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Translational assessment of butyrylcholinesterase activity as a diagnostic biomarker for depression using a chemiluminescent probe

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Depressive episode is a globally prevalent psychiatric condition with a complex, multifactorial etiology involving genetic and environmental factors and despite advances in biomarker research, clinical applications remain limited due to methodological inconsistencies and population variability. Butyrylcholinesterase (BChE), an enzyme involved in cholinergic transmission, has been less studied in the context of depression compared to acetylcholinesterase. Utilizing a BChE-responsive chemiluminescent probe (BCC), BChE activity was measured in a translational approach: human serum, rat plasma, and a cell culture model. First, BChE activity was assessed in patients with depression (32 unipolar depression (UD), 21 bipolar disorder with depressive episode (BD-D)) compared to controls, then changes with respect to remission were reassessed ($n = 15$). Second, a chronic unpredictable mild stress (CUMS) model in rats was used to assess BChE activity in response to fluoxetine treatment. Lastly, BChE activity in response to corticosterone, norepinephrine and fluoxetine was measured in a cell culture model. Results demonstrated significantly lower BChE activity in UD and BD-D patients compared to healthy controls, with restoration following remission. In rats, BChE activity correlated with stress-induced anhedonia, and fluoxetine increased BChE levels although not reaching significance. In vitro, corticosterone exposure reduced BChE activity while fluoxetine reversed this effect and norepinephrine increased BChE activity in solely exposure and subsequent fluoxetine. These findings suggest that BChE activity may serve as a diagnostic biomarker for depression and a potential indicator of treatment response.

Depressive episode is a prevalent psychiatric condition with significant global impact[1], affecting approximately 300 million people worldwide and projected to become the leading cause of disease burden by 2030, resulting in an estimated $1 trillion economic burden[2]. The etiology of depressive episode is complex and multifactorial, involving genetic predispositions (35% heritability) and environmental influences[1]. Multiple pathological mechanisms contribute to its development, including catecholamine neurotransmitter deficiencies, hypothalamic-pituitary-adrenal axis dysregulation, oxidative stress, chronic inflammation, immune dysregulation, and impaired synaptic plasticity[3]. Biomarker research in this field aims to unravel the complex pathophysiology of depression and develop objective diagnostic tools. Ideally, these tools would be reliable, safe, easily measurable, highly sensitive and specific, cost-effective, and useful for diagnosis, staging, and prognosis[4]. However, despite proposed promising candidates such as inflammatory markers (e.g., IL-1, IL-6, TNF-α)[5], glutamate system alterations[6], and metabolic dysfunction biomarkers[7–9], the clinical use of biomarkers in psychiatry remains limited due to inconsistent protocols, patient parameters neglect, and population variability[7].

Cholinergic transmission, crucial for arousal, attention, memory, and motivation, involves acetylcholine (ACh) as a primary neurotransmitter and neuromodulator[10]. Accumulative findings suggest that dysregulation

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of ACh levels is linked to the pathology of depressive episode[11–13]. ACh is degraded by two enzymes: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Research has mainly focused on AChE due to its significant presence in the brain and specificity towards ACh[14]. However, BChE, which is more abundant in the liver, plasma, and serum, also contributes to cholinergic transmission in critical brain regions and compensates for AChE deficiencies in need[15–17]. BChE is also involved in neurite growth, cellular proliferation, lipid metabolism, apoptosis, xenobiotic detoxification, and proteolytic activity[14]. Recent reviews have highlighted its role as a bioscavenger and a metabolic regulator, particularly in the hydrolysis of ghrelin and fatty acid esters, linking it to obesity and systemic inflammation[18,19]. Additionally, serum BChE activity has recently emerged as a robust prognostic marker for severity and mortality in acute inflammatory states like COVID-19, where its decline reflects both systemic inflammation and the depletion of the cholinergic anti-inflammatory pathway[18,20]. However, research on BChE's role in depressive episode has been limited and inconsistent, partly due to small sample sizes, methodological differences, and inefficient tools for measuring BChE activity[21–27].

Several methods have been developed to measure BChE activity, including spectrophotometric, fluorometric, and electrochemical assays. Ellman's colorimetric assay, the most widely used method, indirectly measures ChE activity through the formation of 5-thio-2-nitrobenzoic acid (TNB) anion[28]. However, this method has limitations in both sensitivity and specificity such as instability of the DTNB reagent and interference from free thiols like glutathione[29]. Even if optimized versions such as paper-based models, electrochemical techniques, enzyme-linked immunosorbent assay (ELISA), and pH indicator assays, offer improvements but often require complex sample preparation and are not cost-efficient[30–32]. Fluorescence imaging with selective cyclopropyl-masked probes enables real-time, high-resolution monitoring of BChE activity[33–36]. Despite their success in biological environments, these probes face inherent limitations such as limited light penetration and photobleaching. Recent advances include phenoxy 1,2-dioxetane-based chemiluminescent probes, which offer high sensitivity and signal-to-noise ratios under physiological conditions[37]. Recently, one of our groups introduced the first-ever example of a BChE-responsive 1,2-dioxetane derivative (BCC) and demonstrated its competency for in vitro and in vivo visualization of BChE activity[38]. Usage of cyclopropyl masking unit, which was first proposed by Yang et al.[34] and, combined with the advantages of chemiluminescence imaging created a highly sensitive monitoring probe with a very low detection limit[38]. However, its activity in serum or plasma, where BChE is most abundant, has not yet been determined.

In this study, we utilized BCC to investigate the relationship between BChE activity and depressive episode from a translational perspective. We assessed BCC's effectiveness in measuring BChE activity in human serum, later we compared BChE activity among healthy controls (HC), individuals with a depressive episode [UD and BD-D]. Additionally, we evaluated BCC's utility in detecting BChE activity in rat plasma, and measured BChE activity in a chronic unpredictable mild stress model with and without fluoxetine treatment. We also explored BChE activity in PC-12 Adh cells, in relation with corticosterone, norepinephrine and fluoxetine for an in vitro comparison. Our findings advance the use of chemiluminescent probes in biomarker research and highlight BChE's potential as a novel diagnostic and therapeutic biomarker for UD (Fig. 1).

Materials and methods

Determination of BChE activity in human serum using BCC

The synthesis of BCC was reported in our previous study [35]. To assess the feasibility of BCC for detecting luminescence, a human serum from a healthy control was diluted with DPBS in various concentrations (0–4%) and 50µL of each was added in an opaque (black- sided) 96-well plate. 100µM BCC (50µL, 2% DMSO) was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2 h 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 50µM (1% DMSO in 100µL).

Inhibition assays were conducted to confirm that the observed luminescence was attributable to BChE activity. For donepezil inhibition study, the healthy control serum was diluted with DPBS to 0.2% and 50µL of each was added in an opaque (black-sided) 96-well plate. Then, various concentrations of donepezil (D6821, Sigma-Aldrich) (0–25 mM) were added to wells in 40µL total and the plated was incubated for 1 h. Afterward, 10µL (100 µM in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently monitored for 2 h 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 10µM (0.5% DMSO in 100µL), 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 50µL of each was added in an opaque (black-sided) 96-well plate. Then, various concentrations of tacrine (A79922, Sigma-Aldrich) (0–250 mM) were added to wells in 40µL total and the plate was incubated for 1 h. Afterward, 10µL (100 µM in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently monitored for 2 h 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 10µM (0.5% DMSO in 100µL), and tacrine had a final concentration in the interval of 0–100 mM (0, 2.5, 5, 10, 20, 50, 100 mM).

