChE\_score was assessed by multiple linear regression. The regression model accounted for 32.9% of the variance and was significant. Age's negative effect on BChE score was not significant. This could indicate that within the range of ages studied, age does not have a discernible impact on BChE activity. Even there would be a significance, it will be negative which confirms our findings in human serum samples. On the other hand, the positive effect of sucrose preference% on BChE score was found significant. This suggests that rats with a higher preference for sucrose, potentially indicating lower levels of anhedonia, also exhibit higher BChE activity. This also confirms our findings that healthy controls show more BChE activity than clinical group in human serum sample.

In this section, we evaluated BCC's capability to monitor real-time BChE activity in rat plasma and examined BChE activity differentiation in rats subjected to chronic unpredictable mild stress (CUMS). We found that CUMS significantly induced

anhedonia, evidenced by reduced sucrose preference, while fluoxetine partially mitigated these effects, though not to a statistically significant degree. The forced swim test revealed increased climbing behavior with fluoxetine, suggesting its serotonergic and noradrenergic implications, but no significant differences in immobility times, indicating existence of potential confounding variables. Weight comparisons showed expected trends: Control+Saline group gained more weight as being young and not exposed chronic stress (except saline injection), while CUMS+Fluoxetine groups lost weight, consistent with reported effects of fluoxetine. Correlations indicated decreased BChE activity associated with anhedonia, aligning with human serum sample results. Multiple regression confirmed that reduced sucrose preference (anhedonia) was a significant predictor of decreased BChE activity compared to age, consistent with our findings in the previous section. Overall, it can be concluded that we demonstrated the main finding from human studies, suggesting BChE activity as a potential marker for diagnosing depression, corroborated by animal behavior studies. Further animal research in more consistent CUMS protocol and other chronic stress models such as foot-shock, chronic social defeat, prenatal stress or olfactory bulbectomy is needed to elucidate the underlying mechanism and to determine which symptoms of depression are more dominant in this association.

#### ***4.4 Validating the Link Between BChE Levels and Chronic Stress: Utilizing PC-12 Adh Cells and Modulating with Corticosterone, Noradrenaline and Fluoxetine***

After having a promising connection between human and rat samples, a significantly decreased BChE activity in depressive phenotypes, we investigated its validity *in vitro* conditions by establishing psychological stress models with corticosterone and norepinephrine exposure to PC-12 Adh cell line. We observed a significant increase in corticosterone exposure, a significant decrease with norepinephrine exposure in terms of BChE activity. Also, fluoxetine showed an increased effect on both model similar to previous findings.

Corticosterone (CORT), a corticosteroid hormone produced in the adrenal cortex of mice, rats, birds, and reptiles, is closely related to HPA axis responsiveness and cortisol-binding globulin levels.<sup>289–291</sup> Under normal conditions, the HPA axis regulates the production of CORT through negative feedback mechanisms. However, prolonged exposure to stress can disrupt this feedback loop, resulting in dysregulation of the HPA

axis and sustained elevation of CORT levels.<sup>292,293</sup> Chronic stress has been implicated in damaging the nervous system through mechanisms such as oxidative stress and inducing neurobiochemical alterations in the brain been associated with the development of neurodegenerative diseases, including Alzheimer's Disease and Parkinson's Disease.<sup>294–297</sup> PC-12 Adh cells, derived from mouse pheochromocytoma, is an ideal model for investigating neuronal damage associated with chronic stress as exhibiting typical neuronal properties, including the expression of catecholamines (dopamine and norepinephrine) and high levels of glucocorticoid receptors.<sup>298–302</sup> Treatment of PC12 cells with high concentrations of CORT induces neuronal damage that mimics that observed in depressive physiology and stimulates metabolic pathways such as purine metabolism, glycerolipid metabolism, and phenylalanine metabolism which are also observed in patients with depression.<sup>302–305</sup> Therefore, CORT treated PC-12 Adh model is commonly used for cell based psychological stress model and anti-depressant screening studies.<sup>162,306–309</sup>

The noradrenergic system's involvement in stress responses is essential, with sustained activation of brain norepinephrine (NE) being a hallmark of stress. This activation, initiated parallel to the hypothalamic-pituitary-adrenal (HPA) axis, facilitates both excitatory and inhibitory responses in target cells.<sup>310,311</sup> NE release in stress-related limbic forebrain regions, such as the amygdala and medial prefrontal cortex, mediates anxiety-like behaviors and defensive responses<sup>311</sup>. Dysregulation of this system may contribute to vulnerability to anxiety disorders, stress-related disorders and mood disorders.<sup>312–314</sup> Prolonged stress pathology reduces norepinephrine and locus coeruleus activity, increasing  $\alpha 2$  receptors, necessitating norepinephrine reuptake inhibitors to restore balance.<sup>315</sup> Additionally, the regulatory role of NE in stress and the impact of chronic antidepressant treatment highlights the importance of NE in adaptive and pathological stress responses.<sup>314</sup> Therefore, using PC-12 Adh cells treated with NE could provide valuable insights into the cellular mechanisms of stress, mimicking the neurochemical and behavioral responses observed *in vivo*.

