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Chronic Nasal Administration of Kisspeptin-54 Regulates Mood-Related Disorders Via Amygdaloid GABA in Hemi-Parkinsonian Rats

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Background: Depression and anxiety, the most prevalent neuropsychiatric manifestations in Parkinson's disease (PD), negatively impact their quality of life.

Aims: To determine whether the chronic nasal administration of kisspeptin-54 (KP-54) could. Alleviate symptoms of anxiety and depression in hemi-Parkinsonian rats.

Study Design: Experimental study.

Methods: This study included adult Sprague Dawley male rats who were administered either a vehicle (artificial cerebrospinal fluid) or 6-hydroxydopamine (6-OHDA) unilaterally into the medial forebrain bundle. The vehicle, or KP-54 (3 nmol/kg, applied topically to the rhinarium), was administered daily for a seven-day period. The sucrose preference test (SPT), elevated plus maze test (EPMT), and open field test (OFT) were implemented to evaluate depression- and anxiety-like behaviors, respectively, seven days following the lesion surgery. Gamma-aminobutyric acid (GABA) concentrations in the amygdala were quantified using mass spectrometry. Tyrosine hydroxylase in substantia nigra was analyzed using immunohistochemistry.

Results: The nasal delivery of KP-54 significantly reduced depression- and anxiety-like behaviors that were induced by 6-OHDA, as indicated by the results of the SPT, OFT, and EPMT. Moreover, it was observed that nasal KP-54 effectively mitigated 6-OHDA-induced motor deficits and the loss of nigral dopaminergic neurons. The nasal administration of KP-54 augmented the decline in GABA levels in the amygdala induced by 6-OHDA. Furthermore, effective correlations were established between GABA concentrations and behavioral parameters.

Conclusion: The nasal delivery of KP-54 could function as a viable therapeutic alternative for treating mood-related disorders in PD.

INTRODUCTION

Parkinson's disease (PD) is characterized by the progressive degeneration of dopamine-secreting neurons within the substantia nigra (SN), which leads to primary motor symptoms like akinesia, tremors, postural instability, and rigidity.[1,2] Depression and anxiety, which are non-motor symptoms observed in approximately 35% of patients with PD, represent some of the most debilitating complications associated with the progression of these symptoms.[3,4] Although a diverse array of treatment modalities is available for alleviating PD-related motor symptoms, evidence regarding effective management of depression and anxiety in PD patients remains limited. Therefore, it is essential to develop alternative protective strategies for treating mood-related disorders in PD patients.

Kisspeptins (KPs) are amidated neuropeptides that play a pivotal role in the central mediation of the hypothalamic-pituitary-gonadal axis.[5,6] The *KiSS1* gene encodes a propeptide consisting of 145 amino acids, from which the peptide known as kisspeptin-54 (KP-54) is derived.[7,8] Numerous studies highlighted the role of KPs in behavioral and mood changes.[9-13] Research reveals that intracranial administration of KP to zebrafish results in enhanced exploratory behavior and reduced anxiety.[9] In rodent studies, researchers have observed increased social interaction among the juvenile conspecific male mice when the posterodorsal medial amygdala (MePD) KP neurons are selectively stimulated using a chemogenetic approach.[10] Selective activation of KPergic neurons in the MePD significantly increases the exploratory duration in the open arms of an elevated plus maze (EPM) test when this experimental methodology is

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A portion of this study was presented as a poster at the American Physiology Summit 2023 in Los Angeles. The preliminary findings of the mentioned portion of the study were conducted with the approval of the Institutional Animal Care and Use Committee of the Faculty of Medicine at Akdeniz University (approval number: 125, date: 18.10.2021).

employed, indicating a potential anxiolytic effect.[10] Conversely, there are reports that suggest that the forced swim test demonstrates antidepressant effects in mice following central administration of KP-13.[11] This is corroborated by another study that discovered that intravenous administration of KP-54 in humans led to a decrease in negative mood states.[12,13] Moreover, a recent publication has demonstrated that the nasal delivery of KP-54 effectively alleviates motor impairments in 6-hydroxydopamine (6-OHDA)-injected rats.[14] Considering the effects of KPs, it is hypothesized that they may be effective in treating mood-related disorders that develop in experimental PD.

