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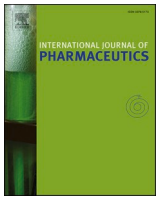

Venlafaxine-PLGA nanoparticles provide a fast onset of action in an animal model of depression via nose-to-brain

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ABSTRACT

Background: Current treatment of depression is hindered by the delayed onset of the action of antidepressant drugs, often resulting in treatment failure. Therefore, new therapeutic solutions are imperative.
Methodology: Venlafaxine-loaded poly(lactic-co-glycolic acid) nanoparticles were produced by a double emulsion-solvent evaporation method. Cellular safety assessment and internalization assays were carried out *in vitro* in human olfactory neuroepithelium cells. The antidepressant effect of intranasal (nose-to-brain) nanoparticle administration was assessed in animals submitted to an animal model of depression by behavioral tests, including open-field, sucrose preference test and tail suspension test.
Results: The drug entrapment efficiency (55–65 %), particle size (190–210 nm), polydispersity index (<0.2), and zeta potential (−20 mV) of Venlafaxine-loaded poly(lactic-co-glycolic acid) nanoparticles were determined to be adequate. Nanoparticles did not show cytotoxic effects. Cell viability was more than 90 % for all formulations and concentrations assayed. The results of the quantitative and qualitative cell uptake assays were consistent, showing an evident internalization of the nanoparticles into the cells. Furthermore, venlafaxine-loaded nanoparticles administered for just 7 days were able to reverse the phenotype induced by a depressive-like model, showing a significant antidepressant-like effect compared to those treated with free venlafaxine.
Conclusions: These findings indicated that intranasal venlafaxine-loaded poly(lactic-coglycolic acid) nanoparticles could become a viable technique for improving venlafaxine brain uptake via nose-to-brain. It could also be a promising nanoplatform for enhancing the treatment of depression.

1. Introduction

One of the major hurdles in developing effective treatments for central nervous system (CNS) disorders is the accessing the brain due to the presence of the blood–brain barrier (BBB)(Dong, 2018). The BBB is mainly composed of endothelial cells tightly sealed by tight junctions which prevent the entry of harmful substances in the blood to the CNS (Wang et al., 2019). The restrictive structure of this barrier results in a therapeutic failure for many compounds because 100 % of large and hydrophilic molecules and 98 % of small compounds are unable to cross it (Pires and Santos, 2018).

Fortunately, a recent reliable route has been investigated. This route, called nose-to-brain can bypass the BBB and the neurotherapeutics could reach the brain directly. Although the route is not completely described, it is known that the access of nanoparticles to the brain could be through extracellular and intracellular pathways (Formica et al., 2022; Huang

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et al., 2024) using trigeminal and olfactory nerves (Crowe et al., 2018; Giunchedi et al., 2020). After intranasal administration, actives are transported to olfactory region by different pathways (Mittal et al., 2014). This area is the only exposed section of the CNS to the external environment, and it is responsible for drug delivery to different locations of the brain (Shukla et al., 2021). Moreover, the route offers potential properties compared to oral or intravenous administration. This non-invasive and painless technique enhances patient compliance by allowing drugs to pass directly to the brain. This bypasses hepatic first-pass metabolism, resulting in a relatively fast onset of action (Erdő et al., 2018; Szabó-Révész et al., 2022).

The use of nanomedicine for intranasal administration is being widely investigated due to the advantages of these administration systems can offer for the nose-to-brain route. Among the main advantages, it can be mentioned: access to remote places; reduction of toxicity and side effects; greater dose–response; enhanced solubility; improvement of pharmacokinetics profiles compared with conventional medicines or the control of drug release (Caban et al., 2014; Choi and Han, 2018; Gänger et al., 2018; Patra et al., 2018). Then, a great variety of drugs have been encapsulated and tested for numerous brain pathological situations such as glioblastoma (Ahmad et al., 2022; Bruinsmann et al., 2022; Ferreira et al., 2021; De Fraga et al., 2021), brain injury (Li et al., 2022; Wang et al., 2021), epilepsy (Serralheiro et al., 2015; Shah et al., 2021; Yousfan et al., 2021), schizophrenia (Pandey et al., 2022; Ruby and Pandey, 2016), Parkinson's disease (Dimiou et al., 2022; Trapani et al., 2022), migraine (Jha and Mishra, 2022; Yadav et al., 2022), acquired immunodeficiency syndrome (AIDS) (Kakad and Kshirsagar, 2021), Alzheimeŕs disease (Sunena and Mishra, 2018), attention deficit hyperactivity disorder (ADHD) (Sharma et al., 2023), amyotrophic lateral sclerosis (ALS) (Lu et al., 2023) or depression (Liu et al., 2023; Sorrentino et al., 2020; Vitorino et al., 2020).

Moreover, recent studies have demonstrated that intranasal administration of nanoparticle-based formulations can produce antidepressant-like effects in animal models, suggesting potential efficacy in humans. Nanoparticles can be engineered to deliver antidepressant agents directly to specific brain regions involved in mood regulation, potentially enhancing therapeutic outcomes (Xinchen et al., 2023).

Focusing on depression disease, Alberto et al., 2022 compiled 11 studies (three of them using venlafaxine-nanosystems) carried out using lipid and polymeric nanosystems for the intranasal administration of antidepressant substances, among them, a previous study from the authors Cayero-Otero et al., 2019 which findings were protected by a patent application (WO2020193838A1) (Martín-Banderas et al., 2020) .

The revision by Alberto et al. 2022 described the ideal characteristics of nanoparticle formulation for intranasal administration and summarized the main studies carried out with various active antidepressant ingredients administered intranasally, including venlafaxine. More recent, Upadhyay et al. 2024 collected the most relevant papers related to the administration of antidepressant drugs administered via the nose-to-brain. Authors analyzed the total number of 18 published articles. Six of them described the use of venlafaxine encapsulated into different nanosystems (Upadhyay et al., 2024). These recent reviews clearly highlight the need for novel therapeutic strategies for treatment of depression. Moreover, authors point out the interest in the nose-to-brain route as an alternative for drug delivery to the central nervous system and emphasize the ability of various nanosystems to reach the brain after intranasal administration. In all the studies included in both reviews, encapsulation of antidepressant actives led to improved delivery of the drug to the brain, regardless of the study design. This translates into a greater antidepressant effect and faster onset of action of encapsulated antidepressants, whether comparing different routes of administration or different formulations (free or encapsulated drug) after intranasal administration.