Although BCC has been demonstrated to monitor BChE activity in different cell lines such as SH-SY5Y and HepG2,<sup>118</sup> its efficacy in PC-12 Adh cells remained untested. Therefore, first, we verified BCC's capacity in PC-12 Adh cells with cell viability test, dose-dependent titration, and subsequent tacrine inhibition respectively. Our findings

determined that BCC is a non-toxic, dose-dependent, BChE-specific chemiluminescent probe to monitor BChE activity in PC-12 Adh cells.

Second, following with cell viability test of corticosterone, a psychological stress model, which mimics the outcome of HPA-axis, was established with 200 $\mu$ M corticosterone for 24h and BChE activity change was determined. As consistent with our findings in both human and rat samples, it was observed that BChE activity was significantly decreased in cell with corticosterone exposure. Then, we tested whether the antidepressant's effect can reverse that decrease or not by treating stressed cell with 5 $\mu$ M fluoxetine for 24h. The significant increase in BChE activity with fluoxetine supports our findings in previous findings.

In the second part of the cell model, rather than mimicking chronic stress with corticosterone exposure, we created cell by mimicking the findings of increased noradrenaline after depression treatment in humans and animals.<sup>316</sup> The model was established with 1mM norepinephrine for 24h and BChE activity change was determined. It was observed that BChE activity was significantly increased in cell treated with norepinephrine compared to controls. Considering that norepinephrine levels increase with treatment, the increase in BChE activity observed as a result of human serum remission and as a result of rat antidepressant treatment may coincide with this finding. Also, by considering norepinephrine involvement in anxiety and stress-related disorders, this finding aligns with our results in human sample where BChE activity is positively correlated with PSS's subitem, namely anxiety for coping and anger. Furthermore, it is also consistent with literature where increased level of pseudocholinesterase was observed in the state of anxiety in human and animals.<sup>317–319</sup> Then, we tested whether the antidepressant treatment change BChE activity or not by treating stressed cell with 5 $\mu$ M fluoxetine for 24h. Again, a significant increase in terms of BChE activity was achieved. This is also consistent with studies reported that fluoxetine increases norepinephrine level.<sup>320,321</sup>

In this section, we explored the validity of BChE activity as a biomarker for depression using *in vitro* models of cell damage based psychological stress with corticosterone and norepinephrine exposure in PC-12 Adh cells. Consistent with our findings in human and rat samples, corticosterone treatment, which mimics the outcome of HPA-axis activation, significantly reduced BChE activity, resonates the depressive

physiological state. Fluoxetine treatment reversed this decrease, supporting our previous antidepressant effects findings. Similarly, norepinephrine exposure increased BChE activity, aligning with stress-induced noradrenergic activation observed *in vivo*. Fluoxetine also enhanced BChE activity in this model, corroborating its role in elevating norepinephrine levels. Overall, these results support our essential finding, BChE's potential as a biomarker for depression and the efficacy of fluoxetine in modulating BChE activity under stress conditions.

Future studies could consider replicating these findings in other cell lines, such as human pheochromocytoma-derived KNA cells, rat neuroblastoma-derived B35 cells, and human neuroblastoma-derived SH-SY5Y cells, to compare origin differences with PC-12 cells and investigate BChE activity changes in the central nervous system mimicking environment. Additionally, experiments examining the acute effects of corticosterone and norepinephrine on BChE activity with shorter exposure times could provide further insights. Furthermore, studies employing combined agonist or antagonist drugs, such as alpha blockers or beta blockers, could elucidate the receptors associated with increased BChE activity in norepinephrine. Finally, exploring the effects of other antidepressants, such as tricyclic antidepressants, selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors, or N-methyl-D-aspartate (NMDA) antagonists like ketamine, could broaden our understanding of the modulation of BChE activity in response to antidepressant treatment.

#### **4.5 Overall Discussion**

This study examines the association between butyrylcholinesterase (BChE) activity and depression from a multidimensional and comprehensive perspective, also emphasizing the potential of chemiluminescent probes in molecular psychiatry research. The experimental workflow was as follows: synthesizing a recently reported butyrylcholinesterase-selective chemiluminescent probe (BCC)<sup>118</sup>; assessing the efficacy of BCC in a human serum environment; determining BChE activity in human serum samples from healthy controls, patients with unipolar depression, and patients with bipolar disorder with depressive episode; evaluating BCC's efficacy in the rat plasma environment; measuring BChE activity in rat plasma samples from control rats exposed to saline injection, rats subjected to chronic unpredictable mild stress (CUMS) with saline

injection, and rats subjected to CUMS with concurrent fluoxetine injection; testing BCC's efficacy in the PC-12 Adh cell line; measuring BChE activity in psychological stress models created using the PC-12 cell line; and assessing the effect of fluoxetine on BChE activity in psychological stress models developed in the PC-12 cell line.

