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Experimental Research Article

The role of Bufexamac in reducing anxiety levels: focus on HPA axis dysfunction and neurotransmitter regulation in a rat model of Alzheimer's disease

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ABSTRACT

Introduction: Alzheimer's disease (AD) is a neurocognitive disorder characterized by neuropsychiatric symptoms (NPS), particularly anxiety. The underlying mechanisms involve disruptions in the hypothalamic-pituitary-adrenal (HPA) axis, and altered serotonergic signaling due to amyloid-beta (Aβ) accumu-lation. This study investigates the effects of Bufexamac, a Cyclooxygenase-2 (COX-2), and HDAC Class IIb inhibitor, on anxiety-like behaviors and neurochemical changes in a rat model of AD induced by Aβ.

Methods: 18 adult Wistar rats were divided into three groups: Saline, Aβ, and Aβ + Bufexamac. Aβ25-35 was administered via intracerebroventricular injection, followed by daily Bufexamac treatment for eight days. Anxiety-like behaviors were assessed using the open-field test, while Western blotting and ELISA measured levels of glucocorticoid receptors (GR), corticotropin-releasing factor (CRF), and serotonin in the amygdala.

Results: Bufexamac significantly mitigated Aβ-induced anxiety-like behaviors, as evidenced by increased line crossings and time spent in the center of the arena ($P<0.05$). Western blot analysis revealed that Bufexamac reduced elevated GR levels in the Aβ group ($P<0.05$). Additionally, Bufexamac treat-ment significantly regulated serotonin ($P<0.01$) and CRF levels ($P<0.05$) in the amygdala compared to the Aβ group.

Conclusion: Bufexamac effectively alleviates anxiety-like behaviors and restores neurochemical alterations in a rat model of AD, suggesting its potential as a possible therapeutic agent targeting neuropsychiatric symptoms associated with AD. Further research is warranted to explore its clinical applicability.

Keywords:
Alzheimer's disease
Bufexamac
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Serotonin
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Introduction

Alzheimer's disease (AD) is an irreversible and progressive neurocognitive disorder (Lucey 2020; Mendez 2021). However, it also manifests a range of psychiatric

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symptoms known as neuropsychiatric symptoms (NPS), which include apathy, depression, anxiety, restlessness, aggression, and psychosis (Lyketsos et al., 2011; Woolley et al., 2011). Among these, anxiety disorders are notably the most common NPS associated with AD (Mendez 2021). Research indicates that anxiety in Alzheimer's is associated with damage in key subcortical brain regions, including the amygdala, locus coeruleus, hypothalamus, and anterior mid-cingulate cortex(Chen et al., 2021).

From a pathological standpoint, the accumulation of amyloid-beta (Aβ) protein deposits disrupts the hypothalamic-pituitary-adrenal (HPA) axis, which plays a crucial role in regulating anxiety (Aminyavari et al., 2019). This disruption links anxiety to memory impairment during the natural aging process, positioning it as a potential risk factor (Gulpers et al., 2019). The HPA axis is essential for the body's response to stress; upon recognizing a stressor, it activates a cascade that leads to the increased release of corticotropin-releasing factor (CRF) and, subsequently, cortisol—a glucocorticoid. This cascade also stimulates the release of adrenocorticotropic hormone, which prompts the secretion of cortisol or corticosterone in humans and rodents (Vale et al., 1981).

Cortisol exerts its effects by attaching to glucocorticoid receptors (GR) (Saklatvala 2002). In response to stress, glucocorticoids, such as cortisol, pass through the cell membrane to bind with GR in the cytoplasm. Once bound, the GR translocates to the nucleus, where it regulates gene expression by attaching to glucocorticoid response elements (GREs) in the regulatory regions of specific target genes. This signaling pathway is vital in determining how individuals physiologically respond to stress (Hinds and Sanchez 2022).

Additionally, CRF and the CRF family of peptides play crucial roles in regulating the stress response by integrating physiological reactions to stressors and functioning as both hormones and neuromodulators.