Evaluation of BChE activity in depressive episode in human serum samples

Human serum samples and psychometric data were obtained from the reference study[39]. 100 participants' serum samples were used for the baseline analysis (52 patients (32 individuals with unipolar depression (UD), 20 bipolar disorder depressive episode (BD-D) and 48 controls). Samples of 15 patients (11 UD, 4 BD-D) have also been retested for the change of BChE activity compared to baseline after 8 weeks of treatment. The participant flowchart is detailed in Supplementary Figure S1 and more detailed methods are given in Supplementary

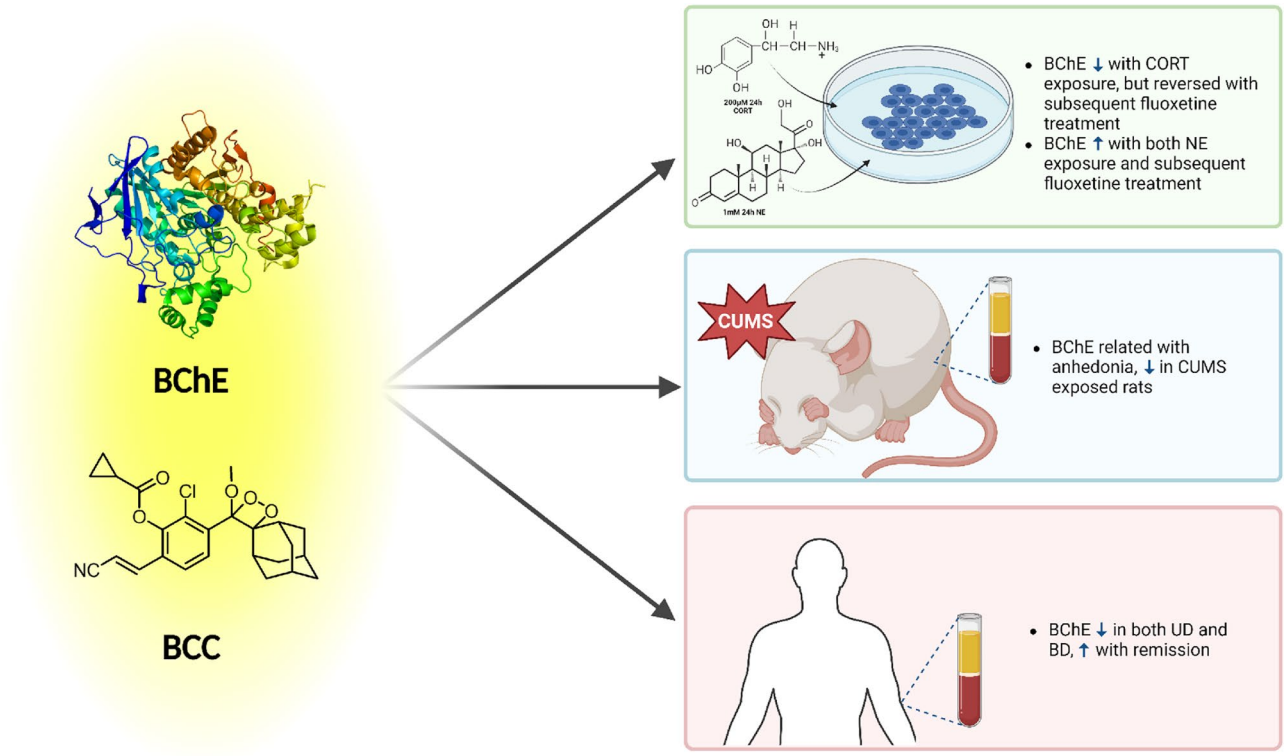


Fig. 1. Translational assessment of butyrylcholinesterase (BChE) activity across in vitro, animal, and human models of depression. Left panel illustrates the BChE protein structure and the chemiluminescent BChE probe (BCC) used for activity assessment. Right panels summarize findings across translational levels. **Top**: In vitro experiments show that BChE activity decreases following corticosterone (CORT) exposure and increases after norepinephrine (NE) exposure in PC12 cells, followed by an increase by subsequent fluoxetine treatment. **Middle**: In the chronic unpredictable mild stress (CUMS) rat model, reduced BChE activity is associated with anhedonia, with significantly lower levels observed in CUMS-exposed animals. **Bottom**: In human samples, BChE activity is reduced in individuals with a depressive episode and increased during remission. Together, these results support BChE activity as a translational biomarker linked to depressive phenotypes and treatment response.

Methods. Hamilton Depression Scale scores of participants at each time point were also used for the further analysis.

This study was conducted under the ethical guidelines approved by the Koç University Ethical Committee (protocol number 2023.282.IRB2.062). Human serum samples from three groups were diluted with DPBS to 1% concentration and 50µL of each was added in an opaque (black-sided) 96-well plate. Afterward, 100 µM BCC (2% DMSO in 50µL DPBS) BCC was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2 h 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 50µM (1% DMSO in 100µL). Same procedure was used for the 8th week samples.

Determination of BChE activity in rat plasma using BCC

To determine if BCC produces a luminescence signal in rat plasma, 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 2 h 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 10µM (0.5% DMSO in 100µL).

To confirm the luminescence signal originated from BChE, inhibition experiments were conducted. For donepezil inhibition, a Control + Saline plasma was diluted with DPBS to 0.1% and 50µL 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 40µL total and the plated was incubated for 1 h. Afterward, 10µL (100 µM in 5% DMSO) BCC was added to each well with multichannel and time-dependent luminescence response was subsequently monitored for 100 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 10µM (0.5% DMSO in 100µL), and donepezil had a final concentration in the interval of 0–10 mM (0, 1, 2, 5, 10 mM).

For tacrine inhibition, a Control + Saline plasma was diluted with DPBS to 0.1% and 50µL 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 40µL total and the plate was incubated for 1 h. Afterward, 10µL (100 µM 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 10µM (0.5% DMSO in 100µL), and tacrine had a final concentration in the interval of 0–10 mM (0, 2.5, 5, 10, 20, 50, 100 mM).

Evaluation of the relationship between BChE activity in a chronic stress rat model of depression

Rat plasma samples and associated behavioral measurements were sourced from pre-registered study (TUBITAK 1001, 122Z771). A 35-day Chronic Unpredictable Mild Stress (CUMS) protocol was implemented (detailed in Figure S2)[40–44], involving 33 male Wistar rats divided into a non-stressed control group receiving saline (NaCl 0.9%, 1.5 ml) (Control + Saline, $n = 9$) and two CUMS-exposed groups receiving daily intraperitoneal injections of either saline (CUMS + Saline, $n = 9$) or 10 mg/kg fluoxetine (CUMS + Fluoxetine, $n = 15$; 0750722, Satyadivis Pharmaceuticals) starting from day 15. The procedure was terminated at day 35, with behavioral analysis using the sucrose preference test (SPT)[45], forced swim test (FST)[46], and percentage weight change from baseline for each rat. Blood samples were taken from the heart and stored into the anticoagulant EDTA tube. Tubes were immediately stored on ice. 3 h 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 until further analysis.

All samples of rat plasma from three groups (Control + Saline, CUMS + Saline, and CUMS + Fluoxetine) were diluted with DPBS to 2% concentration and 50µL of each was added in an opaque (black-sided) 96-well plate. Afterward, 40 µM BCC (2% DMSO in 50µL DPBS) BCC was added to each well with multichannel. Subsequently, time-dependent luminescence response was monitored for 2 h 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 20µM (1% DMSO in 100µL).

Evaluation of BChE activity determination in PC-12 adh cells in relation to corticosterone, norepinephrine, and Fluoxetine

PC12 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. PC12 Adh 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 170 S incubator (humidified, 37 C, 5% CO2). The cells were passaged with DPBS and 0.05% Trypsin/EDTA at ~70% confluency.