Overall, the findings of this translational study propose the potential of butyrylcholinesterase (BChE) activity as a biomarker for diagnosing depression and monitoring treatment outcomes. The observed decrease in BChE activity in depression may be associated with oxidative stress, which disrupts various biological systems implicated in depression.<sup>322</sup> Elevated levels of reactive oxygen species (ROS) linked to oxidative stress can interfere with normal cellular functions, trigger inflammatory responses, alter neurotransmitter metabolism, inhibit neurogenesis, and impair synaptic plasticity, all contributing to depressive symptoms.<sup>322–324</sup> Additionally, evidence suggesting that oxidative stress byproducts, like hydrogen peroxide, can significantly inhibit BChE activity underscores the intricate relationship between BChE and oxidative stress.<sup>325</sup> Moreover, BChE's role in lipid metabolism, coupled with the findings of decreased BChE levels in diseases characterized by lipid peroxidation, further supports this theory.<sup>326,327</sup> Further studies such as employing hydrogen peroxide combined BChE responsive probes in chronic stress conditions or employing ROS-related biomarkers additional to BChE level in clinical samples might elucidate this multifaced association between oxidative stress and BChE activity.

The study has some limitations. First, in the study, we used the total luminescence score of BCC to evaluate BChE activity, but it lacks direct correspondence to a physical quantity. Although the reference article provides a calibration curve for BCC linking luminescence to BChE enzyme concentration<sup>118</sup>, further comparative studies with other detection methods like Ellman's method, electrochemical techniques, or enzyme-linked immunosorbent assay (ELISA) are necessary to determine the degree of correlation between BCC's luminescence signal and the physical presence of BChE levels in human serum and rat plasma.<sup>99,328,329</sup> Second, due to its unstable nature, dioxetane molecules could lose its efficacy in a long-term. To overcome this, we freshly synthesized BCC just before carried out experiments; however, this fact should be taken into consideration for further studies. Third, in the reference human sample study, patients with mild depression were excluded. At what extent BChE activity is influenced with mild depression should

be investigated for further studies. Fourth, we assessed mainly the effects of fluoxetine as a prototype medication of selective serotonin reuptake inhibitors (SSRI) and general antidepressant treatment. However, our patient sample may be using various types of medications. Therefore, strength of these medications and assessing their effects on BChE activity could also be beneficial. Fifth, besides there is a consistent findings between anhedonia and BChE activity, the animal model was not ideal according to behavioral assessments. Additional animal studies using consistent CUMS protocols or other chronic stress models, such as foot-shock, chronic social defeat, prenatal stress, or olfactory bulbectomy could be beneficial to replicate findings and determine the predominant symptoms of depression in this context. Lastly, further studies could replicate these findings in diverse cell lines to explore origin differences and investigate BChE activity changes in brain mimicking environments. Also, examining acute effects of corticosterone and norepinephrine on BChE activity and exploring other antidepressants could broaden our understanding.

## Chapter 5:

**CONCLUSION**

In this study, we presented a comprehensive examination of butyrylcholinesterase activity as a biomarker for depression and treatment response using the BChE selective chemiluminescence probe (BCC).

In human serum, we demonstrated BCC's competency to monitor BChE activity in human serum and found a significant decrease in BChE activity among patients with unipolar depression and bipolar disorder with depressive episodes compared to healthy controls. Also, BChE activity was correlated with psychometric scales with a negative correlation with HDRS and positive correlation with PSS. Notably, BChE activity significantly increased in patients in remission as consistent with decrease in HDRS, indicating its potential in monitoring treatment response.

In rat plasma, we validated BCC's capability to monitor BChE activity in rat plasma and found that rat subjected to chronic unpredictable mild stress (CUMS) induced anhedonia and decreased BChE activity, aligning with our human serum findings. Although fluoxetine mitigated these effects, statistical significance was not achieved. Our findings suggest that BChE activity could serve as a marker for depression and treatment efficacy, though further animal research is needed to explore other chronic stress models and refine CUMS protocols.

*In vitro* studies using PC-12 Adh cells exposed to corticosterone and norepinephrine corroborated our *in vivo* findings. Corticosterone reduced BChE activity, mimicking chronic stress related HPA-axis physiology, while fluoxetine treatment reversed this decrease. Norepinephrine exposure increased BChE activity, consistent with stress-induced noradrenergic activation observed *in vivo*, and fluoxetine further enhanced BChE levels. These results affirm BChE's potential as a biomarker for depression and highlight fluoxetine's role in modulating BChE activity under stress conditions.

Overall, our findings propose BChE as a promising biomarker for diagnosing depression and assessing treatment response, warranting further research to elucidate the underlying mechanisms.