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According to studies, high levels of glucocorticoids in plasma and cerebrospinal fluid have been recorded in the early stages of AD pathology. (Swaab et al., 1994). A study has shown that central administration of Aβ disrupts the HPA axis and induces anxiety-like behaviors in animal models(Grazia Morgese et al., 2014). In rodent models of AD increased corticosterone levels correlate with heightened expression of GR and CRF in critical areas such as the hippocampus and cerebral cortex (Reyna et al., 2023). Additionally, chronic stress and stress-related conditions are associated with hyperactivation of the HPA axis, directly linking dysfunction of the CRF system to Alzheimer's pathology (Vandael and Gounko 2019).

The monoaminergic system, particularly the serotonergic system, has also been implicated in the activation of the HPA axis, the pathogenesis of AD, and cognitive function. The serotonergic system is crucial for the manifestation of anxiety and depression symptoms in both animal and human models. In rodent models of AD, studies suggest that the raphe nucleus, an area significantly impacted by Aβ and tau pathology, may disrupt serotonin (5-HT) signaling and receptor density. Notably, the density of 5-HT1A receptors is reduced in AD, potentially contributing to the anxiety and depression symptoms observed in affected individuals (Reyna et al., 2023).

Cyclooxygenase-2 (COX-2) is an enzyme essential for the inflammatory response and is associated with neuropsychiatric disorders like depression and anxiety. Elevated levels of pro-inflammatory cytokines, which are often associated with COX-2 activity, can impair serotonin transporter function, leading to decreased serotonin levels and contributing to mood disorders (He et al., 2022). On the other hand, Histone deacetylases, especially HDAC6, are enzymes that remove acetyl groups from histones, leading to chromatin condensation and reduced gene expression. HDAC6 has been implicated in the regulation of stress resilience and emotional responses. Studies show that inhibition of HDAC6 can enhance the acetylation of heat shock protein 90 (Hsp90), which is involved in the chaperoning of GR. This process is critical for modulating the body's response to stress and has been linked to improved resilience against stressors. Furthermore, HDAC6 is enriched in serotonin neurons, and its downregulation following stress exposure correlates with resilience and antidepressant responses. This suggests that targeting HDAC6 may offer therapeutic potential for stress-related disorders by influencing serotonin signaling and

HPA axis dynamics (Jochems et al., 2015; Park et al., 2021). On the other hand, the activation of the HPA axis during severe acute stress is linked to a notable rise in glucocorticoid receptor (GR) levels, The results indicate that HDAC6 plays a vital role in mediating the impact of acute stress on synaptic function, and its inhibition results in a relative decrease in phosphorylated GR levels.(Liu et al., 2023).Also, a study indicates that valproic acid, an HDAC inhibitor, may partly exert its mood-stabilizing effects through modifications in CRF neuronal activity (Stout et al., 2001).

The inhibition of COX2 and HDAC6 appears to have beneficial effects on alleviating anxiety associated with AD. Bufexamac (as a NSAID), is proposed as a COX-2 inhibitor. Bufexamac has also been identified as a specific inhibitor of class IIb histone deacetylases, namely HDAC6 and HDAC10 (Bantscheff et al., 2011). In this context, we investigated the impact of Bufexamac administration on the expression levels of GR, CRF, and serotonin—key regulators of the HPA axis involved in maintaining homeostasis— following intraventricular injection of $A\beta_{25-35}$ in the amygdala of rats.

Materials and Methods

Animals

This study was done with 18 adult male Wistar rats (weighing 250-300 g). The rats were maintained under controlled conditions with a 12h light-dark cycle. All animal experiments in this study were approved by the animal care committee and carried out at Shahid Beheshti University of Medical Sciences. (IR.SBMU. PHNS.REC.1399. 001).

Surgery

Initially, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Their heads were then stabilized using a stereotaxic device, and injection coordinates were set at -0.8 mm posterior to the bregma, ±1.5 mm lateral to the midline, and -4.0 mm deep. A small skull incision was made for the insertion of pre-prepared injection cannulas, which were secured with dental cement.

The rats were randomly divided into three groups: Saline, Aβ, and Aβ + Bufexamac. The Aβ and Aβ + Bufexamac groups received a bilateral intracerebroventricular (ICV) injection of $A\beta_{25-35}$ (15 nmol/rat) on the day of surgery. One week later, either Saline or Bufexamac (20 µg/rat) (Jia et al., 2015; Mansouri et al., 2025) was administered ICV daily for eight days (days 7 to 14).