A potential target for modulating mood-related responses is the amygdala's gamma-aminobutyric acid (GABA)-mediated neurotransmission.[15] The administration of GABA or GABA receptor agonists into the amygdala has been demonstrated to reduce indicators of fear and anxiety in various animal species, whereas infusions of GABA antagonists typically exhibit anxiogenic effects.[16,17] Similarly, the anxiolytic effects that are typically induced by benzodiazepines are eliminated by selectively inhibiting the production of the GABA synthetic enzyme in the amygdala.[18] In a separate study, KP-8 was shown to significantly enhance GABA release from nucleus accumbens slices, indicating that it has a modulator effect on GABAergic neurons.[7] These findings suggest that exogenous KP may enhance GABA release in the amygdala, potentially alleviating mood disorders associated with PD. Therefore, this study investigated whether long-term nasal delivery of KP-54 regulates anxiety- and depression-related behaviors in rats with unilateral 6-OHDA lesions.

MATERIALS AND METHODS

Experimental protocol

The male Sprague Dawley rats (aged 10-12 weeks) were confined in groups and were allowed unrestricted access to food and water. They were maintained on a 12-hour light/dark cycle. For the experimental hemi-Parkinsonian rat model, the catecholaminergic neurotoxin 6-OHDA was stereotaxically administered into the right medial forebrain bundle of anesthetized animals. The rats received either a nasal administration of KP-54 (3 nmol/kg) or a vehicle (artificial cerebrospinal fluid) daily for seven days. On the 7th day, the rats were administered their final dose of KP-54 nasally, 30 minutes prior to subjecting them to behavioral assessments. During the nasal treatment, conscious animals were securely restrained, and 20 µl of the drug was evenly distributed over the rhinarium (which is densely innervated by free nerve endings), with 10 µl applied to each side.[14,19] The open field test (OFT) was employed to evaluate motor performance, and the extent of the lesion induced by 6-OHDA was evaluated through immunohistochemistry for tyrosine hydroxylase (TH) in sections of the SN. Seven days following surgery for the lesion, depression- and anxiety-like behaviors were assessed using the sucrose preference test (SPT), OFT, and the EPM test. Mass spectrometry analysis was employed to quantify GABA levels in the amygdala tissues. All experimental procedures related to animal care and treatment were conducted in accordance with the guidelines and ethical standards established by the Institutional Animal Care and Use Committee of the Faculty of Medicine at Akdeniz University (approval number: 17, date: 15.04.2024). Figure 1 illustrates the experimental procedures.

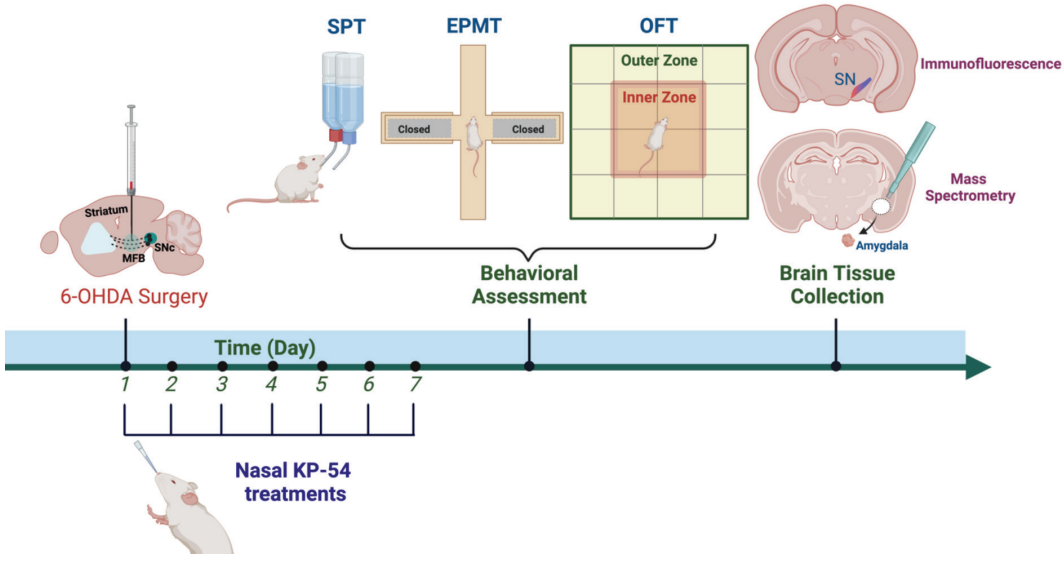

FIG. 1. An overview of the study timeline. This figure was created using the BioRender software (BioRender.com, Toronto, Canada).
SPT, sucrose preference test; EPMT, elevated plus maze test; OFT, open field test; 6-OHDA, 6-hydroxydopamine; KP-54, kisspeptin-54.