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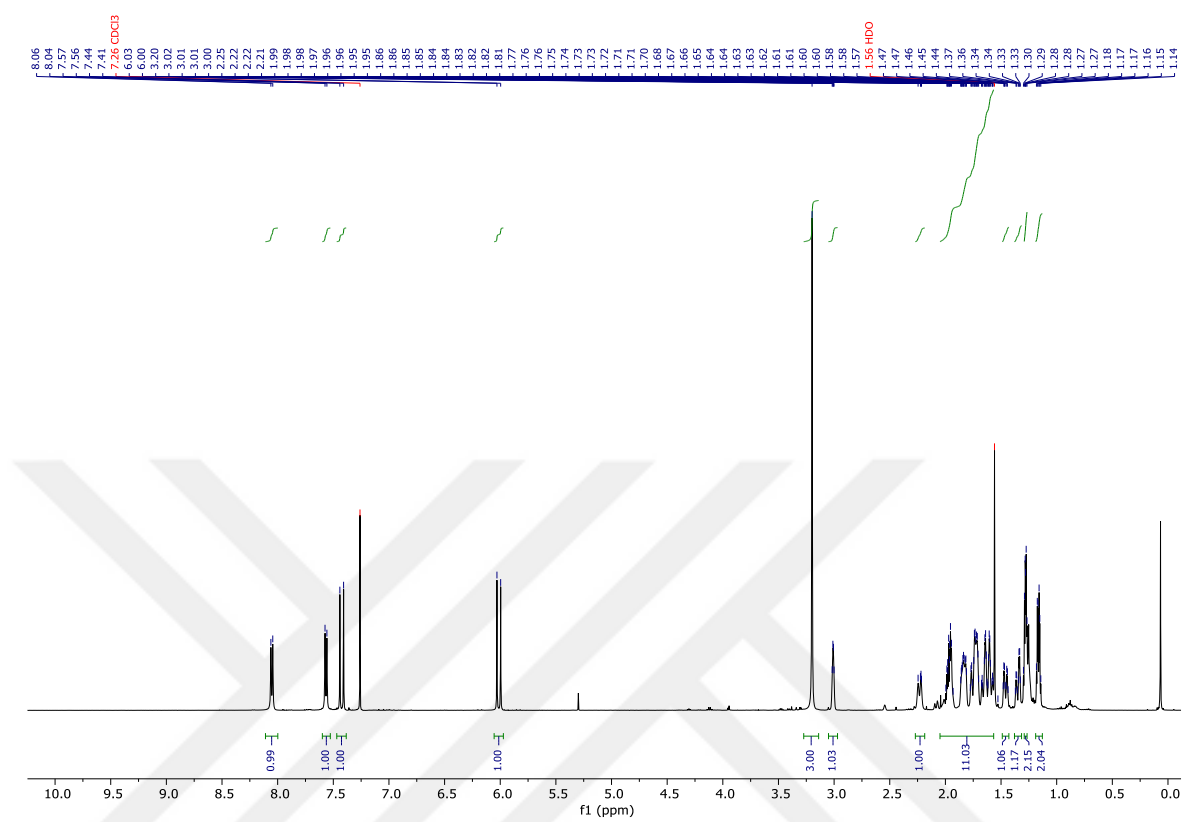
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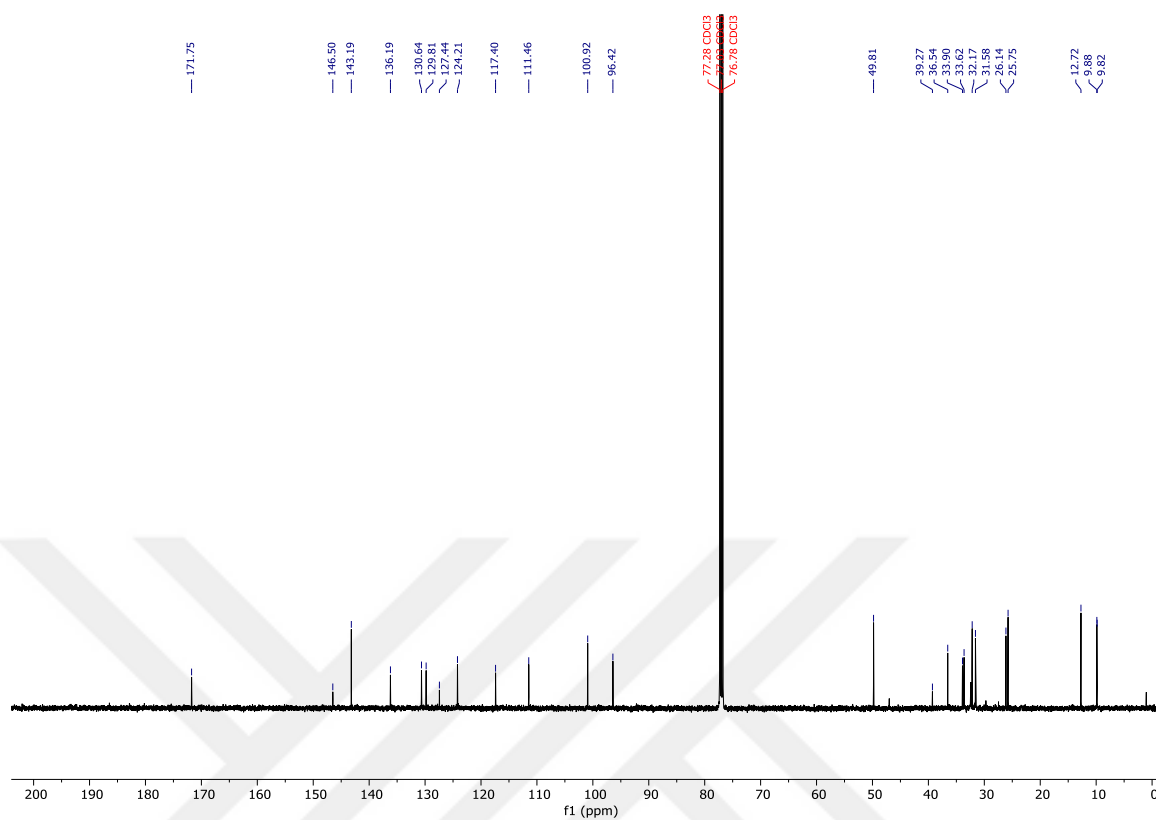