As it is well known, the design of nanocarriers is a key point. Our group carried out an *in vivo* biodistribution study in mice for different types of nanoparticles (NPs): plain NPs, transferrin surface-modified poly(lactic-co-glycolic) acid (PLGA) NPs (Tf-PLGA NPs) and specific peptide of transferrin receptor surface-modified PLGA NPs (TfRp-PLGA NPs). Our study concluded that the simplest approach, plain NPs, showed the highest ability to reach the brain after intranasal administration (Cayero-Otero et al., 2019), avoiding receptor-mediated endocytosis.

According to WHO, major depression will be the first global cause of burden in 2030 and more than 300 million people worldwide are affected (Malhi and Mann, 2018). Depression is a state of low mood characterized by hopelessness and sadness, which can influence emotions, feelings, acts or relationships leading, in many cases, to suicide (Jani et al., 2019). Many types of antidepressants are currently available, such as selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs), or tricyclic antidepressants (TCAs), however the lack of a complete response in some patients remains a significant challenge for global healthcare (Jaffe et al., 2019). The limited effectiveness of antidepressant drugs, combined with their delayed onset of action, contributes to unsatisfactory therapeutic results in patients with depression, highlighting the importance of exploring new medications or alternative approaches to address these challenges (National Institute for Health and Care Excellence, 2022; Oliveira-Maia et al., 2023; Posternak and Zimmerman, 2005). In our study, venlafaxine (VLF), a widely used drug, has been used as a model drug. VLF is a SNRI has dual-monoaminergic action can cause several side effects, including nausea, insomnia, drowsiness, constipation, tachycardia, and dry mouth, among others (Lipid et al., 2022). Furthermore, like other monoamine reuptake inhibitors, it has a slow onset of therapeutic action following oral administration, typically taking around 2–4 weeks for patients to experience mood improvement (Entsuah et al., 1998). Although the underlying pharmacodynamic or pharmacokinetic reason for this delay are not well understood, it is clinically significant because it prolongs patients associated disabilities and increases the likelihood of treatment discontinuation (National Institute for Health and Care Excellence, 2022).

Moreover, oral bioavailability of VLF is low (40–45 %) and it shows a short half-life (4–5 h), which results in frequent administration to maintain therapeutic levels (Xu et al., 2020). Although there are commercial controlled-release tablets and capsules, VLF is a hydrophilic compound and presents a limited BBB permeability. For all this, VLF becomes a suitable candidate to be encapsulated into NPs. Moreover, VLF NPs administrated via nose-to-brain route could bolster intranasal VLF delivery to the brain.

The purpose of the present study was to investigate the brain-targeting potential of VLF-loaded PLGA NPs via a non-invasive intranasal nose-to-brain route. Thus, the study explores the use of nanoparticles for antidepressant drug delivery, with the goal of enhancing the effectiveness, targeting, and safety of treatments such as venlafaxine. A major challenge in antidepressant therapy is the difficulty many drugs face in crossing the blood–brain barrier. By leveraging the nose-to-brain route, nanoparticles can significantly improve drug delivery to the brain, increasing bioavailability and reducing the time required to reach therapeutic concentrations. As a result, the slow neuroadaptive changes—such as receptor regulation and neuroplasticity—that typically take weeks with monoaminergic antidepressants could potentially be accelerated. Additionally, nanoparticles offer controlled and sustained drug release, minimizing dosing frequency and side effects. By enhancing brain uptake, they may also lower the required dosage, further reducing the risk of adverse effects associated with conventional oral antidepressants. Thus, here we explored if VLF-loaded PLGA NPs produced a fast and robust antidepressant-like responses after intranasal administration in mice submitted to an animal model of depression.

2. Materials and methods

2.1. Materials

Resomer® RG 504H, PLGA terminated was obtained from Evonik (Germany). VLF (Venlafaxine hydrochloride) and NHS (N-hydroxysuccinimide) were obtained from Sigma-Aldrich (USA), ethyl acetate (HPLC grade); dimethyl sulfoxide (DMSO) (HPLC grade) and polyvinyl alcohol (PVA) (MW = 72000 g/mol) were purchased from Panreac (Spain).

For *in vitro* experiments, DMEM-F12 medium from Biowest (France) was supplemented with inactivated fetal bovine serum (FBS) from Biowest (France), GlutaMAX from GibcoBRL (USA) and Primocin from InvivoGen (USA). Penicillin-streptomycin, FITC (Fluorescein 5-isothiocyanate), trypsin-EDTA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PBS (pH = 7.4 ± 0.1), nile red (NR), and paraformaldehyde (PFA) were provided by Sigma-Aldrich. Collagen I rat tail was purchased from Gibco (Thermo Fisher, USA). Hoechst® 22,242 from Invitrogen (USA) and WGA (Wheat Germ Agglutinin) Alexa Fluor™ 555 Conjugate was obtained from Thermo Scientific (USA).

2.2. VLF-PLGA NPs preparation

For the preparation of the NPs, a double emulsion-solvent evaporation method (DE-SEV) was used with slight modifications (Martín-Banderas et al., 2013). Briefly, 80 mg of PLGA were dissolved in ethyl acetate (4 % w/v) and were emulsified by addition drop to drop of 100 uL of an aqueous solution of PVA (0.5 % w/v) containing 25 % w/w of drug (VLF) under ultrasonication for 1 min at RT in an ultrasonic bath (JP Selecta, Barcelona, Spain. 50 W). The resulting emulsion (W_1/O) was quickly dropped (with a pipette) into 10 mL of aqueous PVA solution at 0.5 % w/v (previously placed in the homogenizer on ice) employing a high-speed homogenizer (Ultraturrax® T25 with S25N-8G dispersing tool, IKA, Germany) for 1 min to obtain a double $W_1/O/W_2$ emulsion at 24.000 rpm. This emulsion was obtained using ice during the emulsification process to prevent the overheating of the emulsion. Afterwards, the emulsion was left under magnetic stirring employing a magnetic stirrer (IKA RT-15, IKA Germany) until complete evaporation of the organic solvent at room temperature (6 rpm). Then, NPs suspension was collected (first centrifugation) and washed twice with 45 ml of Milli-Q water by ultracentrifugation at 14.610 RCF for 30 min at 4 °C (Eppendorf 5804R centrifuge, Eppendorf AG). Finally, NPs were resuspended in trehalose solution (5 % w/v) (final volume = 1 ml) used as cryoprotectant and freeze-dried to obtain a fine and stable powder (frozen and lyophilized at − 80 °C and 0.078 mbar; Telstar Cryodos, Spain).