As depicted in the Supplementary Figure 1, dose-response curves for nasal KP-54 (logarithmically increasing doses; 0.1, 0.3, 1, 3, 10 nmol/kg) were constructed using the OFT, measured by the time spent in the center, and the EPM test, assessed by the time spent in the open arms. Based on these evaluations, the effective dose at which 50% of the maximal effect is observed (ED50) for nasal KP-54 treatment was determined to be 3 nmol/kg.

6-OHDA surgery

In accordance with the technique outlined in a previous study,[14] anesthesia was achieved via an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (12 mg/kg). The animals were then placed in a stereotaxic apparatus (Kopf Instruments, Tjunga, CA, USA), and 6-OHDA (3 x 4 µg/µl) was injected at a rate of 1 µl/min using coordinates AP + 2.2, ML + 1.5, and DV -8.0 mm relative to bregma, as described in previous studies. Each day the neurotoxin was freshly prepared in a sterile saline solution containing 0.1% ascorbic acid and administered using a 30 G Hamilton syringe. The control rats were administered a vehicle injection containing 0.1% ascorbic acid solution. To ensure the neurotoxin diffused, the cannula was left in the injection site for 1-3 minutes following administration.

Behavioral assessment

An OFT was administered in a square arena made of black plexiglass, which was divided into 16 equal sections, to assess anxiety-related behaviors and motor performance (Figure 1). The animals were placed at the center of the arena, and their movements were recorded for five minutes using the Noldus Ethovision XT System. The total distance traveled, average velocity, the frequency of entries into the inner zone, and the duration of time spent within the inner zone were recorded for each rat to evaluate the motor performance and anxiety-related behaviors.[20,21]

The EPM test was implemented to evaluate anxiety-related behaviors in animals subsequent to the administration of the final dose of KP-54. The EPM test apparatus comprised two open arms and two enclosed arms, arranged perpendicularly, along with a central square, and was elevated 40 cm above the ground (Figure 1). The animals were initially positioned in the center, facing an open arm, and their behavior was observed over a 5-minute period using the Noldus Ethovision XT System. The frequency of entries into the open arms and the duration of time spent within them were used to quantitatively evaluate anxiety-like behavior.[22,23]

The SPT was conducted to assess anhedonic behavior in rats (considered an index of depression). The rats were initially acclimatized to the sweet taste of sucrose by placing a 200 ml bottle containing a 1% sucrose solution in their cages for 24 hours. Subsequently, each rat was granted access to two bottles: one containing 1% sucrose solution, while the other containing tap water. Sucrose and water consumption was quantified by determining the difference in the initial and final weights of each bottle at 24 hours.[22,23] Sucrose preference was determined using the formula SPT = [sucrose intake/(water intake + sucrose intake)] × 100.

Quantitative assessment of GABA using mass spectrometry

After the behavioral assessments, the brain tissues from five rats from each group were meticulously obtained. After obtaining 200 µm-thick coronal sections that included the amygdala region using a cryostat, the micro-punch technique was employed to extract amygdala tissue. The amygdala tissues were subsequently homogenized in 0.1 M formic acid, centrifuged, and the supernatant was collected and stored at -80 °C. Stock GABA standard solutions were prepared, and GABA quantification was conducted using ultra-fast liquid chromatography coupled with tandem mass spectrometry. The precursor and product ions were targeted at m/z 104 and 87, 68.8, respectively, and detected through multiple reaction monitoring in positive electrospray ionization mode, with a retention time of 1.26 minutes. The calibration curve was linear from 50 to 800 ng/ml, enabling a rapid analysis of each sample within five minutes, as previously described.[24]

Immunofluorescence

For immunofluorescent staining, brain tissues (n = 4) from each experimental group were harvested post-mortem and fixed in 4% PFA with 20% sucrose for two days. A cryostat (Leica, Germany) was employed to produce consecutive coronal sections, each of which was 40-50 µm thick. The nigral sections were identified using landmarks from a rat brain stereotaxic atlas. Following this, the sections were then stained with sheep anti-TH (Abcam, UK, 1:1000) and donkey anti-sheep Alexafluor 568 (ThermoScientific, USA; 1:1000), respectively. Fluorescent imaging was performed using an Olympus BX43 microscope (Olympus, Japan). At least five stained sections per brain, encompassing the entire SN, were roughly counted. Detailed methodologies have been delineated in previous publications.[14,22]

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software. Data normality was evaluated using the Shapiro-Wilk test. The behavioral differences across the groups were analyzed employing One-Way ANOVA followed by Tukey's post-hoc test. GABA concentrations and TH-positive cell counts were assessed using the Kruskal-Wallis test with Dunn's post-hoc analysis. Spearman's correlation coefficient was employed to investigate the correlations between GABA levels and behavioral outcomes. The results are presented as the mean ± SEM, and a p-value of less than 0.05 was considered statistically significant. The detailed outcomes of all statistical analyses are reported in the "results" section.