Cell viability was assessed using the MTT assay to measure mitochondrial dehydrogenase activity as an indicator of cellular health. For all assays, cells were seeded at a density of 2×10^4 cells/well in opaque 96-well plates and allowed to attach overnight. After washing twice with DPBS, cells were exposed to various treatments: BCC (0–50 µM, 1% DMSO, 100µL total) tacrine (0, 2, 5, 10, 20, 50, 100, and 200 µM, 100µL total) were administered for 1 h followed by a 23-h recovery in fresh medium, while corticosterone (0, 50, 100, 200, 400, and 500 µM, 1% DMSO, 100µL total) and norepinephrine (0, 10, 20, 50, 100, 200, 400,500, and 1000 µM, 100µL total) were administered for a continuous 24-h period. Following treatment, the medium was replaced with MTT solution (0.5 mg/mL) for 4 h. The resulting formazan crystals were solubilized in 100 µL of DMSO with orbital shaking, and absorbance was measured at 570 nm using a Biotek Synergy H1 microplate reader. All experiments were performed with six technical replicates.

BChE activity in PC-12 Adh cells was monitored across several treatment conditions using opaque, black-sided 96-well plates with an initial overnight incubation for cell attachment. In the in vitro comparison experiments, treated groups were seeded at 2×10^4 cells/well and exposed to either 200 µM CORT (1% DMSO) or 1 mM NE for 24 h, while control groups were seeded at 1×10^4 cells/well and received a medium replacement containing 1% DMSO. For experiments investigating subsequent fluoxetine treatment, all cells were seeded at 1×10^4 cells/well and initially stressed with either 200 µM CORT or 1 mM NE for 24 h; following this period, cells were washed twice with DPBS and incubated for an additional 24 h with 0 or 5 µM fluoxetine. After the completion of all respective treatment protocols, the media were discarded and the cells were washed twice with DPBS before being treated with 10 µM BCC (1% DMSO in 100 µL DPBS). Kinetic luminescence measurement was initiated immediately and monitored for 2 h using a Biotek Synergy H1 MF microplate reader, with all experiments performed using six technical replicates.

Statistical analysis

Statistical analyses were performed using SPSS (Version 28.0.0), GraphPad Prism (Version 9.5), and Jamovi (Version 2.3.26.0). For human serum baseline data analysis of 100 participants (48 healthy controls, 32 unipolar depression patients, and 20 bipolar disorder depressive episode patients), normality was assessed using Shapiro-Wilk tests. BChE_0W and PSS Total data were found to be normally distributed across groups (Shapiro-Wilk $p > .05$), and homogeneity of variances was confirmed using Levene's test ($p > .05$). Group means for these normally distributed variables were compared using one-way ANOVA with Tukey's HSD post-hoc tests. For HDRS Total, YMRS, age, BMI, and education data, which showed deviations from normality in at least one group (Shapiro-Wilk $p \leq .05$), group differences were analyzed using the non-parametric Kruskal-Wallis test,

with Mann-Whitney U post-hoc tests and Bonferroni correction for pairwise comparisons. To determine any potential differences in terms of parameters stated above between group, nonparametric Kruskal-Wallis test was used.

For cell culture analyses, due to the small sample size in each group ($n = 6$), the non-parametric Mann-Whitney U test was employed. For all 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). Statistical significance was set at $p < .05$ for each analysis.

Results

Evaluation of the relationship between BChE activity in human serum and depressive episode

The performance of BCC in human serum was assessed by comparing luminescence signals from 0.005% human serum with 50 μM BCC (1% DMSO) to those from serum alone and BCC alone. Only the combination of serum and BCC produced a significant luminescence, peaking at 27 min with a 20-fold increase (Figure S3.a). The total luminescence was 17-fold greater than serum alone and 15-fold greater than BCC alone (Figure S3.b). A dose-dependent titration of human serum (0.005%–2%) with BCC showed that peak luminescence was delayed with higher serum concentrations, and a linear response was observed between 0.005% and 0.1% serum ($R^2 = 0.9906$) before saturation (Figures S4). Inhibition experiments with donepezil and tacrine demonstrated that the luminescence signal was specific to BChE, as luminescence decreased with increasing inhibitor concentrations (Figures S5-S6). Upon demonstrating BCC can sensitively detect BChE activity in human serum lower than 0.005% in a single step, human serums from clinical and non-clinical participants at 0th week were screened with BCC (Figure S7). The one-way ANOVA revealed a significant main effect of group on BChE_0W activity, $F(2, 97) = 18.4$, $p < .001$, indicating a significant overall difference in BChE_0W activity across the healthy control, unipolar depression, and bipolar depression groups. Tukey's HSD post-hoc comparisons revealed that healthy controls (HC) had significantly higher BChE_0W activity compared to both the unipolar depression (UD) group (Mean difference = 239593, $t(97.0) = 5.70$, $p < .001$) and the bipolar depression (BD-D) group (Mean difference = 190176, $t(97.0) = 3.881$, $p < .001$). The post-hoc comparison between the unipolar depression (UD) and bipolar depression (BD-D) groups for BChE_0W was not statistically significant (Mean difference = −49418, $t(97.0) = -0.942$, $p = .615$) (Fig. 2). The detailed comparison of groups in terms of sociodemographic, total score psychometric scales, and total luminescence score of BCC at 0th week (BChE_0W) is given in Table 1.

As the distribution of age and BMI varied across groups, each preliminary assumption has been tested and multiple linear regression analysis was employed to test if age, BMI, and group significantly predicted BChE_0W. The group variable was recoded into nonclinical (healthy control) and clinical (unipolar depression and bipolar disorder with unipolar depression) groups. The model explained 37.6% of the variance and was a significant predictor of the total luminescence score at BChE_0W ($F(3, 96) = 19.25$, $p < .001$) (Table 2). Being in the clinical group significantly lowered BChE activity despite the effects of age and BMI. Same analysis was done by dividing clinical groups into unipolar depression and bipolar disorder with depressive episode (Table S1) and found that having unipolar depression has more substantial effect.

A Pearson correlation coefficient was computed to assess the relationship between BChE_0W and psychometric scales for both the entire sample and the clinical group. In the overall group, there was a significant negative correlation between HDRS and BChE_0W, $[r(98) = -0.496, p < .001]$. In the clinical group, a significant positive correlation between PSS and BChE_0W was observed, $[r(40) = 0.359, p = .019]$ (Table S2, S3). In the clinical group, when the correlation coefficient between BChE_0W and subitems of HDRS was determined, there was a significant negative correlation between BChE_0W and the HDRS suicide item (#11), $[r(50) = -0.364, p = .008]$, and a significant positive correlation between BChE_0W and the HDRS somatic anxiety item (#13), $[r(50) = 0.277, p = .047]$ (Table S4, S5). Analysis of PSS subitems showed a significant negative correlation between BChE_0W and PSS#7, $[r(44) = -0.369, p = .014]$ and a significant positive correlation between BChE_0W and PSS#11, $[r(44) = 0.430, p = .004]$ (Table S6, S7).

After the 8-week antidepressant treatment period, remission was assessed using an HDRS score of 7 or lower and the absence of depression criteria in the SCID-I Interview. When the change in the activity of BChE in clinical group ($n = 15$: 11 UD, 4 BD-D) between baseline (0th week) and the 8th week, a significant increase in BChE levels was observed ($n = 15$, $p < .001$) (Fig. 3). While the mean total luminescence BChE at baseline was $1.24 \times 10^6 \pm 1.53 \times 10^5$ (min:1.00×10^6 max: 1.50×10^6), it became was $1{,}57 \times 10^6 \pm 1.34 \times 10^5$ (min:1.25×10^6 max:1.75×10^6) at the 8th week.