2.3. FITC-PLGA NPs production

For cell uptake studies, FITC was used as a fluorophore and covalently bonded to PLGA to evaluate the internalization of NPs in cells. The fluorescent polymer was synthetized through carbodiimide method employing the protocol used in previous experiments (Cayero-Otero et al., 2019). Then, fluorescent NPs were prepared by DE-SEV following the same protocol followed to prepare VLF-PLGA NPs.

2.4. Nps characterization

NPs were characterized for mean hydrodynamic diameter, size distribution (PdI) and surface charge (ZP). The mean particle size and polydispersity index of the blank of both VLF-loaded and FITC-PLGA NPs was measured by photon correlation spectroscopy and the ZP was analysed by Laser Doppler using a Zetasizer ZS90 (Malvern Instruments Ltd, Malvern, UK). Measurements were carried out in triplicate after diluting an aliquot of recently prepared NPs suspension with distilled water at room temperature.

For the encapsulation capabilities studies, the drug content of the NPs was evaluated by the previously validated HPLC method (Haque et al., 2012) with slight modifications. Chromatographic separation was achieved with a LiChroCART® 250–4 HPLC Cartridge LiChrospher® 100 5 µm column. First, aqueous supernatant was collected and filtered (Millex GV syringe filter, 0.22 µm) and, then 20 µL of the supernatant was injected into the HPLC system for drug detection and quantification using a Hitachi LaChrom (D-7000) series HPLC equipped with a L-7200 automatic injector and a quaternary pump (model L-7100). Column was kept at 25 °C (L2350 column oven, Elite LaChrom) and the measurements were performed at 225 nm.

The amount of drug encapsulated in the development nanosystems was calculated indirectly (Jain and Datta, 2014), measuring the amount of active in the supernatant after NPs collection by ultracentrifugation and compared with an initial amount of drug used to prepare the NPs. The drug content into NPs was expressed as encapsulation efficiency (EE %) and drug loading (DL%) according to the equations (1) and (2) respectively:

EE% = ((total drug amount − unencapsulated drug amount)/total drug amount) x 100 (1).

DL% = ((total drug amount − unencapsulated drug amount)/total mass of NPs) x 100 (2).

2.5. In vitro cell studies

2.5.1. Cell culture

It is well known that the structure of the olfactory bulb comprises different types of cells, including the cells used in this study derived from the olfactory neuroepithelium (ONE) (Lavoie et al., 2017). ONE cells were used for preliminary *in vitro* studies as they are one of the cell types that the particles may initially encounter on their journey through the intranasal route. ONE cells were obtained from a healthy volunteer by nasal brushing as described previously (Delgado-Sequera et al., 2021). This healthy subject provided written, informed consent, approved by the Ethics Committee (PI-151019). Cells obtained by the nasal brushing included highly proliferative neural precursors that were grown at 37 °C with 5 % CO_2, in Dulbecco's Modified Eagle Medium/Ham F-12 (DMEM/F12) supplemented with 10 % FBS, 2 % GlutaMAX and 0.2 % Primocin. When the confluence was reached, cells were detached with 0.25 % trypsin-EDTA and replated in DMEM/F12 medium supplemented with FBS (10 % v/v), antibiotic–antimycotic (1 % v/v) and Glutamax® (2 % v/v) in humidified environment (AutoFlow NU-4750 Water Jacket CO_2 incubator; NUAIRE, USA). The medium was replaced every day, and the *in vitro* experiments were carried out in passage 5.

2.5.2. In vitro cell cytotoxicity assay

The cytotoxicity of the NPs was evaluated by the MTT proliferation assay, determining mitochondrial dehydrogenase activity (Mosmann, 1983). The MTT (yellow) is cleaved to formazan (violet) by a system of the mitochondrial respiratory chain of living cells. This change of colour can be quantified by spectrophotometry.

Cells from the ONE were plated at a density of 4 x 10^4 cells/well in a 96-well plate (Nunclon®) and incubated for 24 h at 37 °C to allow cell attachment. Then, the medium was completely removed, and cells were washed twice with 200 µL of PBS before the incubation with different concentrations of NPs (from 0.1 to 1000 µg/mL, approximately equivalent to 0.0155—155 µg/ml of VLF) for 24 h. NPs suspension was removed after incubation time and cells were washed twice with PBS. 50 µL of MTT solution (1 mg/ml in medium) was added to each well for 2.5 h in dark. Finally, MTT solution was discarded and MTT formazan crystals were dissolved with 100 µL of isopropanol, shaking in dark for 10 min at room temperature. Absorbance was measured in a microplate reader (Synergy HT, BioTech, USA) at 570 nm and cell viability was calculated following equation (Cayero-Otero et al., 2019):

Viability (%) = ((experimental value – negative control) / (positive

control – negative control)) x 100 (Eq. 3).

Cells incubated only with DMEM/F12 medium were used as negative control and DMSO as positive control.

2.5.3. *In vitro internalization studies*

The uptake of NPs into the cells was determined by qualitative and quantitative analysis and FITC-PLGA NPs were used for those purposes.

Analysis by confocal laser microscopy.