RESULTS

Nasal application of KP-54 ameliorated the motor deficits induced by 6-OHDA and mitigated the loss of nigral dopaminergic neurons

An analysis of TH-immunoreactivity was conducted on the coronal brain sections at the SN level to evaluate the extent of degeneration in dopaminergic neurons within the SN as a consequence of 6-OHDA injection. Compared to the vehicle-treated control group

(88.7 ± 2.6, n = 4), there was a significant ($p < 0.01$) reduction in TH-positive neurons in the 6-OHDA group (20.7 ± 3.1, n = 4), indicating substantial neuronal loss. Conversely, KP-54 treatment (3 nmol/kg, nasally for 7 days) preserved 6-OHDA-induced loss of TH-positive neurons (79 ± 3.9, $p < 0.05$, Kruskal-Wallis statistic = 8.7, n = 4) as reported previously,[14] (Figure 2a-d).

In the OFT, compared to control groups (distance: 1872 ± 117.4 cm; velocity: 6.5 ± 0.5 cm/s, n = 9), 6-OHDA injection resulted in a significant decrease in the distance traveled by the rats (1082 ± 122.7 cm, $p < 0.0001$, n = 9) and their velocity (3.8 ± 0.5 cm/s, n = 9), reflecting motor deficits typical of Parkinsonian symptoms. These impairments were effectively ($p < 0.05$) alleviated by KP-54 treatment [distance: 1455 ± 56 cm, F (2, 24) = 14.6; velocity: 5.6 ± 0.3 cm/s, F (2, 24) = 8.2, n = 9] as reported recently,[14] (Figure 2e, f).

Nasal application of KP-54 improved mood-related disorders in hemi-Parkinsonian rats

The OFT demonstrated that rats who were administered 6-OHDA exhibited significantly reduced time spent in the center (16.2 ± 1.2 s, $p < 0.001$, n=9) and entry rate into the center zone (9.3 ± 1.1 count, n = 9) compared to the control groups (time: 29.9 ± 2.4 count, $p < 0.01$, n=9); entry: 15.7 ± 1.5, n = 9), suggesting increased anxiety. The reduction in time spent in the center zone induced by 6-OHDA was mitigated by the nasal administration of KP-54 [24.3 ± 1.8 s, $p < 0.05$, F (2, 24) = 12.8, n = 9]. However, although not statistically significant, the nasal KP-54 treatment exhibited a propensity to increase the entry of the rats with 6-OHDA-induced lesions into the central area (13.6 ± 1 count, n = 9) (Figure 3a, b).

In the EPM test, 6-OHDA injection was found to effectively attenuate the time spent in open arms (27.8 ± 2.3 s, $p < 0.0001$, n = 9) and decreased the number of open-arm entries (5 ± 0.7 count, $p < 0.001$, n = 9), reflecting heightened anxiety levels when compared to the control group (time: 61.1 ± 5.8 s; entry: 12.8 ± 1.3 count, n = 9). Nasal KP-54 treatment significantly ($p < 0.01$) mitigated the reduction in time spent in open arms [49.6 ± 2.7 s, F (2, 24) = 17.9, n = 9] and the number of open-arm entries [11.3 ± 1.2 count, F (2, 24) = 13.3, n = 9] in 6-OHDA-injected groups (Figure 3c, d).

Together, these results verify that KP-54 administration mitigates motor impairments and mood-related behavioral deficits in hemi-Parkinsonian rats.

Nasal KP-54 treatment decreased depression-like behavior in hemi-Parkinsonian rats

The control group rats demonstrated a strong preference for sucrose, with an average preference of approximately 79.9 ± 1.3%. The treatment with 6-OHDA significantly ($p < 0.0001$) diminished sucrose preference in the rats to around 57 ± 3.1%, indicating a possible induction of anhedonic behavior (considered an index of depression). The administration of KP-54 mitigated this effect, partially restoring sucrose preference to approximately 68.2 ± 2.4% [F (2,24) = 22.3, p < 0.01, n=9], (Figure 4).

The findings indicate that the anhedonic effects of 6-OHDA are reduced by the nasal administration of KP-54.