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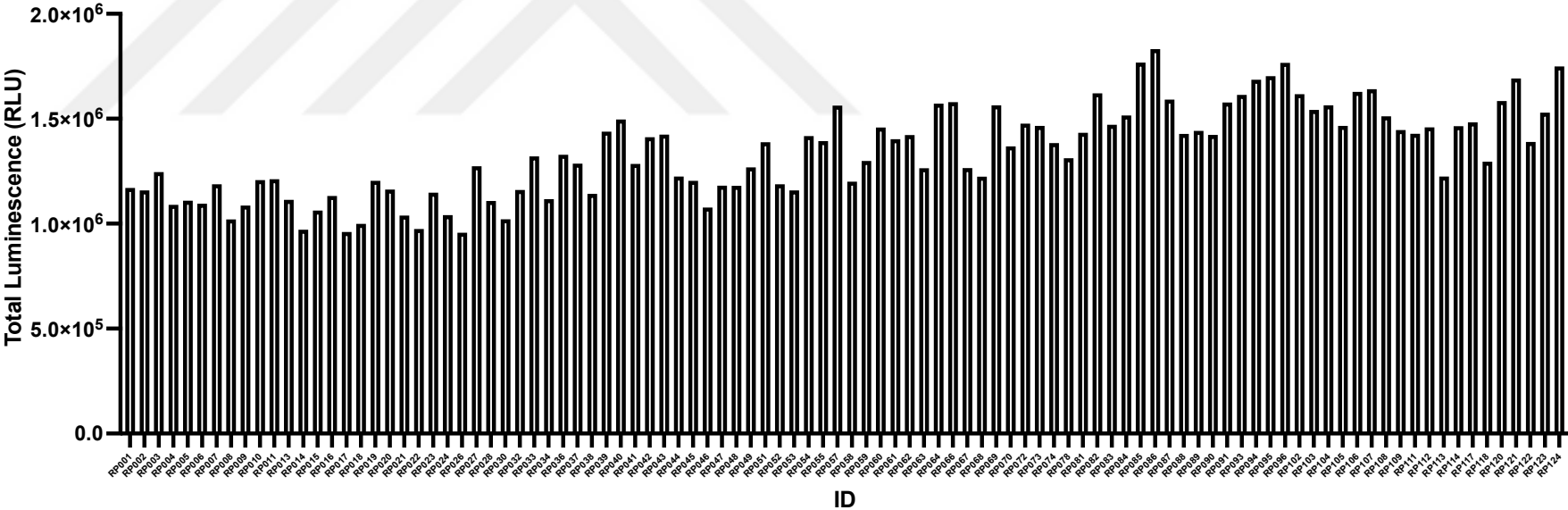
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## Appendix A: NMR Results of BCC