For qualitative studies of NPs internalization, ONE cells were seeded (4×10^4 cells/well) in a μ-Slide 8 well (iBidi, Germany) and were allowed to attach overnight. After that, cells were washed twice with PBS (pH 7.4) and incubated with FITC-PLGA NPs suspension (250 μg/mL in culture medium without FBS) for 1 and 2 h at 37 °C. At the end of incubation time, cells were washed twice with PBS to remove the remains of NPs suspension and the nuclei were counter-stained with Hoechst® 33,342 for 10 min. Then, cells were washed twice with PBS and the cytoplasms were stained with WGA-Alexa Fluor 555. Finally, cells were washed twice with PBS and fixed with PFA (4 % w/v in PBS)

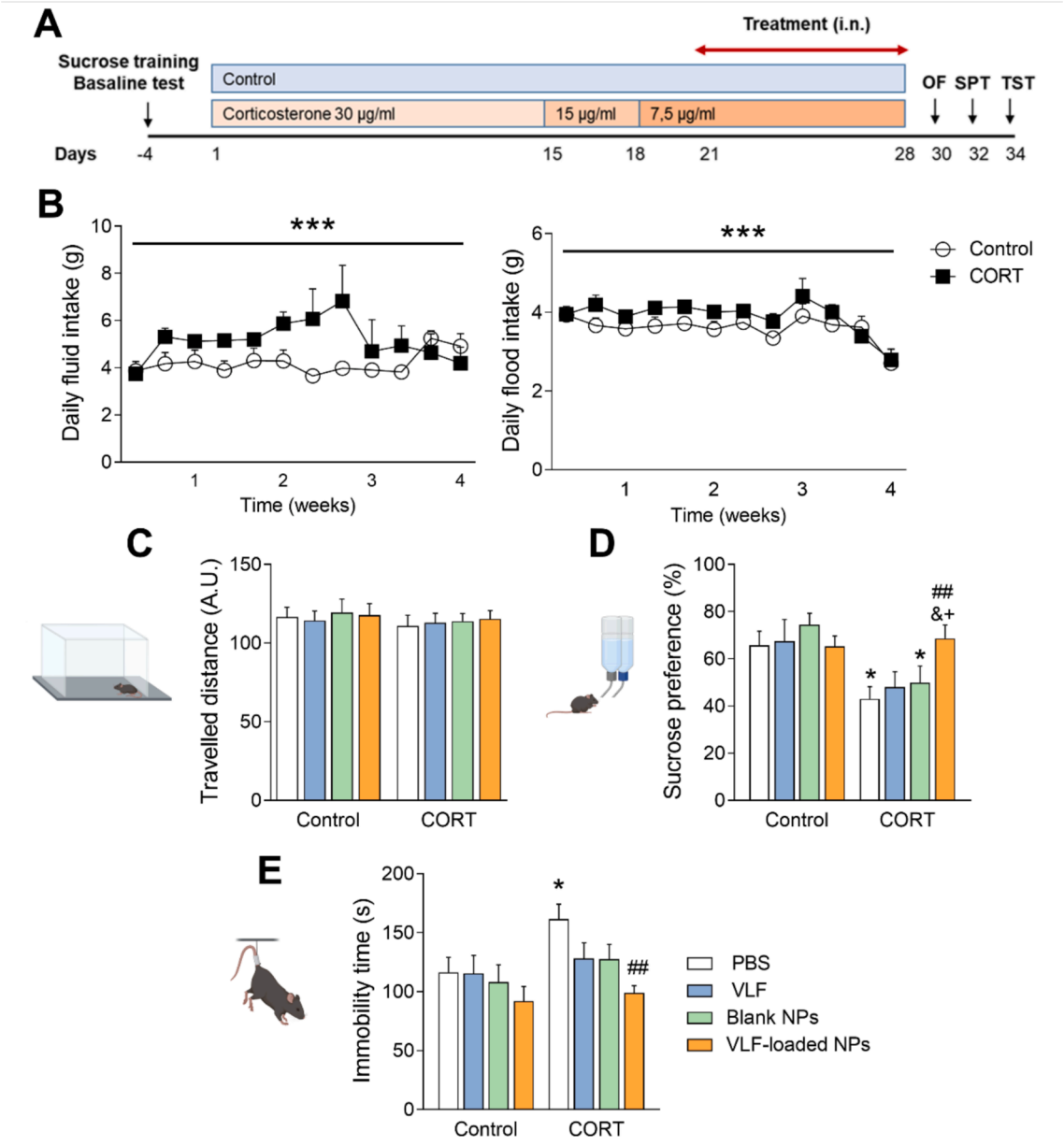


Fig. 1. **(A)** Experimental design of the *in vivo* experiments. Mice underwent a baseline sucrose training test four days prior to the start of the CORT regimen. Animals were exposed to the tap water (control animals) or CORT via drinking water for 28 days in decreasing doses equivalent to a daily dose of 6.6 mg/kg/day for 15 days, followed by 2.7 mg/kg for 3 days and 1.1 mg/kg for 10 days. Treatments (VLF-loaded NPs, Blank NPs, VLF free drug or PBS) were administered intranasally daily during the last 7 days of the CORT regimen. Behavioural experiments including the open-field test (OF) the sucrose preference test (SPT) and the tail suspension test (FST) were performed. **(B)** Effects of CORT administered orally via the drinking water upon fluid and food consumption. The intake was tracked three times a week over the 28 days of the CORT regimen. Estimation of daily intake of fluid per mice determined by weighing bottles and estimation of food intake per mice measured in grams of pellet consumed. All values are presented as mean ± SEM. Statistical significances displayed are results of depression model factor obtained by a repeated measure ANOVA analysis (n = 9–10 per group). **(C)** Effect of treatments on locomotor activity in the OF. Graph represents the mean of the total distance travelled expressed in arbitrary units (A.U.) ± S.E.M. Two-way ANOVA test (n = 13–17 per group). **(D)** Effect of treatments on the SPT. Graph represents the mean of the percentage of sucrose preference ± S.E.M. Two-way ANOVA followed by Bonferroni post-hoc test (n = 12–21 per group). **(E)** Effect of treatments on the tail suspension test. Graph represents the mean of the immobility time expressed in seconds ± S.E.M. Two-way ANOVA followed by Bonferroni post-hoc test (n = 5–10 per group). * $p < 0.05$ vs respective control group, ## $p < 0.01$ vs PBS-treated group, & $p < 0.05$ vs VLF-treated group, + $p < 0.05$ vs Blank NPs- treated group.

for 20 min and maintained in PBS to be observed by confocal laser scanning microscope (Leica Stellaris 8 Falcon; Leica; Germany) with imaging software (Las X). Cells without NPs were used as control.

Quantitative study by flow cytometry.