Open field test

The anxiety-like behaviors of rats were assessed using the open-field test. The rats were placed individually in a black wooden arena (50 × 50 × 40 cm). Total distance moved and behaviors such as grooming, rearing, crossing, and time spent in the center were recorded using a camera for a 5-minute duration. Total distance and time spent in the center of the box were quantified using EthoVision software (Zhang et al., 2023). Additionally, grooming, crossing, and rearing behaviors were assessed by an observer who was unaware of the groups (Seemiller et al., 2021).

Western blot

The amygdala tissues were homogenized and centrifuged in a lysis buffer. Then, the supernatants were assayed for total protein concentration using the Bradford protein assay (Kruger 2009). The proteins were separated by 12.5% SDS- polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to a PVDF membrane. To block non-specific binding, the membranes were treated with 2% non-fat dried milk for 2 hours. To continue they incubated with primary antibodies anti-GR (1/1000) and anti-β-actin (1/2500) and secondary antibody (1:5000), respectively. The protein bands were detected using a chemiluminescence kit and recorded on X-ray films. Finally, the band densities were analyzed and quantified with ImageJ software.

Neurochemical biomarkers assay

The animal's amygdala area was isolated to measure serotonin and CRF levels. Amygdala samples were homogenized in phosphate-buffered saline (PBS, pH 7.4; 100 mg/ml) and then centrifuged at 300 g and 4°C for 5 minutes. The resulting supernatants were collected and quickly used for analysis. Levels of CRF (CSB-E08038r) and serotonin (CSB-E08364r) in the amygdala were determined using the protocol of commercial rat ELISA kits from Cusabio (USA). The optical density was read at 450 nm with a microplate photometer (Abd Elkader et al., 2024).

Statistical analysis

Statistical analyses were conducted using GraphPad

Prism software version 8.0. The Shapiro-Wilk test was used to evaluate data normality. One-way ANOVA, accompanied by Tukey's post-hoc test, was applied for comparisons. Results are presented as mean ± SEM, with significance set at $p<0.05$.

Results

Bufexamac mitigated anxiety-like behaviors induced by Aβ

The analysis showed no significant differences in the total distance traveled among the different groups, and they had normal functions. Consequently, we can conclude that the surgical procedure and the administered treatments did not have a meaningful impact on the animals' movement (Figure 1a).

There were significant differences in the number of line crossings and time spent in the center among the groups (Figure 1b, c). The Aβ group exhibited fewer line crossings ($P<0.05$) and spent less time in the center ($P<0.001$) compared to the Saline group; however, Bufexamac treatment enhanced this behavioral parameter ($P<0.05$). Similarly, notable disparities were observed in grooming and rearing behaviors (Figure 1d, e), with the Aβ group exhibiting higher frequencies of both compared to the Saline group ($P<0.05$). Bufexamac treatment also reduced these behavioral parameters ($P<0.05$).

Bufexamac reversed Aβ-induced amygdala GR impairment

To investigate Bufexamac's potential to modulate GR impairment, we performed a Western blot analysis. As shown in Figure 2, GR expression levels were higher in the Aβ group compared to the Saline group ($P<0.01$). However, treatment with Bufexamac for 8 days significantly decreased this elevated expression ($P<0.05$).

Bufexamac changed CRF and serotonin levels affected by Aβ

To examine the effects of Bufexamac on the activity of the HPA axis in an AD rat model, we measured the levels of serotonin and CRF in the amygdala using ELISA (Figure 3 a, b). Our results indicated that the expression levels of serotonin ($P<0.001$) and CRF ($P<0.01$) in the Aβ group were significantly lower compared to the control group. Conversely, administration of Bufexamac resulted in a marked increase in the levels of both CRF ($P<0.05$) and serotonin ($P<0.01$).