A linear mixed model was used to investigate whether changes in BChE_0W to BChE_8W were predicted by changes in HDRS. The model included total luminescence of BChE as the dependent variable, with time and HDRS as fixed effects, and ID as a random effect. Results indicated a significant negative relationship between HDRS and BChE ($p = .024$), while time did not significantly influence BChE activity ($p = .913$) (Table S8).

The analysis for HDRS suicide item (#11) revealed a significant negative relationship between its severity and BChE activity ($p = .004$), while the effect of time on BChE activity was initially significant ($p = .031$) but not after Bonferroni correction ($p = .051$), indicating that as suicide severity decreases, BChE activity increases, with minimal variability between individuals (Table S9, S10). However, same effect was not observed for HDRS somatic anxiety item (#13) (Table S11,S12).

Evaluation of the relationship between BChE activity in a chronic stress rat model of depression

The model was terminated at 35th day, and the details (behavioral tests and animal characteristics) were provided at Table S13 and Figure S8-12. The final sample composition was 9 Control + Saline, 7 CUMS + Saline, and 12 CUMS + Fluoxetine rats.

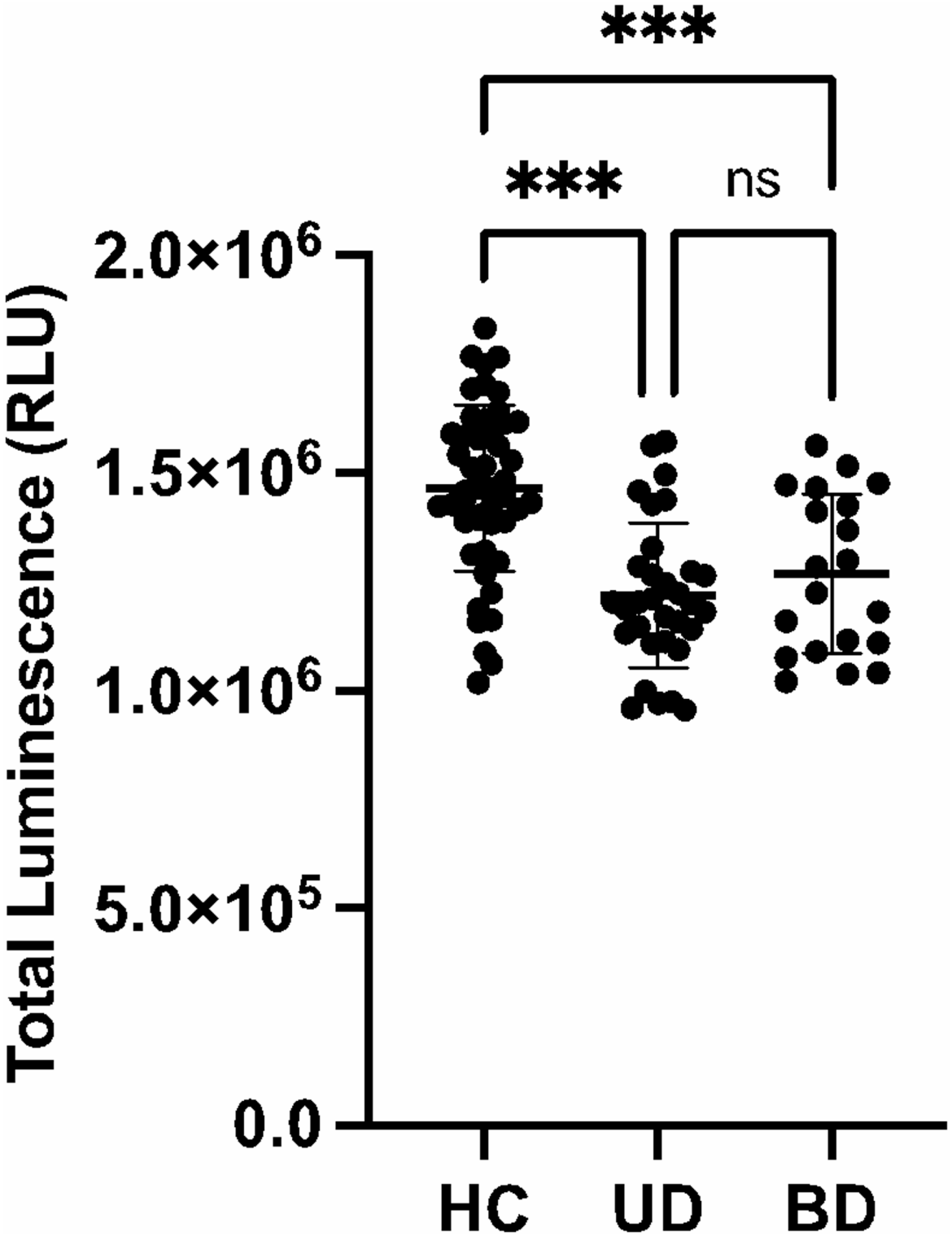


Fig. 2. Total luminescence score comparison of healthy controls (HC), unipolar depression (UD), and bipolar disorder with depressive episode (BD-D). There is a significant difference between HC with both UD ($p < .001$) and BD-D ($p < .001$), but no significant difference between UD and BD-D ($p = .615$) (number of technical replicates = 3). Data were presented as mean ± SD. *** represents $p < .001$.

BCC's ability to monitor BChE in rat plasma was tested. Luminescence from 0.005% rat plasma with 10 µM BCC (0.5% DMSO) was significantly higher compared to plasma or BCC alone, peaking at 30 min with a 60-fold increase (Figures S13a, b). Dose-dependent titration with rat plasma (0.005%–2%) and BCC showed peak luminescence at 30 min for lower concentrations, shifting to 36 min at higher concentrations (Figure

Groups	HC ($n=48$) (n%)	UD ($n=32$) (n%)	BD-D ($n=20$) (n%)	p	
Sex (female%)	31 (64.6%)	24 (75.0%)	14 (70.0%)	χ^2: 0.986, $p=.611$	
Smoking (smoker%)	18 (37.5%)	20 (62.5%)	7 (35.0%)	χ^2: 5.859, $p=.053$	
Working Status (Yes%)	22 (45.8%)	15 (46.9%)	13 (65.0%)	χ^2: 2.258, $p=.323$	
Alcohol (No%)	20 (41.7%)	20 (62.5%)	10 (50.0%)	χ^2: 5.697, $p=.223$	
Exercise (Yes%)	20 (41.7%)	6 (18.8%)	6 (30.0%)	χ^2: 4.615, $p=.099$	
	Mean ± SD (min-max)	**Mean ± SD (min-max)**	**Mean ± SD (min-max)**	p^*	p^{**}
Age	26.5 ± 7.49 (18–45)	32.8 ± 8.85 (18–45)	33.00 ± 7.91 (19–45)	**0.001**	**0.006 (HC- UD)** **0.011 (HC- BD-D)** 1.00 (UD- BD-D)
Education (year)	14.9 ± 2.67 (5–20)	12.6 ± 4.82 (5–20)	15.8 ± 3.68 (8–23)	0.086	
BMI	22.4 ± 3.58 (15.6–32.4)	25.2 ± 5.45 (17.1–41.0)	25.4 ± 4.72 (16.9–33.6)	**0.013**	**0.047 (HC- UD)** **0.048 (HC- BD-D)** 1.00 (UD- BD-D)
HDRS	0.417 ± 1.13 (0–5)	21.7 ± 4.07 (18–32)	22.9 ± 5.78 (18–35)	**<0.001**	**<0.001 (HC- UD)** **<0.001 (HC- BD-D)** 1.00 (UD- BD-D)
YMRS	0.167 ± 0.595 (0–3)	1.38 ± 1.43 (0–4)	1.45 ± 1.93 (0–6)	**<0.001**	**<0.001 (HC- UD)** **0.004 (HC- BD-D)** 1.00 (UD- BD-D)
PSS*	22.3 ± 6.60 (11–35)	32.6 ± 6.73 (19–52)	30.9 ± 5.83 (20–40)	**<0.001**	**<0.001 (HC- UD)** **<0.001 (HC- BD-D)** 0.666 (UD- BD-D)
BChE_0W (RLU)	$1.46\times10^6 \pm 1.95\times10^5$ (1.02×10^6–1.83×10^6)	$1.22\times10^6 \pm 1.67\times10^5$ (9.57×10^5–1.57×10^6)	$1.27\times10^6 \pm 1.82\times10^5$ (1.02×10^6–1.56×10^6)	**<0.001**	**<0.001 (HC- UD)** **<0.001 (HC- BD-D)** 0.615 (UD- BD-D)