For this purpose, cells were plated in a 6-well plate (9.6 cm^2/well) at a density of 1 x 10^6 cells per well (2 ml) and were allowed to attach overnight. The next day, medium was retired, and cells were washed twice with PBS and incubated with FITC-PLGA NPs suspension (500 μg/ml in culture medium without FBS) for 2 h. After incubation, the suspension was removed, and the wells were washed twice with PBS to eliminate the particle suspension residues. The cells were detached with trypsin-EDTA (1 mL) for 5 min. Finally, the cell suspension was washed and resuspended with PBS and placed directly in cytometer tubes. The fluorescent NPs inside of cells were measured using a flow cytometer (MACSQuant VYB; Miltenyi Biotec; Germany) and the data were analyzed with MACS Quantify software). Cells non-incubated with fluorescent particles were used as a control of basal autofluorescence.

2.6. In vivo studies

2.6.1. Animals

Adult male C57BL/6J mice (10 weeks old at the beginning of the experiments) were housed under controlled conditions (22 ± 1 °C; 12-h light/dark cycle) with food and water available ad libitum. All procedures were performed in accordance with the European Guidelines (2010/63/EU) and Spanish Law (RD 53/2013) regulating animal research. All the experimental protocols were approved by the Ethical Committee for Animal Experimentation of the University of Cádiz.

2.6.2. Experimental design

In brief, the depression-like model was established by oral corticosterone (CORT) chronic administration. Animals were presented with water (control group) or CORT (CORT group) diluted in the drinking water for 28 days. In order to assess the baseline behaviour, a sucrose preference test (SPT) was performed 3 days before the CORT regimen. Drinking and food consumption was followed three times a week during the 4-weeks of CORT administration in each cage. Additionally, mice were administered daily from the 21st to the 28th day of the CORT regimen with i.n. administration of VLF-loaded NPs, Blank NPs, VLF free drug or PBS. After that, to examine the possible antidepressant-like effect of these treatments animals were tested in the open-field test (OF), the sucrose preference test (SPT) and tail suspension test (TST) with an interval of 2 days between tests (Fig. 1).

2.6.3. Mouse model of depression

The animal model of depression used is the one induced by chronic CORT administration. This model induces a depressive-like phenotype that effectively mimics both the behavioral symptomatology and the neurobiological mechanisms typical of depression (Gourley and Taylor, 2009; Zhao et al., 2008). The mice model of depression was induced as described previously (Ferrés-Coy et al., 2016). CORT (Sigma-Aldrich, Spain) was dissolved in regular tap water and brought to a pH of 7.4.

Mice were exposed to CORT for 28 days in decreasing doses equivalent to a daily dose of 6.6 mg/kg/day for 15 days, followed by 2.7 mg/kg for 3 days and 1.1 mg/kg for the last 10 days until the start of the behavioural evaluation. CORT was prepared freshly every 72 h and kept light protected in opaque bottles. Animals in the control group received water only.

2.6.4. Treatment

Treatment was initiated on day 21 post-exposure to CORT and was maintained for 7 consecutive days. All treatments were applied daily intranasally in both nostrils, 5 μL drop in each nostril (total volume 10 μL/day). For i.n. administration, animals were lightly anaesthetized with 2 % isoflurane (Farmavet, Spain) and placed in a supine position. VLF-PGLA NPs at a dose of 0.06075 mg/day or an equivalent amount of blank NPs were administered. Free VLF was diluted in PBS and administered daily at 0.06075 mg/day. Control mice received PBS.

2.6.5. Behavioural assessment

Open-field test (OF).

Locomotor activity was measured using an open-field apparatus to assess the spontaneous movement and exploratory behavior of the mice. The apparatus consisted of a square plexiglass box (45 × 45 cm) with 35 cm high walls. Mice were placed in one corner of the box and allowed to roam freely for a period of 15 min. During this time, the movement of the animals was recorded and tracked using a camera connected to the S.M.A.R.T system (Spontaneous Motor Activity Recording and Tracking: Panlab, S.L.), which measures the total distance travelled in arbitrary units. This test allows for the quantification of locomotor activity, providing valuable insights into the effects of the treatments on general motor function and exploratory behavior.

Sucrose preference test (SPT).

The SPT is a reward-based test, used as an indicator of anhedonia, a core symptom of depression. SPT was assessed in a two-bottle paradigm for 8 h (9:00–17:00 h), where the mice received two bottles, one filled with 1 % sucrose solution and one with tap water. After half of the time (4 h), the places of the bottles were changed to avoid side bias. No previous food or water deprivation was applied before the test. The weight of each bottle was determined before and after the test to accurately determine the volume of liquid consumed. The sucrose preference was calculated in percentage as the amount of consumed sucrose solution divided by the total liquid intake (Liu et al., 2018). This measurement provides an index of the animal's motivation for a sweet reward, with a decrease in sucrose preference compared to water indicating an anhedonic-like behavior, a hallmark of depressive states (Liu et al., 2018). The SPT is performed both before (as baseline) and after the CORT regimen to assess any changes in reward sensitivity and behavior following the experimental treatment.

Tail suspension test (TST).

The TST is one of the most widely used preclinical tools to study antidepressant activity in mice (Steru et al., 1985). This test is based on the behavioral response of mice to an inescapable situation, specifically assessing the duration of immobility as a proxy for depressive-like behavior. In the TST, mice were individually suspended by the tail 20 cm above the floor using adhesive tape placed 1 cm from the tip of the tail. This position forces the animal to remain suspended in a helpless state, leading to a natural tendency to either attempt to escape or remain immobile. The test session lasted for 6 min, and they were videotaped and subsequently scored by a trained observer. The total duration of immobility during the test was measured. A reduction in immobility in this test was considered to indicate antidepressant activity (Berrocoso et al., 2013; Can et al., 2012).

2.7. Statistical analysis

For the *in vitro* experiments, data were expressed as the mean ± S.D. of the parameter measured. For the *in vivo* experiments, data were analysed by two-way analysis of variance (ANOVA) followed by the Bonferrroni post-hoc test. The factors evaluated (between subjects) were the depression model and treatment. Data were analysed using Prism 7.0 GraphPad and Statistica 10.0 software. All p-values < 0.05 were considered statistically significant.

3. Results

3.1. Nps production and physicochemical characterization

The production of blank and VLF-loaded NPs (VLF-PLGA NPs) was carried out by DE-SEV method and the mean particle size, polydispersity index (PdI) and zeta potential (ZP) were measured as previously were described (subsection 2.4). The data are expressed as the mean value ±

SD.