Discussion

Acknowledging the critical role of anxiety as a common psychological disorder in AD and the strong link between stress systems and AD progression, we induced cognitive impairment through bilateral intraventricular injection of $A\beta_{25-35}$ and evaluated the possible anti-anxiety effect of Bufexamac in neurotoxicity caused. Our findings show that Bufexamac significantly improves the levels of key factors involved in stress regulation associated with Alzheimer's pathogenesis in the $A\beta_{25-35}$ rat model, while also reducing anxiety-related behaviors. AD is the most common type of dementia in the elderly, characterized by two main histopathological features: senile plaques made up of aggregated Aβ peptides and neurofibrillary tangles formed by hyperphosphorylated tau protein. In the human brain, the major soluble Aβ oligomers include $A\beta_{1-40}$ and $A\beta_{1-42}$, along with shorter peptides like $A\beta_{25-35}$. $A\beta_{25-35}$ is linked to key changes in AD, including cognitive deficits, abnormal processing of APP, tau dysfunction, neuroinflammation, oxidative stress, and dysregulation of the HPA axis. Additionally, anxiety is a common symptom among AD patients, especially in the early stages of the disease. AD is the most common form of dementia among the elderly. AD is the most common type of dementia in the elderly y. Its two principal histopathological features are senile plaques, which consist of aggregated Aβ peptides, and neurofibrillary tangles formed by hyperphosphorylated tau protein. In the human brain, the major soluble Aβ oligomers include $A\beta_{1-40}$ and $A\beta_{1-42}$, along with shorter peptides like $A\beta_{25-35}$. The biological activity of $A\beta_{25-35}$ is associated with several critical structural and functional changes observed in AD, such as tau dysfunction, APP misprocessing, cognitive deficits, neuroinflammation, oxidative stress, and dysregulation of the HPA axis (Canet et al., 2023). Furthermore, anxiety is a prevalent symptom in AD patients, especially in the early stages of the disease (Ferretti et al., 2001). Previous studies have demonstrated that animals receiving $A\beta_{25-35}$ oligomer injections, either via ICV or intra-hippocampal methods, display anxious behaviors (Olariu et al., 2001). Also, the effects of intracerebroventricular injection of $A\beta_{25-35}$ in different brain regions, including the hippocampus, amygdala, and hypothalamus, have been examined (Zussy et al., 2011). Consequently, we employed bilat-