Table 1. Groups comparison of sociodemographic data, psychometric data and total BCC luminescence score. Notes. HC: Healthy controls; UD: Unipolar depression; BD-D: Bipolar disorder with depressive episode; BMI: Body-mass-index; HDRS: Total Hamilton depression rating score; YMES: Total young mania rating 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^*: One-way Anova for BChE_0W and PSS; Kruskal-Wallis test for others; p^{**}: Tukey's HSD post-hoc test for BChE_0W and PSS; Bonferroni correction for the others.

Model	B	Coefficients Std. Error	Std. Coefficients (β)	p
(Constant)	1821474.34	98503.36	-	**<0.001**
Clinic_group	−154973.52	37972.07	−0.364	**<0.001**
Age	−6287.05	2230.84	−0.252	**0.006**
BMI	−8820.57	3999.12	−0.192	**0.030**

Table 2. Multivariate linear regression model to assess predictors of BChE_0W by dividing group with clinical and non-clinical samples.

S14a). Linear regression indicated a strong linear response between 0.005% and 0.5% plasma (R^2 = 0.9963) before saturation (Figures S14b, c). Inhibition with donepezil and tacrine reduced luminescence, confirming it originated from BChE (Figures S15,16).

Following BCC's competency was confirmed in rat plasma environment, all group samples were subsequently screened (Figure S17). Rats who exposed the CUMS and saline injection showed significantly lower BChE activity compared to controls only exposed to saline injection ($p=.011$). Besides there was an increase in terms of BChE activity in rats who exposed CUMS and concurrent fluoxetine, which could not reach a significance ($p=.345$) (Fig. 4).

A Pearson correlation analysis showed a significant positive correlation between BChE score and sucrose preference% [$r(28)=0.544$, $p=.003$], but no significant correlations with immobility ($p=.184$), climbing ($p=.448$), or swimming ($p=.875$) (Table S14). Upon noting differences in the distribution of starting age by day among groups, multiple linear regression analysis was conducted to determine if age and sucrose preference% significantly predicted BChE_score (Table 3). Model explained 32.9% of the variance in BChE_score and was a significant predictor of the total luminescence score at BChE_0W ($F(2, 25)=6.14$, $p=.007$).

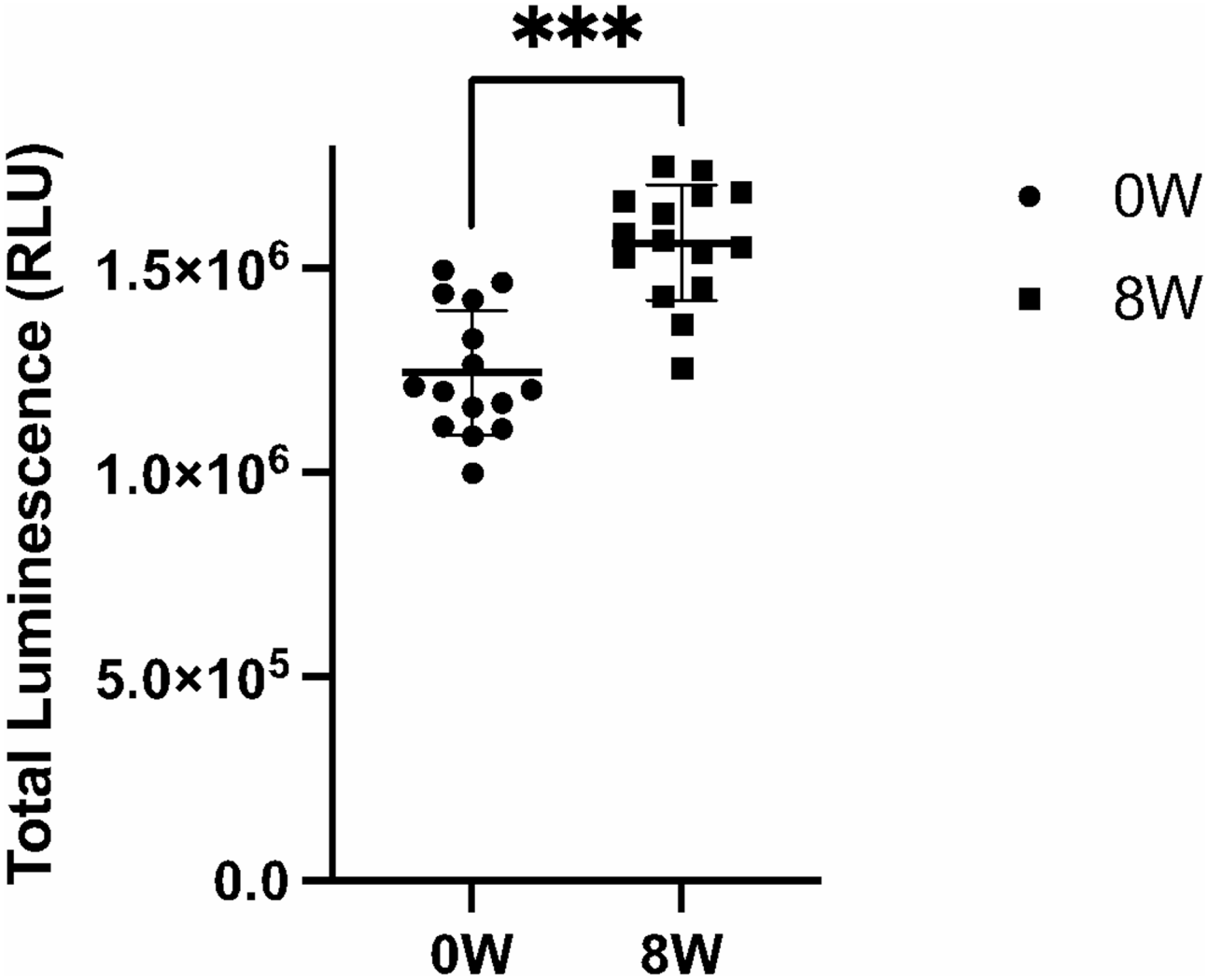


Fig. 3. Total luminescence score comparison of 0th week and 8th week ($n = 15$, number of replicates = 3, $p < .001$) following 8 weeks of antidepressant treatment. Data was presented as mean ± SD.