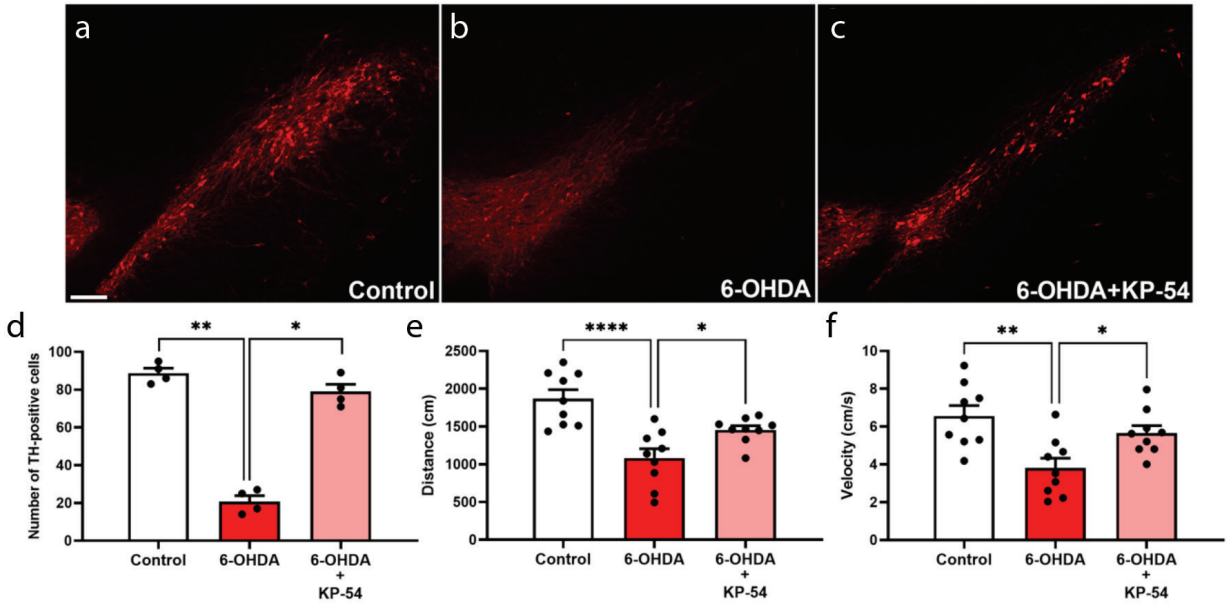

FIG. 2. Effect of nasal KP-54 administration on TH-immunoreactivity in the SN (a-d) and motor performance (e, f) in 6-OHDA-injected rats. (d) Quantitative assessment of TH-positive neurons in the SN. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. The scale bar indicates a measurement of 200 micrometers.

KP-54, kisspeptin-54; TH, tyrosine hydroxylase; SN, substantia nigra; 6-OHDA, 6-hydroxydopamine.

Nasal KP-54 treatment effectively counteracts the reduction of GABA levels in the amygdala induced by 6-OHDA

The 6-OHDA group exhibited significantly lower GABA levels (1388 ± 89.9 ng/mg protein, $p < 0.05$, n = 5) compared to the control group (2138 ± 67 ng/mg, n = 5). Compared to the 6-OHDA-injected rats, nasal administration of KP-54 showed a partial increment in GABA levels in rats with 6-OHDA-induced lesions (2068 ± 117 ng/mg, $p < 0.05$, Kruskal-Wallis statistic = 9.6, n = 5) (Figure 5a). Moreover, a strong positive correlation was observed between GABA levels and the time spent in the open arms of an EPM (r = 0.97, $p = 0.033$), the time rats spent in the center of an OF (r = 1, $p = 0.016$), and sucrose preference percentages (r = 0.97, $p = 0.033$) (Figure 5b-d).

These findings suggest that KP-54 treatment effectively prevents the decrease in GABA levels in the amygdala induced by 6-OHDA.

DISCUSSION

This study offers suggestive evidence for KP-54's therapeutic potential for resolving mood-related disorders in a PD rat model. The long-term nasal administration of KP-54 not only alleviated mood disturbances and motor impairments but also 6-OHDA-induced dopaminergic neuron loss. These findings highlight the multifaceted neuroprotective effects of KP-54, which may be mediated through its interaction with the neuroendocrine axes and neurotransmitter systems (particularly GABA in the amygdala, as demonstrated in this study).