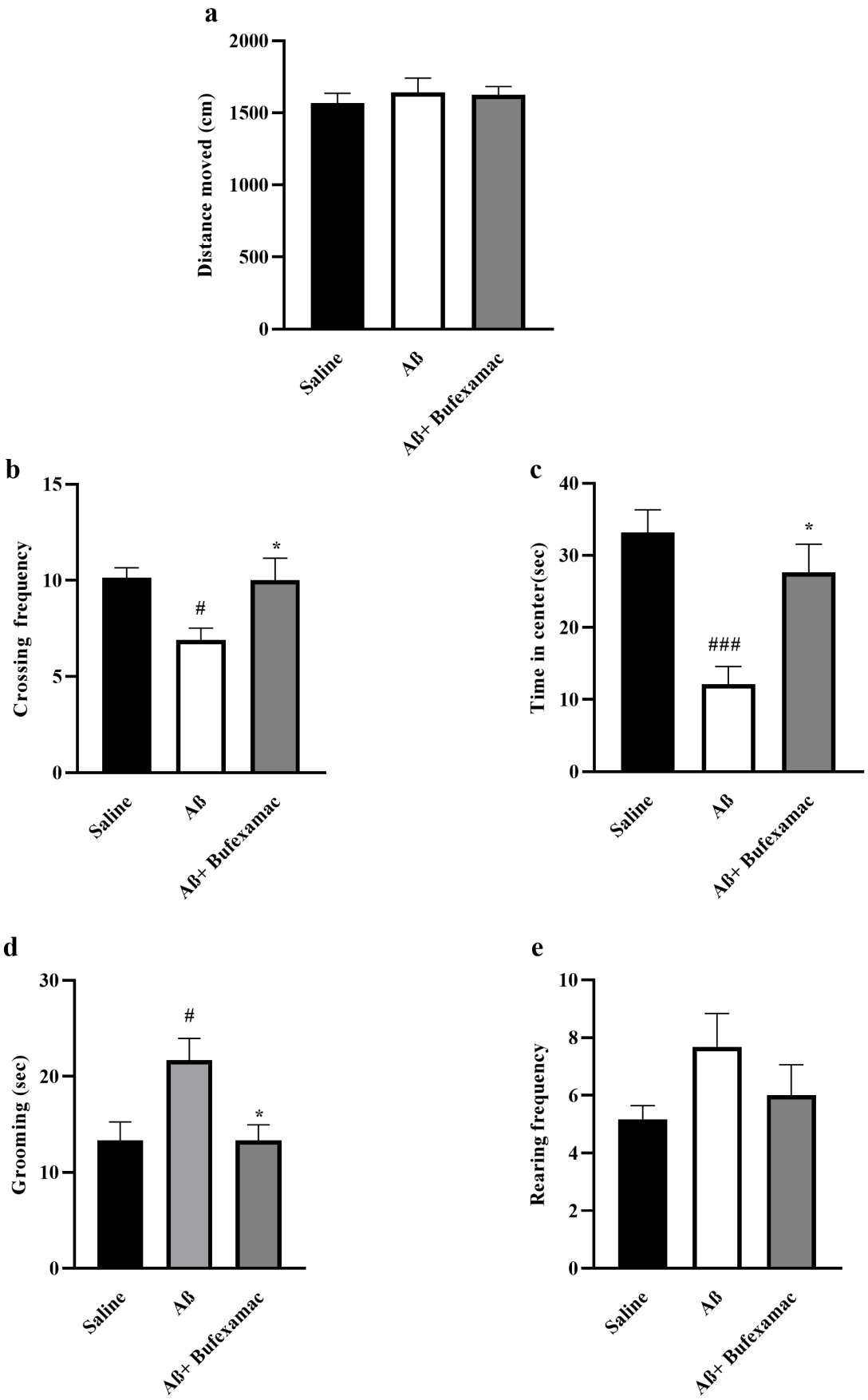

FIGURE 1. Effect of Bufexamac on some rat behavior parameters in the open field test. Total distance moved (a) crossing (b), time spent in the center (c), grooming (d), and rearing (e). Data are ex-pressed as means ± SEM (n = 6). Statistical comparisons were made using one-way analysis of variance with the Tukey post-hoc test. #P<0.05, ###P<0.001 vs Saline group, *P<0.05 vs Aβ group.

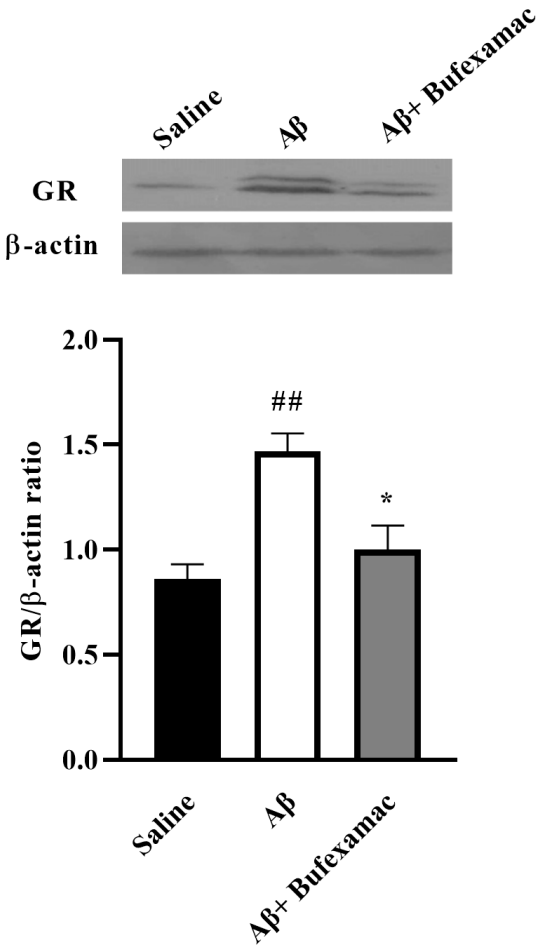

FIGURE 2. Effect of Bufexamac on GR in the amygdala of the Aβ-treated rats. Data are expressed as means ± SEM (n = 3). Statistical comparisons were made using one-way analysis of variance with the Tukey post-hoc test. ##P<0.01 vs Saline group, *P<0.05 vs Aβ group.

eral intracerebroventricular administration of $A\beta_{25-35}$ to establish a model of AD.