Evaluation of BChE activity determination in PC-12 adh cells in relation to corticosterone, norepinephrine, and fluoxetine

After consistent results were obtained in human serum and rat plasma findings, the in vitro validity of BCC was evaluated in PC-12 Adh cells exposed to psychological stress related molecules to further elucidate the mechanism. Toxicity was evaluated using the MTT assay, showing no toxicity up to 50 μM (Figure S18), making BCC suitable for in vitro imaging. The time-dependent and dose-dependent responses of BCC were tested in PC-12 Adh cells over 200 min. BCC exhibited a rapid response to BChE, peaking at 60 min before decreasing due to consumption (Figure S19a). The dose-dependent increase in luminescence was highly linear (Figures S19b, c). To confirm that luminescence originated from endogenous BChE, cells were treated with tacrine, resulting in a dose-dependent decrease in luminescence (Figure S20). This decrease was not attributed to tacrine-induced cell toxicity (Figure S21).

The capacity of BCC was further validated by modeling the psychological stress related molecules' effects. First, a corticosterone model to mimic HPA-axis outcomes was established. PC-12 Adh cells treated with various corticosterone concentrations for 24 h showed decreased viability with increasing corticosterone (Figure S22). A corticosterone concentration of 200 μM, where cell viability dropped to 50%, was selected. BChE activity significantly decreased at this concentration compared to control ($p = .0022$) (Fig. 5a). Next, a norepinephrine stress model was created. Cells treated with varying norepinephrine concentrations for 24 h also showed decreased viability with increasing norepinephrine (Figure S23). A concentration of 1 mM norepinephrine, resulting in 50% toxicity, was chosen. BChE activity increased significantly at this concentration compared to control ($p = .0022$) (Fig. 5c). Fluoxetine's effect was tested in both stress models. In the corticosterone model, cells treated with 200 μM corticosterone were also exposed to 5 μM fluoxetine for 24 h. BChE activity increased significantly in the fluoxetine-treated group compared to control ($p = .004$) (Fig. 5b). Similarly, in the norepinephrine model, cells treated with 1 mM norepinephrine and 5 μM fluoxetine showed a significant increase in BChE activity compared to control ($p = .010$) (Fig. 5d).

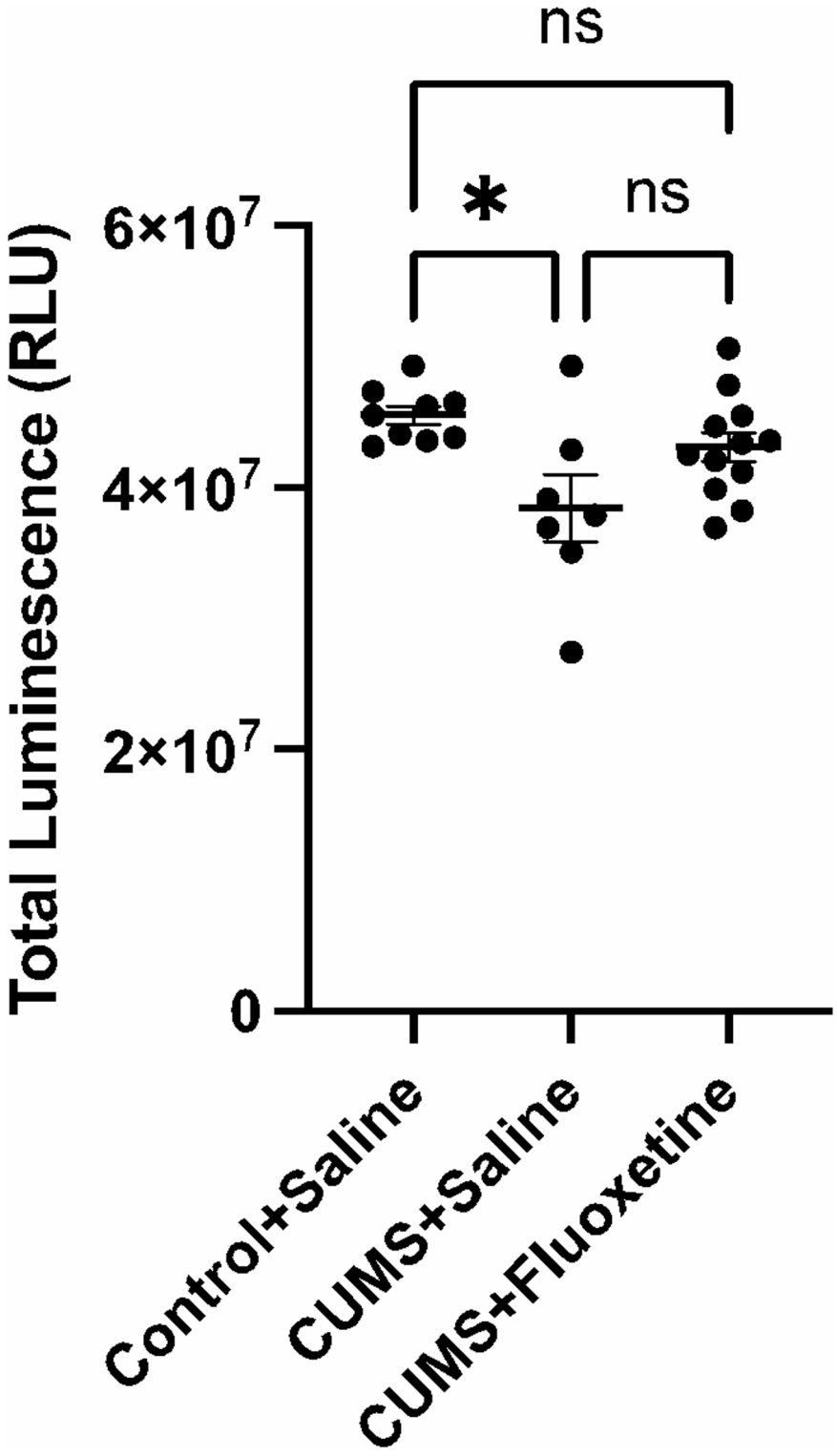


Fig. 4. Total luminescence score comparison of Control + Saline, CUMS + Saline, and CUMS + Fluoxetine plasma samples. While there is a significant difference between Control + Saline and CUMS + Saline ($p = .011$), no significance difference between Control + Saline and CUMS + Fluoxetine ($p = .323$) and CUMS + Saline and CUMS + Fluoxetine ($p = .345$) was observed. ($n = 28$, number of technical replicates = 3). Data were presented as mean ± SEM.

Model	B	Coefficients Std. Error	Std. Coefficients (β)	*p*
(Constant)	1.618×10^9	1.686×10^9	-	0.347
Starting Age	-6.961×10^6	6.216×10^6	−0.217	0.273
Sucrose preference%	3.589×10^7	1.622×10^7	0.428	**0.036**

Table 3. Multivariate linear regression model to assess predictors of BChE score in rat plasma.

Discussion

Although BCC has proven effective in monitoring BChE activity in cell lines and tumor-bearing mice in our previous work[38], its performance in human serum was unvalidated prior to this study. In this study, we addressed this gap by analyzing luminescence in a mixture of 0.005% human serum and 50 μM BCC over 2 h. Our results showed a 20-fold increase in peak luminescence at 27 min and a 15-fold total increase compared to controls, confirming BCC's sensitivity to BChE activity in human serum for the first time. Dose-dependent serum titration (0.005–0.10%) had a high correlation ($R^2 = 0.991$), and inhibition experiments with donepezil and tacrine confirmed BCC's specificity for BChE activity, which is consistent with our previous findings on BCC's selectivity for BChE over other enzymes and serum components[38]. While optimized colorimetric protocols, such as those described by Dingova et al.[29], have successfully mitigated issues regarding reagent instability and thiol interference, they remain fundamentally limited by the optical complexity of biological fluids—specifically background absorbance from hemoglobin and lipids—which the zero-background nature of the chemiluminescent BCC probe effectively circumvents.