The mean hydrodynamic diameter of blank PLGA and VLF-PLGA NPs was 192.1 ± 6.7 nm and 208 ± 2.9 nm, respectively. The PdI was less than 0.2 indicating a high homogeneity and narrow size distribution (Stetefeld et al., 2016). The surface charge was measured after washing NPs twice by ultracentrifugation to remove the PVA residues (Zambaux et al., 1998). ZP were very similar for blank and loaded NPs, –21.4 ± 0.3 mV and –22.4 ± 1.3 mV, respectively.

Regarding FITC-PLGA NPs used for internalization studies, similar results were obtained. The average size of these NPs was 277 ± 7 nm with a PdI = 0.102 ± 0.065 and ZP values were –21.7 ± 0.2 mV.

The encapsulation process was measured indirectly, calculating the amount of drug in the supernatant as described in the Method section (subsection 2.4). The EE% and DL% of PLGA NPs were 55–65 % and 10–12 %, respectively.

3.2. *In vitro cell studies*

3.2.1. *In vitro cell cytotoxicity assay*

VLF was approved in 1993 by FDA (Watanabe et al., 2018) and is known to have a favourable safety profile. However, toxicity cell studies are extremely important in the development of NPs. Thus, an MTT assay was carried out in order to evaluate the metabolic activity of viable ONE cells after incubation with these formulations at different concentrations.

For this assay, we used ONE cells incubated with a range of concentrations (0.1—1000 ug/mL) of blank and VLF-loaded PLGA NPs. Cells incubated with medium (without PLGA NPs) were used as negative and DMSO as positive control. After 24 h of incubation, the metabolic activity of living cells was superior to 85 %, maintaining cell viability in the range of 90—100 % for all the formulations (blank and VLF-loaded NPs) and concentrations assayed. Results obtained are summarized in Fig. 2. The obtained results shown less than 15 % viability loss after incubation, suggesting that unloaded and VLF-loaded NPs are compatible with these cells according to the ISO 10993–5 guidelines (Biological evaluation of medical devices. Part 5: Test for *in vitro* cytotoxicity). Additionally, the cytotoxicity of the cells was also evaluated after incubation with the free drug and the viability was superior to 90 % for all concentrations (Fig. 2).

3.2.2. *In vitro internalization study*

To evaluate the association of NPs to ONE cells, laser confocal microscopy was used. Briefly, FITC-PLGA NPs were incubated (250 μg/mL) for 1 h or 2 h with ONE cells and their behaviour was visually evaluated.

Results (Fig. 3) showed that FITC-PLGA NPs can interact with ONE cells (60 and 120 min) with a greater accumulation after 120 min,

Additionally, in order to check the localization of NPs, a Z-stack (optical section: 2.5 μm) was performed to confirm that NPs are inside of cells. Images obtained confirm NPs internalization and their location in cytoplasm and nucleus (supplementary data).

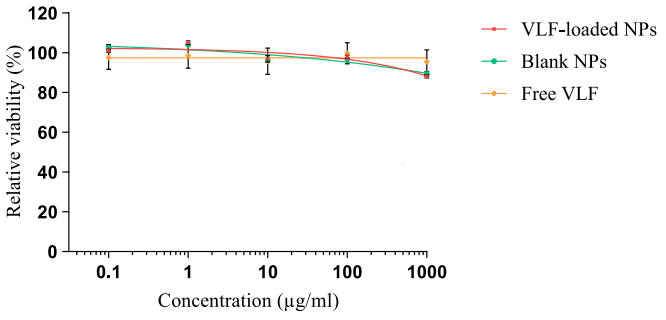


Fig. 2. Viability of ONE cells after 24 h of incubation with different concentrations (0.1—1000 ug/mL) of blank NPs, VLF-loaded NPs and free drug (VLF). Graph represents the mean ± S.D (n = 6).

To support the qualitative assays, a quantitative study was carried out to confirm the internalization of fluorescent NPs (FITC-PLGA NPs) in ONE cells by flow cytometry. For this purpose, cells and fluorescent nanoparticles were incubated under same conditions of visual assay (250 μg/mL, 60 and 120 min, 37 °C) and after incubation, samples were analysed by flow cytometry.

As it shown in Fig. 4, compared with negative control (cells incubated without NPs), the intensity of fluorescence increased around 33 % (46.24 % vs 78.91 %) after 60 min and around 44 % after 120 min (46.24 % vs 90.55 %) confirming the presence of fluorescent NPS inside of the cells. These results suggest that NPs internalization is a time-dependent process.

Beyond the useful information about the tolerable dose, the association studies (NPs cell uptake) could provide an insight into the access mechanism to the brain. In this sense, and due to the physicochemical properties of the nanosystems assayed in this work (diameter > 100 nm and slightly negative zeta potential), the main hypotheses supported by the scientific community to understand the nose-to-brain access of nanoformulations to the brain is the intra-cellular pathway (Chen et al., 2024; Clementino et al., 2021).

4. *In vivo* studies

4.1. *Fluid and food consumption*

The fluid and food consumption were monitored over the CORT regimen to check the induction of the depressive-like behavior. As expected, CORT administration produced a significant increase of both fluid and food intake (Fig. 1B).

4.2. *Locomotor activity*

The OF was performed to evaluate the effect of CORT and the administration of each i.n. treatment on the spontaneous locomotion of the animals. The results showed that none of the treatments significantly modified the animal's motor activity in the OF (Fig. 1C).

4.3. *Sucrose preference test*

No differences between groups in sucrose preference before the CORT regimen (at baseline measurement) were observed (data not shown). However, CORT significantly decreases the sucrose preference in both PBS and Blank NPs- treated animals demonstrating that the depression model produced anhedonia. Regarding the effect of i.n. treatments, none of the treatments induced any effect on the sucrose preference in control animals. However, in animals submitted to CORT while the VLF-free drug was not able to modify the sucrose preference, the VLF-loaded NPs restored the deficit induced by the animal model, showing a significant increase in sucrose preference compared to all PBS-, VLF- and Blank NPs- treated animals. Thus, the dose employed of VLF free drug was not able to normalize the sucrose preference, but the same dose administered through the NPs was able to block the anhedonic effect induced by the depression model (Fig. 1D).