The HPA axis plays a crucial role in how the body reacts to stress by promoting the release of glucocorticoids in both humans and animals. These steroid hormones can cross the blood-brain barrier and bind primarily to mineralocorticoid receptors (MR) and, to a lesser extent, to GRs (Reul and Kloet 1985). When a stressful stimulus is detected, it is processed through sensory neural circuits and relayed to the hypothalamus for further response (Shi and Davis 2001). Upon activation, the hypothalamus engages with the HPA axis, which functions as a hormonal feedback system connecting the hypothalamus, the pituitary gland, and the adrenal gland. This axis not only regulates the body's stress response but also plays a pivotal role in anxiety-related behaviors. The neurochemical dynamics of HPA and anxiety signaling commence when stressors prompt the hypothalamus to release CRF. This release stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) into the bloodstream. In turn, the adrenal cortex detects the elevated levels of ACTH and responds by releasing glucocorticoids, including cortisol. This cascade creates a negative feedback loop, where glucocorticoids attach to receptors in the hypothalamus and pituitary gland, thereby reducing the secretion of CRF and ACTH. Dysregulations in these neurochemical interactions within the HPA axis are associated with a range of stress-related disorders (Curran and Chalasani 2012). AD is marked by dysregulation of the HPA axis, which can contribute to the acceleration of disease progression and cognitive decline. In the early stages of AD pathology, the central HPA axis is activated prior to the onset of cognitive impairments and behavioral symptoms. This dysregulation is thought to be significantly influenced by the early accumulation of pathological forms of Aβ in humans(Ah-

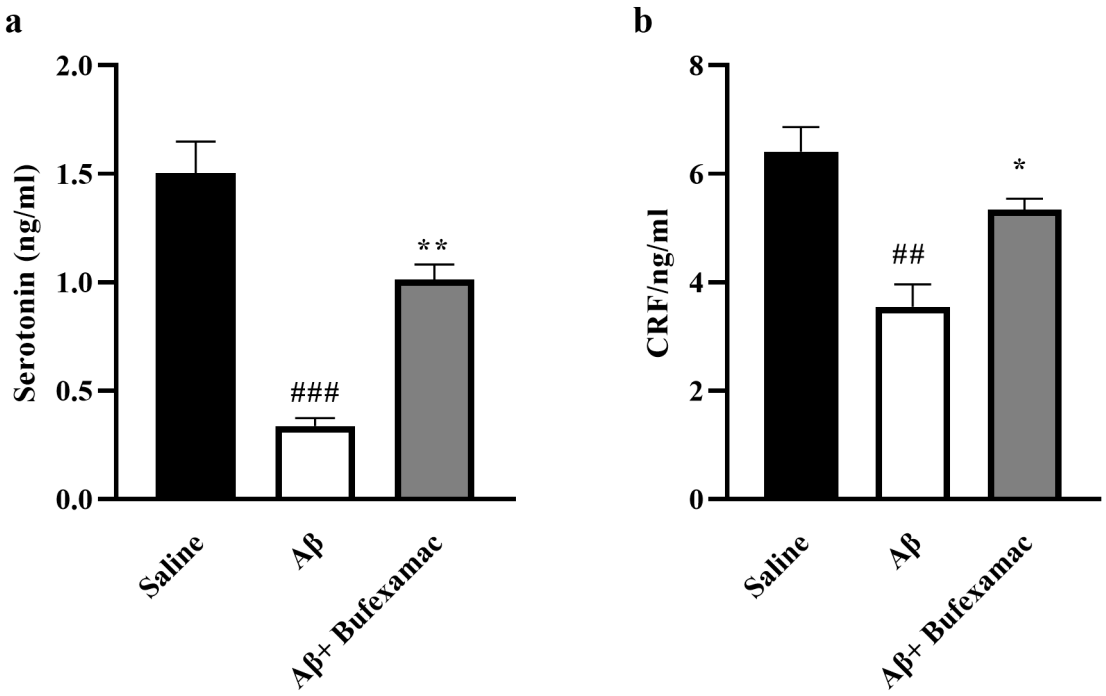

FIGURE 3. Ef Effect of Bufexamac on neurochemical biomarkers in the amygdala of the Aβ-treated rats. serotonin (a) CRF (b). Data are expressed as means ± SEM (n = 3). Statistical comparisons were made using one-way analysis of variance with the Tukey post-hoc test. ##P<0.01, ###P<0.001 vs Saline group, *P<0.05, **P<0.01 vs Aβ group.

mad et al., 2019).