In PD patients, depression and anxiety are prevalent neuropsychiatric manifestations that substantially diminish their quality of life.[25] The conventional therapeutic strategies are primarily focused on motor symptoms and frequently prove insufficient in managing these

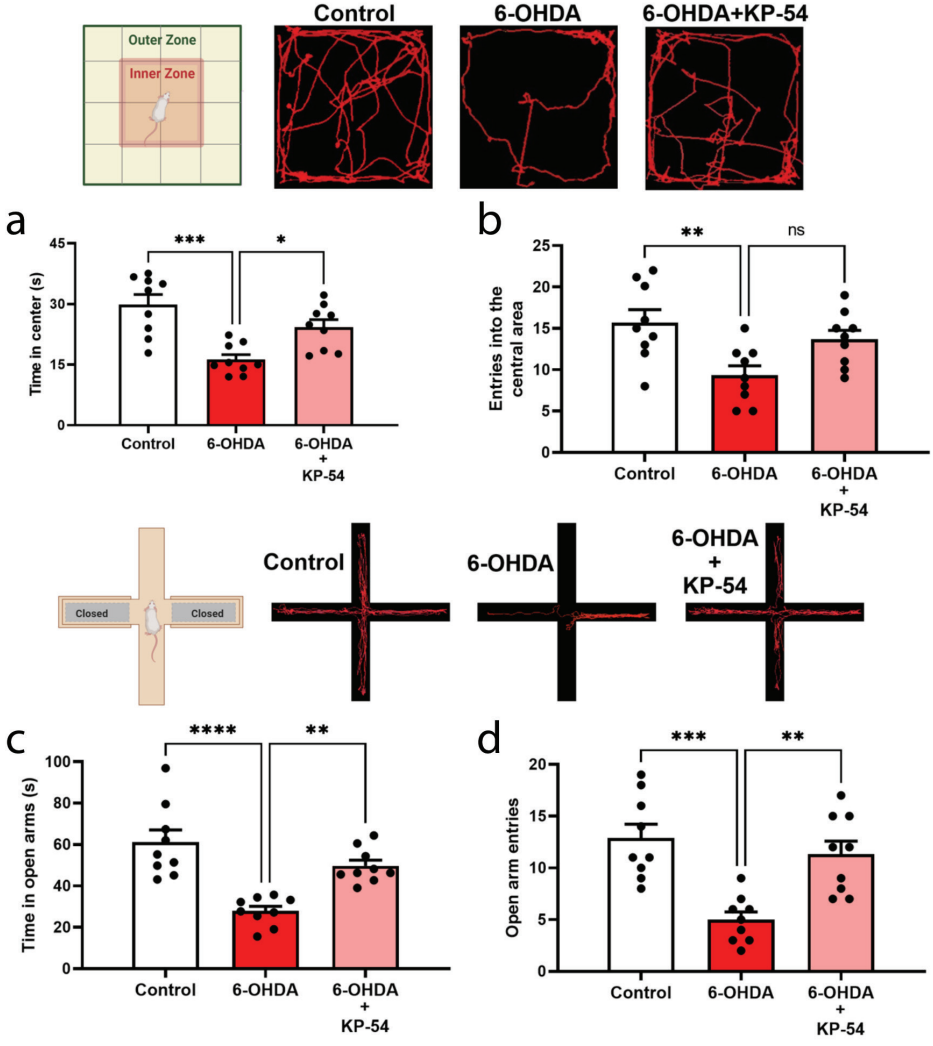

FIG. 3. Effect of nasal KP-54 treatment on anxiety-like behaviors in 6-OHDA-induced Parkinsonian rats. The representative track plots illustrate the recordings acquired during the 5-minute test sessions (OFT or EPM). The central square was designated as the "inner zone," while the periphery was termed the "outer zone." (n = 9) rats per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

KP-54, kisspeptin-54; 6-OHDA, 6-hydroxydopamine; OFT, open field test; EPM, elevated plus maze.

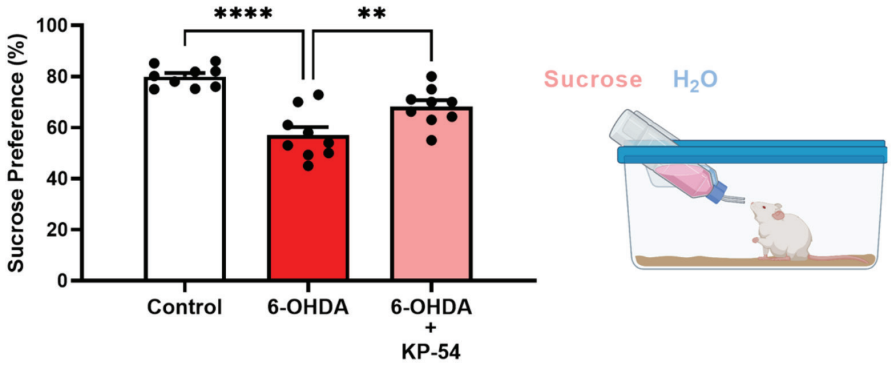

FIG. 4. Effect of nasal KP-54 treatment on depression-like behaviors in 6-OHDA-induced Parkinsonian rats. A schematic illustration displaying a two-bottle choice SPT. (n = 9) rats per group. $^{**}p < 0.01$, $^{****}p < 0.0001$.