4.4. *Tail suspension test*

Regarding the tail suspension test, the results showed that the CORT regimen induced a depressive-like behaviour due to increases in the immobility time in PBS-treated animals. Regarding the effect of i.n. treatments, none of them altered the immobility time in control animals, however, in CORT animals VLF-loaded NPs significantly decreased the immobility time and restored the depressive-like behavior induced by the model. As in SPT, the dose employed of VLF free drug was not able to restore the depressive-like behaviour.

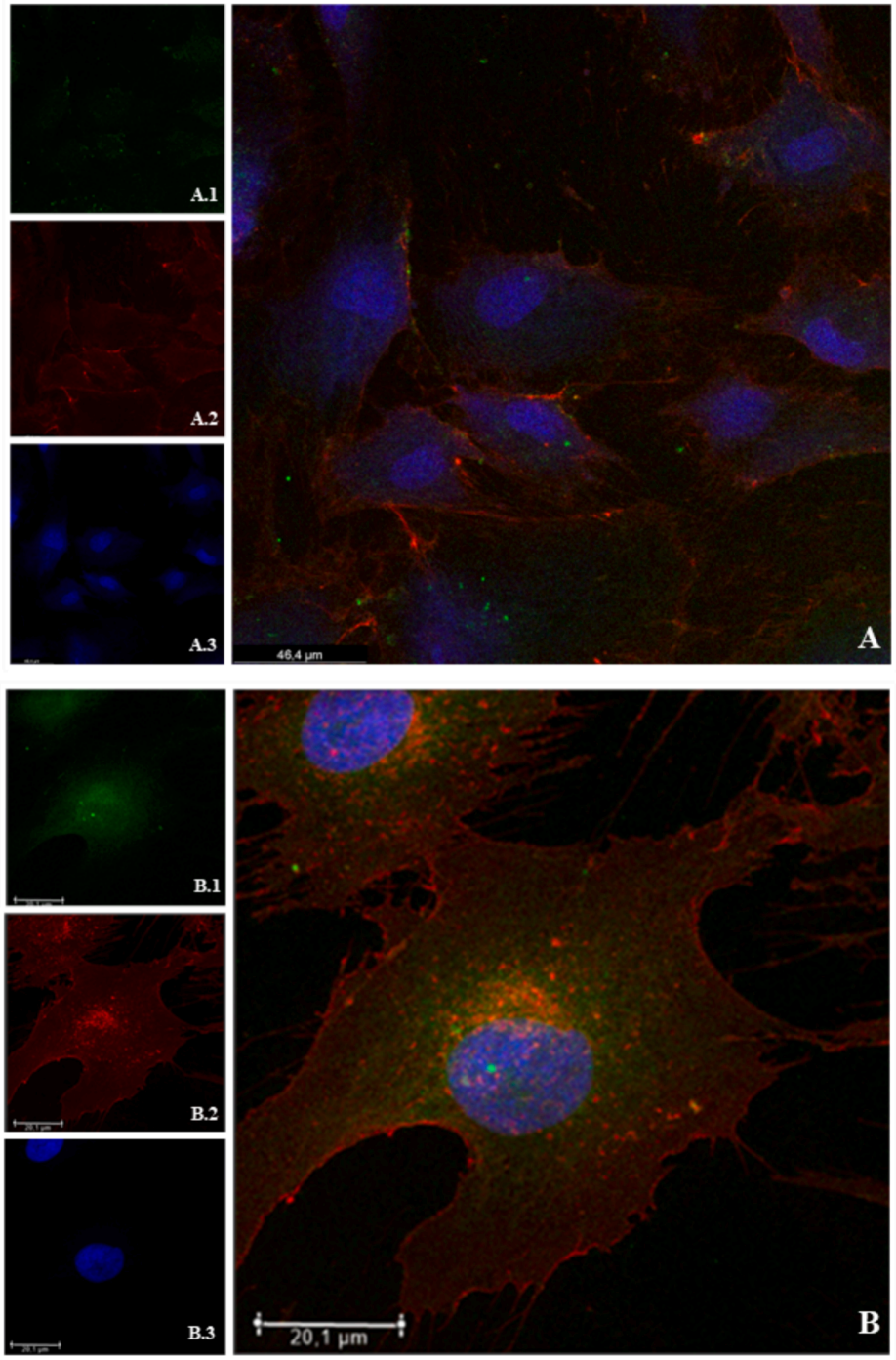


Fig. 3. Merged confocal microscopy images of ONE cells after 60 min (A) and 120 min (B) of incubation with FITC-PLGA NPs (250 µg/mL). Representative immunofluorescence images in ONE cell for FITC-PLGA NPs in green (A1, B1), cytoplasm marked in red with WGA-Alexa Fluor ® (A2, B2), nuclei stained in blue with Hoechst® (A3, B3) and merged (A and B) in ONE cells using 20x objective for A and 60x objective for B.

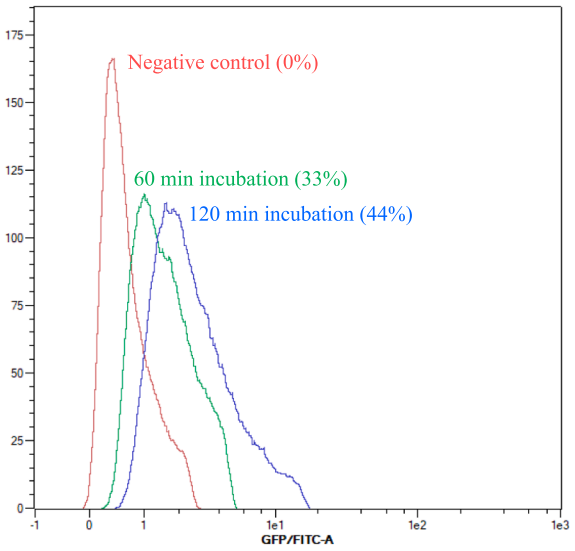
5. Discussion

5.1. Nps production and physicochemical characterization

VLF is a dual action antidepressant which presents a short half-life, narrow absorption window and remarkable hepatic first-pass metabolism, which leads to poor oral bioavailability (Li et al., 2021). In the conventional oral therapy, these disadvantages lead to frequent administration to maintain the therapeutic levels and restricted access to the brain due to the presence of the BBB. Therefore, the encapsulation of the antidepressant in polymeric NPs could improve the current oral therapy compared to traditional dosage forms becoming a promising formulation for depression treatment.