Pentkowski et al. reported that TgF344-AD mice display early signs of heightened anxiety-like behavior even when AD-related neuropathology is minimal (Pentkowski et al., 2018). In another study, TgF344-AD rats spent less time in the center of the OFT than WT rats (Reyna et al., 2023). Grooming is often seen in animal models of stress and anxiety and is considered an anxiogenic response. In one study, anti-anxiety medication normalized the grooming pattern by reducing grooming-related indicators (Smolinsky et al., 2009). In the present study, Bufexamac demonstrated a significant reduction in anxiety-like behavior in Alzheimer's rats compared to the $A\beta_{25-35}$ group. This finding is consistent with earlier research indicating that MS-275, an inhibitor of class I and III histone deacetylases, effectively alleviates anxiety in S1 mice. Furthermore, other histone deacetylase inhibitors such as TSA, which targets class I and II, and SAHA, which inhibits class I, II, and IV, have also been shown to mitigate anxiety behaviors in rats (Peedicayil 2020).

CRF is the key hormone in the HPA axis, initiating a series of stress responses that include the release of corticosteroids from the adrenal cortex. In addition to its regulatory role at the pituitary gland, CRF is found in various brain regions, acting as a neuromodulator or neurotransmitter that influences autonomic and behavioral responses (Vandael and Gounko 2019). It works alongside corticosteroids to adjust stress responses in the short term. However, chronic or elevated stress levels can lead to hyperactivation of the HPA axis and disrupt CRF regulation in AD patients. Postmortem studies have shown a significant decrease in CRF immunoreactivity (CRF-IR) in the cerebral cortex and cerebrospinal fluid of AD patients compared to controls (May et al., 1987; Vandael and Gounko 2019). In the present study, we observed a notable decrease in CRF expression in the amygdala of Alzheimer's rats in the $A\beta_{25-35}$ group. This finding aligns with prior research indicating that low levels of CRF are prevalent in neurodegenerative diseases (Mouradian et al., 1986). Chronic stress results in overstimulation of the HPA axis, leading to elevated levels of CRF and GRs in the bloodstream, which can have detrimental effects on neuronal health and synaptic connectivity. Consequently, these adverse effects may further reduce CRF levels in the brain, impairing its neu-

romodulatory functions that are crucial for memory and cognitive processes (Bisht et al., 2018). Numerous studies have revealed a significant reduction in CRF concentrations in the frontal and temporal cortex, as well as the caudate nucleus, accompanied by an increase in CRF receptor density (Nemeroff et al., 1989). In contrast, dysfunction of the HPA axis is evidenced by increased levels of cortisol in humans and corticosterone in animal models. In AD, glucocorticoids are unable to regulate the HPA axis through the negative feedback mechanisms involving GRs in the hypothalamus, hippocampus, and anterior pituitary. Both impaired corticosteroid negative feedback and elevated corticosteroid levels have been documented in AD patients and preclinical models (Ahmad et al., 2019).