KP-54, kisspeptin-54; 6-OHDA, 6-hydroxydopamine; SPT, sucrose preference test.

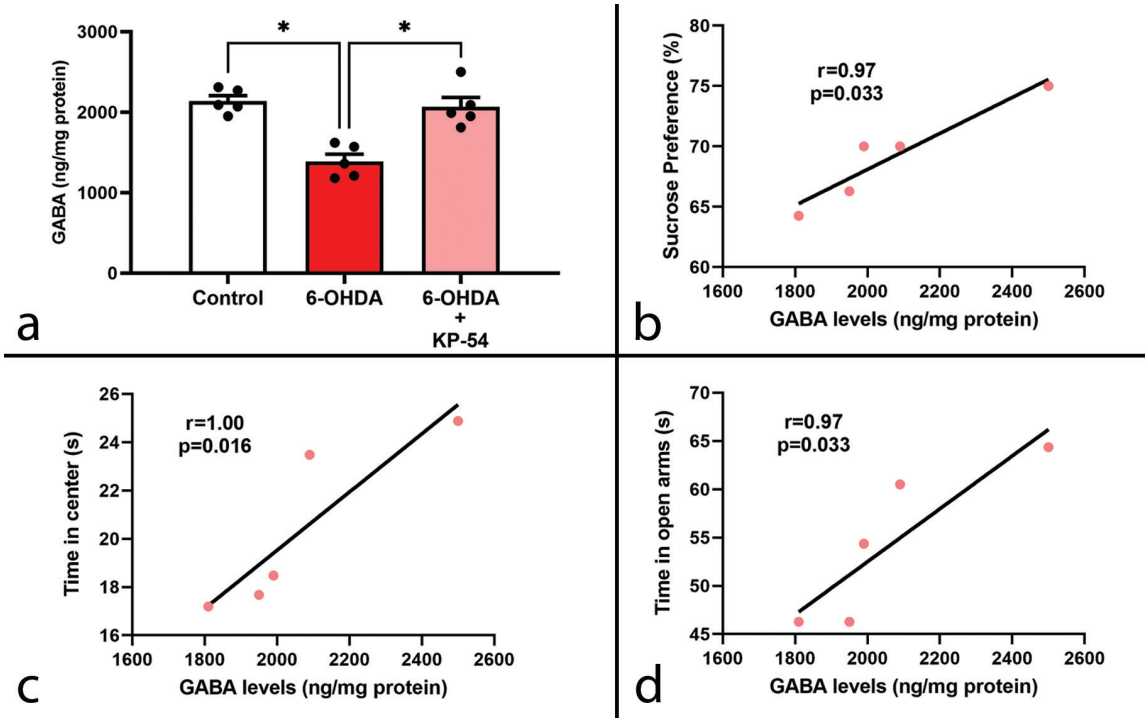

FIG. 5. Effect of nasal KP-54 treatment on GABA levels in the amygdala of 6-OHDA-induced Parkinsonian rats. (n = 5) rats per group. $^{*}p < 0.05$. Correlations between sucrose preference percentages (b), the time rats spent in the center of an OF (c), time spent in the open arms of an EPM (d), and GABA levels in rats with 6-OHDA + KP-54.

KP-54, kisspeptin-54; GABA, gamma-aminobutyric acid; 6-OHDA, 6-hydroxydopamine; OF, open field.

neuropsychiatric consequences. The efficacy of KP-54 in reducing depressive- and anxiety-like behaviors, as revealed by the SPT, OFT, and EPM, is consistent with previous studies that have shown the anxiolytic and antidepressant effects of KP in various animal models.[9-13] Conversely, other research suggests that KP may induce anxiety-like behavior.[7,26,27] Central administration of KP-13 has been demonstrated to decrease the number of entries and the time spent in the open arms of an EPM in male rats in a dose-dependent manner.[26] Furthermore, anxiety-associated behavior has been examined by developing murine models that lack the *KiSS1r* gene.[27] This modification led to the male mice spending twice as much time in the open arms of an EPM without altering their performance in the OFT. This outcome may indicate that intact KP signaling aggravates anxiogenic responses among mice to heights.[27] Furthermore, a recent study explored the impact of central treatment of the KP-8 on anxiety responses in rodents.[7] Contrary to these findings, the present study established the anxiolytic and antidepressant effects of KP-54 in an experimental model of PD. Notably, these effects of KPs may differ based on their specific form, dosage, animal species, and pathophysiological conditions.