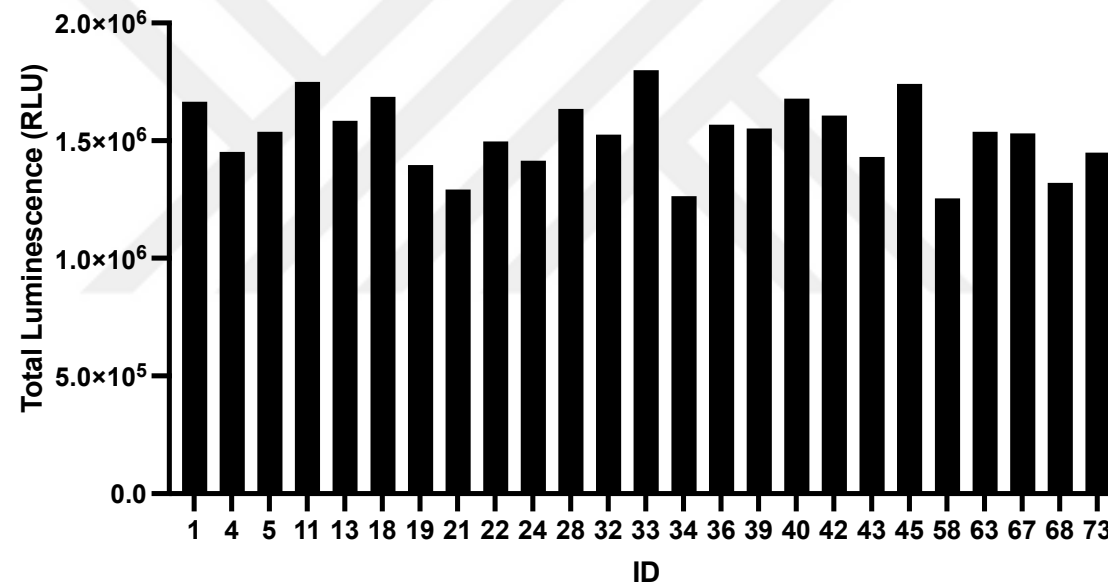
 $^1\text{H}$  NMR spectrum of BCC

 $^{13}\text{C}$  NMR spectrum of BCC

Appendix B: Total Luminescence Score for each Human Serum Samples



Total luminescence score of human serum samples at 0<sup>th</sup> week.



Total luminescence score of clinical serum samples at 8<sup>th</sup> week.

Appendix C: Detailed Pearson Correlation Table of BChE\_0W with Subitems of HDRS

|    |                                         | 1       | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     | 13     | 14     | 15     | 16    | 17    |
|----|-----------------------------------------|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|-------|-------|
| 1  | <b>BChE_0W</b>                          |         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |       |
| 2  | <b>Depressed mood</b>                   | -.467** |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |       |
| 3  | <b>Work and Activities</b>              | -.429** | .860** |        |        |        |        |        |        |        |        |        |        |        |        |        |       |       |
| 4  | <b>Genital symptoms</b>                 | -.374** | .661** | .610** |        |        |        |        |        |        |        |        |        |        |        |        |       |       |
| 5  | <b>Somatic symptoms</b>                 | -.188   | .539** | .488** | .579** |        |        |        |        |        |        |        |        |        |        |        |       |       |
| 6  | <b>Gastro-intestinal</b>                |         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |       |
| 7  | <b>Loss of weight</b>                   | -.044   | .307** | .343** | .389** | .535** |        |        |        |        |        |        |        |        |        |        |       |       |
| 8  | <b>Insomnia: early in the night</b>     | -.310** | .639** | .629** | .283** | .393** | .253*  |        |        |        |        |        |        |        |        |        |       |       |
| 9  | <b>Insomnia: middle of the night</b>    | -.398** | .625** | .478** | .308** | .385** | .407** | .664** |        |        |        |        |        |        |        |        |       |       |
| 10 | <b>Insomnia: early hours of morning</b> | -.334** | .499** | .456** | .380** | .446** | .239*  | .503** | .549** |        |        |        |        |        |        |        |       |       |
| 11 | <b>General somatic symptoms</b>         | -.400** | .811** | .731** | .576** | .526** | .302** | .454** | .537** | .407** |        |        |        |        |        |        |       |       |
| 12 | <b>Feelings of guilt</b>                | -.393** | .711** | .661** | .583** | .494** | .330** | .428** | .406** | .387** | .613** |        |        |        |        |        |       |       |
| 13 | <b>Suicide</b>                          | -.542** | .758** | .731** | .604** | .489** | .245*  | .579** | .510** | .466** | .703** | .535** |        |        |        |        |       |       |
| 14 | <b>Anxiety psychic</b>                  | -.397** | .713** | .548** | .499** | .478** | .243*  | .369** | .457** | .328** | .635** | .647** | .422** |        |        |        |       |       |
| 15 | <b>Somatic anxiety</b>                  | -.336** | .818** | .705** | .433** | .507** | .357** | .531** | .549** | .447** | .725** | .674** | .644** | .664** |        |        |       |       |
| 16 | <b>Hypochondriasis</b>                  | -.181   | .307** | .293** | .200   | .335** | .179   | .206*  | .272** | .089   | .313** | .358** | .208*  | .358** | .242*  |        |       |       |
| 17 | <b>Insight</b>                          | -.032   | .222*  | .206*  | .200   | .308** | .195   | .027   | .029   | -.128  | .174   | .255*  | .141   | .220*  | .158   | .308** |       |       |
| 18 | <b>Retardation</b>                      | -.337** | .446** | .494** | .463** | .183   | .262*  | .224*  | .128   | .163   | .328** | .319** | .346** | .373** | .335** | .134   | .261* |       |
| 19 | <b>Agitation</b>                        | -.200   | .407** | .270** | .329** | .198   | .227*  | .092   | .168   | .074   | .296** | .394** | .122   | .462** | .294** | .039   | .203* | .233* |