GR is a critical factor in stress-related memory deficits that arises under conditions of high cortisol (Lanté et al., 2015). Elevated cortisol levels have been shown to cause Aβ deposition, with recent studies confirming that GR antagonists can effectively prevent this GR-mediated Aβ accumulation (Baglietto-Vargas et al., 2013). In this line, Lanté et al. demonstrated that chronic treatment with RU486, a GR antagonist, can significantly alleviate cognitive decline in the Tg2576 AD mouse model (Lanté et al., 2015). In the current study, Bufexamac significantly decreased the expression of GR in the amygdala of rats with AD, compared to the $A\beta_{25-35}$ group. This highlights the importance of GR in the disease. Additionally, GR is known to interact with histone deacetylase HDAC6 in the brain. Previous findings conducted by Lee et al. demonstrated that acute stress, through GR activation, enhances glutamatergic signaling in the prefrontal cortex (PFC) of rats. Their findings indicated that inhibiting or knocking out HDAC6 effectively prevented the stress-induced increase in glutamatergic signaling (Lee et al., 2012). Moreover, another study found that bilateral injection of a histone deacetylase inhibitor into the central amygdala (CeA) reduced anxiety-like behaviors and alleviated somatic and visceral hypersensitivity associated with elevated corticosterone (CORT) levels (Tran et al., 2015). Studies have shown that injecting sodium butyrate into the medial prefrontal cortex (mPFC) to inhibit HDAC4 could disrupt the GR signaling pathway. This disruption resulted in a decrease in mechanical allodynia and a reduction in anxiety-like behaviors (Zhang et al., 2019). In a study by Athira et al., Vorinostat, an HDAC inhibitor, was shown to improve corticosterone-induced depressive and anxiety-like behaviors. The treatment not only alleviated these behaviors but also reduced dysregulation of the HPA axis, as well as oxidative stress and inflammation (Athira et al., 2018). Administering a selective COX-2 inhibitor decreases anxiety-related behaviors in mice following chronic oral corticosterone treatment. These findings underscore the preclinical effectiveness of COX-2 inhibitors in established animal models (Morgan et al., 2019).

Serotonin, a monoamine neurotransmitter, plays a crucial role in modulating neural circuits, particularly in fear and anxiety. The serotonergic system primarily originates from the brainstem's dorsal and median raphe nuclei, projecting to various forebrain and limbic structures including the hypothalamus, amygdala, hippocampus, thalamus, and frontal cortex. This extensive network forms what is often referred to as the serotonin circuit (Charnay and Léger 2010; Curran and Chalasani 2012). When exposed to fear and anxiety stimuli, serotonergic neurons in the dorsal raphe nucleus become selectively activated. These neurons then project to the amygdala and the hypothalamic region of the HPA axis. Dysregulation within this axis, along with abnormalities in the amygdala, can lead to inappropriate fear responses and anxiety disorders, such as panic attacks and post-traumatic stress disorder (PTSD) (Andrews et al., 2022; Charnay and Léger 2010). Recent discussions have also highlighted the potential of serotonin signaling in the context of neurodegenerative diseases, such as AD. Enhancing serotonin signaling and developing molecules that elevate serotonin concentrations in the synaptic cleft are being explored as therapeutic strategies to slow AD progression (Charnay and Léger 2010; Curran and Chalasani 2012). The administration of SSRIs and serotonin receptor agonists in AD by affecting key pathological features such as Aβ accumulation and neuroinflammation, as well as improving mood and anxiety presents a multifaceted approach to treatment (Cassano et al., 2002; Claeysen et al., 2015). In this study, Bufexamac notably enhanced serotonin expression in the amygdala of Alzheimer's rats when compared to the $A\beta_{25-35}$ group. This finding aligns with previous research indicating that treatment with the TSA (HDAC inhibitor) elevates 5-HT synthesis. These results imply that HDAC inhibitors could improve the functionality of 5-HT neurons through an epigenetic mechanism (Asaoka et al., 2015). Espallergues et al. revealed that selective deletion of

HDAC6 in serotonergic neurons significantly reduced the anxiety-inducing effects of glucocorticoids. Specifically, in mice subjected to chronic social defeat, the deletion of HDAC6 inhibited the emergence of social avoidance behaviors typically observed in these animals (Espallergues et al., 2012). MGCD0103 mitigated the loss of serotonergic neurons in mice treated with oligomeric $A\beta_{25-35}$ and alleviated anxiety symptoms in the AD model mice (Huang et al., 2019).

Conclusion

In summary, Bufexamac significantly reduced neuropsychiatric symptoms, including anxiety induced by bilateral ICV injections of $A\beta_{25-35}$. The positive effects are attributed to several mechanisms, including the modulation of CRF, a decrease in GR expression, and an increase in 5-HT expression in the amygdala of Alzheimer's rats.

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Conflict of interest

The authors declare that they have no competing interests.

Ethics approval

All animal experiments in this study were approved by the animal care committee and carried out at Shahid Beheshti University of Medical Sciences. (IR.SBMU. PHNS.REC.1399. 001).

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