The observed plateau at serum concentrations above 0.5%, particularly in the 1–2% range, suggests that higher BCC concentrations may be required for accurate measurement at elevated serum levels. After confirming BCC's efficacy in human serum, we then measured BChE activity in serum from healthy controls (HC) and patients with unipolar depression (UD) and bipolar depression (BD-D). Healthy controls had significantly higher BChE activity than patients, with no significant difference between UD and BD-D. Although age and BMI influenced BChE levels, the clinical group effect remained significant, with slightly better model fit when differentiating between UD and BD-D. Our findings suggest a decline in BChE activity with age, consistent with some previous studies[27,47–50]. This age-dependent decrease is further corroborated by recent large-scale observational data, which found significantly lower BChE values in COVID-19 patients aged 65 years—a demographic that also represented nearly 96% of the cohort's total mortality[18,20]. Similarly, our finding that BMI also negatively impacted BChE activity, aligning with research linking BChE variants to obesity-related phenotypes[51–55]. Interestingly, while our clinical data shows a negative impact of BMI on BChE activity, studies in COVID-19 populations have reported a positive correlation, though a progressive decline in the enzyme remains a specific indicator of a fatal course regardless of the initial BMI[18,20].

Based on the multivariate linear regression analysis presented in Table S1, we observed that the clinical group negatively affected BChE activity, with UD suggesting a potentially stronger negative effect than BD-D, when controlling for age and BMI. This observation from our regression model may suggest potential neurochemical differences between UD and BD in relation to BChE activity[56]. In line with the oxidative stress theory of depression[57], we propose that the observed decrease in BChE activity in depressive states may be linked to oxidative stress, which disrupts biological systems involved in depression. It is well-established that elevated reactive oxygen species can interfere with cellular functions, trigger inflammation, alter neurotransmitter metabolism, inhibit neurogenesis, and impair synaptic plasticity, all of which contribute to depressive symptoms[57,58]. Therefore, the inhibition of BChE activity by oxidative stress byproducts, such as hydrogen peroxide, and the association of decreased BChE levels with lipid peroxidation further support this hypothesis[59].

Previous literature also includes a few studies assessing BChE activity and UD with conventional and non-translational methods. For example, Jope et al.[21] found lower levels of BChE in patients with mania, UD, and schizophrenia. Supporting these findings, Awan et al. confirmed decreased serum BChE activity in UD[60]. Genetic studies have also provided converging evidence, linking BCHE gene variants to depressive symptoms and altered glucocorticoid secretion[22]. Furthermore, the potential of BChE as a pharmacological agent for stress modulation has been suggested[23]. Interestingly, studies on environmental factors like pesticide exposure have also been associated with reduced BChE levels and increased risk of UD and suicidal behaviors[24]. However, in contrast to the general trend of reduced BChE in depression, Modai et al. reported a significantly higher BChE activity in patients with agitated depressive and acute psychotic episode compared to retarded patients[26]. Similarly, Katewa et al.[25] this seemingly contradictory finding by reporting that the BChE activity is higher in people with UD and BD compared to controls. On the other hand, and further adding to the complexity, Kücükosman et al.[27] found no significant association between BChE level with diagnosed psychiatric diseases in electroconvulsive therapy cases while reporting positive correlation in terms of age and BMI. As noted by previous researchers, these inconsistent findings mainly stem from methodological limitations such as psychiatric diagnosis methods, small sample sizes, medication confounds, and failure to account for relevant parameters (e.g., BMI, smoking, exercise). Moreover, we emphasize that all of those prior studies used conventional analysis techniques to assess BChE activity and, importantly, no repeated analysis with remission was performed. In contrast, in the present study, it has been first proposed a novel technique to assess BChE activity very sensitive and specific in lower than 0.005% human serum in a single step. Using this novel approach, we have shown for the first time that BChE activity is significantly decreased in active depressive episode (both UD and BD-D). Furthermore, and also for the first time, we have shown that the association between BChE activity and depression is dynamic in which BChE activity significantly increases with remission.

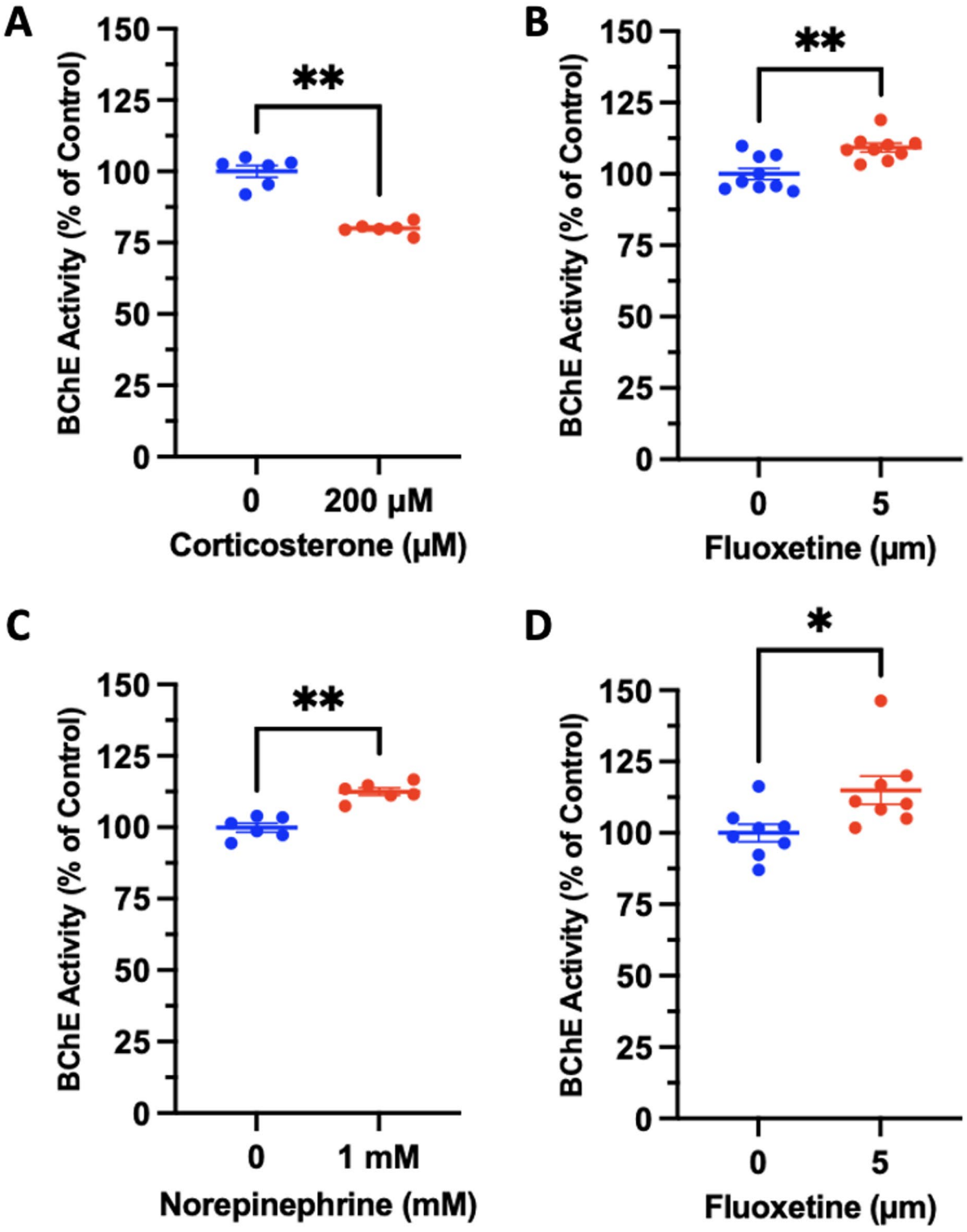


Fig. 5. BChE activity change in corticosterone or norepinephrine exposure and subsequent fluoxetine treatment. BChE activity was significantly decreased with 200 μm 24 h corticosterone exposure (p =.0022) (A); Fluoxetine exposure was significantly increased the BChE activity in corticosterone-exposed cells. (p =.004) (B); BChE activity significantly increased with 1mM 24 h norepinephrine exposure (p =.0022) (C); Fluoxetine exposure even significantly increased the BChE activity in norepinephrine-exposed cells. (p =.010) (D).