Note. N=94, \* $p < .05$ ; \*\* $p < .01$

Appendix D: Detailed Pearson Correlation Table of BChE\_0W with Subitems of HDRS for Only Clinical Group

|    |                                           | 1       | 2     | 3      | 4     | 5      | 6     | 7      | 8     | 9      | 10    | 11    | 12      | 13   | 14    | 15    | 16   |
|----|-------------------------------------------|---------|-------|--------|-------|--------|-------|--------|-------|--------|-------|-------|---------|------|-------|-------|------|
| 1  | <b>BChE_0W</b>                            |         |       |        |       |        |       |        |       |        |       |       |         |      |       |       |      |
| 2  | <b>Depressed mood</b>                     | .237    |       |        |       |        |       |        |       |        |       |       |         |      |       |       |      |
| 3  | <b>Work and Activities</b>                | .059    | .117  |        |       |        |       |        |       |        |       |       |         |      |       |       |      |
| 4  | <b>Genital symptoms</b>                   | -.084   | .158  | .117   |       |        |       |        |       |        |       |       |         |      |       |       |      |
| 5  | <b>Somatic symptoms Gastro-intestinal</b> | .193    | -.017 | .008   | .343* |        |       |        |       |        |       |       |         |      |       |       |      |
| 6  | <b>Loss of weight</b>                     | .215    | -.060 | .122   | .243  | .448** |       |        |       |        |       |       |         |      |       |       |      |
| 7  | <b>Insomnia: early in the night</b>       | .049    | .049  | .174   | -.241 | .051   | .055  |        |       |        |       |       |         |      |       |       |      |
| 8  | <b>Insomnia: middle of the night</b>      | -.143   | -.012 | -.220  | -.192 | .041   | .270  | .423** |       |        |       |       |         |      |       |       |      |
| 9  | <b>Insomnia: early hours of morning</b>   | -.104   | -.170 | -.047  | .039  | .204   | .076  | .235   | .308* |        |       |       |         |      |       |       |      |
| 10 | <b>General somatic symptoms</b>           | .077    | -.165 | -.042  | .069  | .147   | .056  | -.297* | -.034 | -.159  |       |       |         |      |       |       |      |
| 11 | <b>Feelings of guilt</b>                  | -.016   | -.141 | .020   | .186  | .138   | .132  | -.119  | -.157 | -.044  | -.095 |       |         |      |       |       |      |
| 12 | <b>Suicide</b>                            | -.364** | -.035 | .161   | .198  | .103   | -.021 | .150   | .011  | .074   | .136  | -.134 |         |      |       |       |      |
| 13 | <b>Anxiety psychic</b>                    | -.058   | -.038 | -.288* | .036  | .122   | .000  | -.216  | -.036 | -.135  | -.091 | .208  | -.380** |      |       |       |      |
| 14 | <b>Somatic anxiety</b>                    | .277*   | .057  | -.075  | -.264 | .095   | .160  | -.022  | .027  | -.017  | .000  | .131  | -.026   | .117 |       |       |      |
| 15 | <b>Hypochondriasis</b>                    | -.025   | -.042 | .022   | -.017 | .196   | .080  | -.006  | .086  | -.112  | .089  | .181  | -.079   | .187 | -.056 |       |      |
| 16 | <b>Insight</b>                            | .108    | .167  | .085   | .103  | .248   | .143  | -.129  | -.123 | -.281* | .031  | .173  | -.012   | .123 | .000  | .265  |      |
| 17 | <b>Retardation</b>                        | -.194   | .048  | .236   | .253  | -.090  | .136  | -.096  | -.236 | -.109  | -.139 | -.031 | -.008   | .071 | -.078 | -.014 | .200 |
| 18 | <b>Agitation</b>                          | .022    | .021  | -.199  | .086  | -.042  | .106  | -.253  | -.141 | -.198  | -.143 | .139  | -.358** | .258 | -.100 | -.110 | .139 |