GABAergic mechanisms are of crucial significance in the context of anxiety and depression. Studies have revealed that diminished GABAergic activity is linked to increased anxiety- and depression-related symptoms, indicating an imbalance in excitatory and inhibitory neurotransmission as a potential underlying pathophysiological mechanism. For instance, Petty discovered that patients with major depressive disorder exhibit significantly lower GABA levels in the occipital cortex than those of healthy controls.[28] Similarly, anxiety disorders have been associated with altered GABA receptor function and expression, which results in increased neuronal excitability and anxiety symptoms.[29] Numerous studies have demonstrated the efficacy of GABA agonists in alleviating symptoms of anxiety and depression, underscoring the therapeutic potential of targeting GABAergic systems to modulate mood and anxiety disorders.[30] The amygdala is a critical component of emotional processing and is frequently implicated in the pathophysiology of mood disorders.[31] Depressive and anxiety disorders have been specifically linked to GABAergic dysfunction, and it may be feasible to target this pathway as a therapeutic approach.[31,32] In the present study, we observed a substantial decrease in GABA concentrations in the 6-OHDA group compared to the control group, a result consistent with previous research indicating that 6-OHDA induces significant neurochemical alterations. This depletion underscores the vulnerability of the GABAergic system in the context of PD, where the equilibrium between excitatory and inhibitory neurotransmission is essential for normal function.[33] The administration of nasal KP-54 led to a partial increment of GABA levels, which suggests that the peptide's potential has the potential to modulate neurotransmitter systems affected by neurotoxicity. The mechanism responsible for this protection may be related to KP-54's ability to impact neurotrophic factors or its direct interaction with GABAergic neurons, which needs additional investigation. Furthermore, our findings revealed a strong positive correlation between GABA levels and behavioral outcomes measured by the EPM, OFT, and SPT. Conversely, it is postulated that KP may modulate GABAergic neurotransmission either directly by regulating KP receptors expressed in GABAergic neurons or indirectly through modulation of other neurotransmitter systems that affect GABAergic activity. However, direct evidence linking nasal administration of KP-54 to alterations in GABA levels is limited, indicating a substantial gap in the current understanding. Future research could focus on in vivo studies that measure GABA levels before and after KP-54 administration, possibly by employing techniques like microdialysis or functional MRI to detect real-time changes in brain regions associated with mood regulation.

This study provides significant evidence that long-term nasal administration of KP-54 can considerably mitigate depressive and anxiety-like behaviors in rats with 6-OHDA-induced lesions, indicative of potential beneficial benefits for mood disorders in PD. The mitigation of these neuropsychiatric symptoms was accompanied by improvements in motor impairments and the preservation of dopaminergic neurons in the nigrostriatal pathway. Additionally, KP-54 treatment was revealed to regulate the elevated levels of GABA in the amygdala, correlating positively with behavioral improvements. These results suggest that KP-54, when administered nasally, has the potential to be a non-invasive and effective approach to managing mood disturbances and associated motor deficits in PD, warranting additional clinical investigation.

While our study leverages the rat model of hemi-Parkinsonism to uncover novel insights into the therapeutic potential of KP-54, it is important to acknowledge the inherent limitations of this model. The translational relevance of our findings to human PD remains uncertain, necessitating further studies in more clinically representative models to validate our results. Additionally, the long-term efficacy and safety profile of KP-54 remains to be determined. Our findings are promising for short-term application; however, chronic use poses unanswered questions regarding potential side effects and sustained effectiveness. Addressing these gaps through extended longitudinal studies will be crucial for advancing KP-54 toward clinical application.

Ethics Committee Approval: All experimental procedures related to animal care and treatment were conducted in accordance with the guidelines and ethical standards established by the Institutional Animal Care and Use Committee of the Faculty of Medicine at Akdeniz University (approval number: 17, date: 15.04.2024).

Informed Consent: Patient approval has not been obtained as it is performed on animals.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- O.S.; Design- O.S.; Supervision- O.S., N.D.; Materials- O.S., A.G.S., N.D., M.A.A.; Data Collection or Processing- O.S., A.G.S., N.D., M.A.A.; Analysis and/or Interpretation- O.S., A.G.S., N.D., M.A.A.; Literature Search- O.S., A.G.S.; Writing- O.S., A.G.S.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Supplementary FİG 1: https://balkanmedicaljournal.org/uploads/pdf/Supplementary-FIG-1.pdf

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