In our correlation analyses, we found a significant negative correlation between HDRS and BChE activity in the overall sample, and a positive correlation between PSS and BChE activity in the clinical group. Higher HDRS scores linked to reduced BChE luminescence, but PSS correlation was significant only in the clinical group. We interpret this to mean that this may reflect BChE's association with specific symptoms rather than the broad HDRS spectrum. Taken together, this suggests BChE's role in phenotyping depression and its potential elevation in response to increased cholinergic activity linked to stress, regulating acetylcholine levels as a compensatory mechanism as previously reported[10,61].

Specifically within the clinical group, we observed that BChE activity correlated negatively with the HDRS suicide item and positively with the somatic anxiety item. This indicates that patients with higher suicidal ideation had lower BChE activity, suggesting a potential link between reduced BChE and suicide risk, consistent with studies like Zheng et al. on pesticide exposure[24,62–64]. Higher somatic anxiety scores correlated with increased BChE activity, possibly due to elevated cholinergic transmission promoting anxiety-related behaviors[65,66]. It is also important to consider BChE's association with cardiac health and conditions like stroke and myocardial infarction may also play a role[67–70]. We suggest that future studies could benefit from using detailed anxiety scales for further exploration of these relationships.

Interestingly, a study by Chen et al. highlighted BChE's role in attenuating aggression through modulation of the ghrelin axis in mice, where increased BChE expression reduced aggression, while BChE knockout mice exhibited heightened aggression[71]. Although this aggression is not anxiety-driven, further research is needed to clarify BChE's broader role in aggression, especially its differential effects on cholinergic transmission and the ghrelin axis.

Following 8 weeks of antidepressant treatment in our remission analysis, we found that a significant negative correlation between HDRS scores and BChE activity (p =.024), which indicated that reduced depression severity is linked to higher BChE levels. The HDRS suicide item also showed a negative relationship with BChE, suggesting it as a potential biomarker for suicide risk. Notably, the increase in BChE activity during treatment that we observed supports its role in indicating clinical improvement, while the lack of correlation with somatic anxiety suggests the need for more targeted measures of somatic symptoms. Consistent with the human serum findings, in animal models, we also demonstrated that BCC was effective as a probe for BChE activity in rat plasma, showing a 60-fold increase in peak luminescence. Dose-dependent titration confirmed BCC's sensitivity (R^2 = 0.9963), with inhibition experiments verifying that the signal reflected BChE activity, consistent with literature[38,72].

Consistent with findings in stressed rats and human study[73], we found that BChE activity was significantly lower in CUMS + Saline rats. Furthermore, in our study, a positive correlation between BChE activity and sucrose preference highlighted its link to anhedonia, with no significant correlations for other behaviors. Multiple regression analysis revealed that reduced sucrose preference predicted lower BChE activity, while age had no effect. These findings from our rat model support the potential of BChE as a biomarker for depressive phenotypes, particularly anhedonia, though further tests are needed to assess its relation to psychomotor activity.

At the in vitro level, after treating cells with 200 μM corticosterone for 24 h, as hypothesized, BChE activity significantly decreased, mirroring findings in human and rat samples. Fluoxetine treatment (5 μM for 24 h) reversed this effect, increasing BChE activity. This is consistent with the known effects of corticosterone under prolonged stress, potentially causing neurobiological damage linked to neurodegenerative diseases[74–77]. PC-12 Adh cells, with neuronal properties and high glucocorticoid receptor levels, are ideal for studying stress-related neuronal responses[78,79]. Thus, we propose that their response to high corticosterone mimics depressive physiology, making them a suitable model for screening stress and antidepressant effects as shown in metabolomics studies[80,81].

In another cell culture experiment, mimicking the norepinephrine increase seen after UD treatment and acute stress by exposing cells to 1 mM norepinephrine for 24 h, we found that BChE activity significantly increased, aligning with human serum remission and rat antidepressant studies. This is also consistent with findings linking elevated BChE levels to anxiety[82,83]. Fluoxetine treatment further increased BChE activity, consistent with studies showing fluoxetine enhances norepinephrine levels[84]. Given the established role of norepinephrine as a key in stress responses, modulating excitatory and inhibitory signals, especially in the amygdala and prefrontal cortex, affecting anxiety and defensive behaviors[85–87], and the knowledge that chronic stress reduces norepinephrine and locus coeruleus activity, highlighting norepinephrine reuptake inhibitors' role in treatment[88,89], our findings suggest that PC-12 Adh cells treated with norepinephrine could offer insights into cellular stress mechanisms, replicating neurochemical and behavioral responses seen *in vivo*.

Overall our findings, exploring BChE activity's validity as a biomarker of a depressive state using in vitro psychological stress models with corticosterone and norepinephrine exposure in PC-12 Adh cells, indicate that the decrease in BChE activity with corticosterone treatment reflects depressive states, while the reversal by fluoxetine supports its antidepressant effects. The increase in BChE activity with norepinephrine exposure mirrors the increase in antidepressant treatments. Collectively, these results from our translational approach affirm BChE's potential as a depression biomarker and fluoxetine's efficacy in modulating BChE activity under stress.

The development and validation of the highly sensitive and specific BCC probe offers a valuable new tool for investigating BChE activity in psychiatric research. Previous studies relying on less sensitive methods, such as the widely used Ellman's colorimetric assay, may have been limited by sensitivity and specificity issues[29], potentially contributing to some of the inconsistent findings in the literature. For example, while some studies, including Jope et al.[21], have suggested decreased BChE activity in mania, unipolar depression, and schizophrenia, other studies have reported conflicting results, even finding increased BChE activity in some patient subgroups[25,26]. The improved sensitivity of the BCC probe could be instrumental in revisiting these discrepancies. Future research could employ BCC to re-examine BChE activity specifically in patients experiencing mania, comparing

it directly to depressed patients and healthy controls within the same study using this enhanced methodology. If BCC confirms consistently decreased BChE in mania, it would strengthen the argument for BChE as a biomarker relevant across the spectrum of mood disorders. Conversely, if BCC reveals a different pattern, it could refine our understanding of BChE's role, perhaps indicating state-dependent changes. Beyond mania, BCC could also be valuable in reassessing BChE's dynamic changes across different phases of depression (acute, remitted, relapsed) in longitudinal studies, and in more precisely dissecting the influence of confounding factors like medication and lifestyle variables on BChE levels. By providing a more accurate and sensitive measure of BChE activity in serum and plasma, BCC has the potential to significantly advance biomarker research in depression and related psychiatric conditions, helping to clarify BChE's role and enhance its utility in clinical applications.

As a conclusion, this translational study suggests that BChE activity could serve as a biomarker for diagnosing depression and monitoring treatment outcomes. Based on these findings, future research should focus on the determinants of the decrease of BChE in depressive episode and also the neuropathological pathways associated with BChE activity.

Data availability

The data that support the findings of this study are available from the authors upon reasonable request and with the permission of Koç University Institutional Review Board.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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