Note. N=52, \* $p < .05$ ; \*\* $p < .01$

Appendix E: Detailed Pearson Correlation Table of BChE\_0W with Subitems of PSS

|           |                | 1      | 2       | 3       | 4       | 5       | 6      | 7       | 8       | 9      | 10      | 11     | 12     | 13   | 14    |
|-----------|----------------|--------|---------|---------|---------|---------|--------|---------|---------|--------|---------|--------|--------|------|-------|
| <b>1</b>  | <b>BChE_0W</b> |        |         |         |         |         |        |         |         |        |         |        |        |      |       |
| <b>2</b>  | <b>PSS1</b>    | -.027  |         |         |         |         |        |         |         |        |         |        |        |      |       |
| <b>3</b>  | <b>PSS2</b>    | -.169  | .705**  |         |         |         |        |         |         |        |         |        |        |      |       |
| <b>4</b>  | <b>PSS3</b>    | .000   | .491**  | .410**  |         |         |        |         |         |        |         |        |        |      |       |
| <b>5</b>  | <b>PSS4</b>    | .333** | -.297** | -.415** | -.315** |         |        |         |         |        |         |        |        |      |       |
| <b>6</b>  | <b>PSS5</b>    | .121   | -.163   | -.021   | -.218*  | .376**  |        |         |         |        |         |        |        |      |       |
| <b>7</b>  | <b>PSS6</b>    | .077   | -.317** | -.083   | -.270*  | .307**  | .684** |         |         |        |         |        |        |      |       |
| <b>8</b>  | <b>PSS7</b>    | -.104  | -.287** | -.103   | -.374** | .217*   | .399** | .382**  |         |        |         |        |        |      |       |
| <b>9</b>  | <b>PSS8</b>    | -.002  | .403**  | .531**  | .364**  | -.270*  | -.065  | -.286** | -.064   |        |         |        |        |      |       |
| <b>10</b> | <b>PSS9</b>    | .094   | -.281** | -.060   | -.320** | .389**  | .623** | .519**  | .631**  | .048   |         |        |        |      |       |
| <b>11</b> | <b>PSS10</b>   | -.036  | -.128   | .033    | -.107   | .293**  | .654** | .505**  | .408**  | -.073  | .648**  |        |        |      |       |
| <b>12</b> | <b>PSS11</b>   | .235*  | .508**  | .375**  | .576**  | -.162   | -.196  | -.307** | -.358** | .314** | -.282** | -.119  |        |      |       |
| <b>13</b> | <b>PSS12</b>   | .156   | .076    | .102    | -.113   | .203    | .326** | .290**  | .151    | .042   | .187    | .189   | .047   |      |       |
| <b>14</b> | <b>PSS13</b>   | -.040  | -.040   | .017    | .015    | .230*   | .571** | .605**  | .418**  | -.042  | .520**  | .609** | -.118  | .149 |       |
| <b>15</b> | <b>PSS14</b>   | -.225* | .554**  | .648**  | .430**  | -.578** | -.126  | -.272*  | -.166   | .496** | -.251*  | -.192  | .425** | .098 | -.137 |

Note. N=85 for PSS4, PSS10, and PSS14; N=86 for others, \* $p < .05$ ; \*\* $p < .01$

Appendix F: Detailed Pearson Correlation Table of BChE\_0W with Subitems of PSS for Only Clinical Group

|    |                | 1      | 2      | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11     | 12     | 13   | 14   |
|----|----------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------|------|
| 1  | <b>BChE_0W</b> |        |        |        |        |        |        |        |        |        |        |        |        |      |      |
| 2  | <b>PSS1</b>    | .218   |        |        |        |        |        |        |        |        |        |        |        |      |      |
| 3  | <b>PSS2</b>    | .030   | .693** |        |        |        |        |        |        |        |        |        |        |      |      |
| 4  | <b>PSS3</b>    | .280   | .380*  | .264   |        |        |        |        |        |        |        |        |        |      |      |
| 5  | <b>PSS4</b>    | .227   | -.045  | -.319* | .038   |        |        |        |        |        |        |        |        |      |      |
| 6  | <b>PSS5</b>    | -.033  | -.005  | .259   | .019   | -.212  |        |        |        |        |        |        |        |      |      |
| 7  | <b>PSS6</b>    | -.026  | -.099  | .169   | .000   | -.223  | .713** |        |        |        |        |        |        |      |      |
| 8  | <b>PSS7</b>    | -.369* | -.174  | .074   | -.285  | -.094  | .388** | .353*  |        |        |        |        |        |      |      |
| 9  | <b>PSS8</b>    | .257   | .323*  | .463** | .264   | .038   | .153   | -.094  | .082   |        |        |        |        |      |      |
| 10 | <b>PSS9</b>    | -.067  | .013   | .295   | -.031  | -.075  | .548** | .432** | .656** | .433** |        |        |        |      |      |
| 11 | <b>PSS10</b>   | -.275  | .087   | .360*  | .208   | -.317* | .512** | .376*  | .364*  | .157   | .579** |        |        |      |      |
| 12 | <b>PSS11</b>   | .430** | .422** | .292   | .577** | -.038  | -.119  | -.242  | -.242  | .277   | -.113  | -.016  |        |      |      |
| 13 | <b>PSS12</b>   | -.036  | .132   | .084   | -.090  | .112   | .250   | .244   | .180   | .034   | .102   | .047   | .031   |      |      |
| 14 | <b>PSS13</b>   | -.064  | .058   | .187   | .297   | -.093  | .568** | .660** | .429** | .184   | .536** | .622** | -.017  | .147 |      |
| 15 | <b>PSS14</b>   | .020   | .417** | .596** | .092   | -.244  | .377*  | .116   | .208   | .360*  | .258   | .216   | .399** | .290 | .071 |

Note. N=43 for PSS4, and PSS14; N=44 for others, \* $p < .05$ ; \*\* $p < .01$

Appendix G: Total Luminescence Score of Each Rat Plasma Samples