Taking into account the solubility of the polymer (hydrophobic) and the active (water-soluble drug), NPs were prepared by double emulsion-evaporation method (Ding et al., 2019; Iqbal et al., 2015) using PLGA, a polymer that is approved for human use by FDA and EMA. The type of nanoparticle to employ in the *in vivo* studies was based on the results obtained in previous work, as mentioned above (Cayero-Otero et al., 2019).

For VLF NPS, results obtained by Malvern Zetasizer showed a low PdI (<0.4) indicated the homogeneity of the population and the



Path	%-T
Cells\Singlets\Control\Control	46,24
Cells\Singlets\FITC-PLGA NPs\60 min	78,91
Cells\Singlets\PLGA-FITC NPs \120 min	90,55

Fig. 4. Flow cytometry analysis of internalization evaluation of FITC-PLGA NPs after 60 min (green) and 120 min (blue) at 250 μg/mL of concentration. Negative control is illustrated in red.

aerodynamic diameter not exceeding 200 nm. This particle size, according to the literature, is the limit for an efficient brain targeting efficiency through olfactory region (Tong et al., 2017). Then, a low PdI is essential to ensure a monodisperse population because size is a critical factor for access to the CNS by the intranasal route, determining the mechanism of entry into the brain (Bonaccorso et al., 2017).

FITC-PLGA NPs exhibited a slightly larger but compatible size (around 270 nm) for their use in internalization studies with a highly homogeneous population.

Regarding the zeta potential, negative values of all formulations indicated a stable system without aggregation and easy redispersion and the absence of PVA on the surface of nanosystems (Zambaux et al., 1998). It is essential to remove the PVA residues to ensure a sufficient negative charge and prevent aggregation. Zeta potential also influences both nanoparticle stability in biological fluids and the risk of immune clearance and longer circulation times in the bloodstream. This is mainly due to their reduced interaction with plasma proteins and decreased uptake by the mononuclear phagocyte system (MPS) (Blanco et al., 2015). Authors previously demonstrated an accumulation of less than 5 % of nanoparticles in spleen or liver, in an *in vivo* biodistribution assays, indicating minimal clearance.

Finally, EE% and DL% values obtained by DE-SEV method were remarkably reproducible, with more than 50 % of initial amount encapsulated into the NPs suggesting the particles production method was optimal.

5.2. *In vitro MTT and internalization studies*

As mentioned above, although the mechanism of nose-to-brain route is not completely elucidated (Yokel, 2022), most studies suggest that there are several possible routes may be implicated (Borrajo et al., 2022) in the nose-to-brain pathway and it is well-known that trigeminal and olfactory nerves are involved (intracellular and extracellular pathway) (Giunchedi et al., 2020)(Kumar et al., 2014). Therefore, the study of cellular uptake of PLGA NPs can be a useful tool to determine the ability of them to reach the brain across the olfactory cells in future *in vivo* experiments.

In the *in vitro* experiments, cell uptake assays (qualitative and quantitative) of fluorescent NPs (FITC-PLGA) and cytotoxicity test of formulations were evaluated after the incubation with olfactory cells.

On the one hand, cell viability analysis indicated that incubation with the NPs assayed did not result in significant cytotoxicity, as cell viability remained above 85 %. According to the ISO 10993–5:2009 guidelines, these results suggest that the tested formulations are non-toxic within the studied concentration range. On the other hand, in order to establish a correlation between *in vivo* and *in vitro* assessments and elucidate the access of the fluorescent NPs to the brain, we studied the internalization of NPs in ONE cells for 60 and 120 min. Our results showed that fluorescent NPs can interact with olfactory cells, suggesting that NPs could cross the cells in nose-to-brain route in case of intracellular pathway. The quantitative uptake studies of NPs, confirm the results obtained for qualitative analysis. These results are in concordance with recent literature. It is believed that particles with sizes greater than 100 nm could access the brain through the intracellular route, which would reinforce the need of efficient cellular internalization (Clementino et al., 2021)(Chen et al., 2024).

5.3. *Antidepressant-like effect*

In the present study, we use a depressive model in rodents based on the chronic administration of CORT. The exposure to exogenous CORT produces a persistent depression-like state sensitive to antidepressant treatment in rodents. This behavioral phenotype is accompanied by brain changes inherent to depressive-like states, which overall makes this model an appropriate tool to assess the antidepressant-like effect of novel drugs (Gourley and Taylor, 2009)(Zhao et al., 2008). First, we controlled the proper induction of this animal model by the evaluation of the food and drinking consumption during the entire protocol. In agreement with previous findings, the chronic administration of CORT induces an increase in liquid and food consumption (David et al., 2009; Ferrés-Coy et al., 2015). Additionally, at the end of the protocol we demonstrated the depressive-like state in CORT–treated animals as measured by the decrease in the sucrose preference as well as with the increase in the immobility time in the TST.

To evaluate the behavioral effect of the different intranasal treatments, we evaluate the animals in the OF, the sucrose preference test

and the TST. Spontaneous locomotor activity assessed in the OF is a sensitive measure in rodents of sedative and stimulant effects induced by drugs. Our results showed that neither VLF-loaded NPs nor VLF-free drug produced any effect on the spontaneous activity. Indeed, previous studies demonstrated that acute high doses of VLF produced an increase in locomotor activity while chronic administration did not affect or even induce a sedative effect measured in the OF (Redrobe et al., 1998) (Karlsson et al., 2011), (Zhang et al., 2022). The low potential for stimulant or sedative effects after VLF-loaded NPs administration suggests a potentially favorable side-effect profile of this compound. In addition, these results rule out the possibility that basal activity levels are confounding the results obtained in other tests, such as TST.

Anhedonia is one of the core symptoms of depression. In the present study, we use the SPT, which is one of the most common tests for assessing anhedonia in rodents. Our results showed that VLF-loaded NPs restored the deficit found in CORT–treated animals, while VLF-free drug was not able to modify the sucrose preference. Similar results were obtained in the total time spent immobile scored as a measure of behavioral despair. In the TST, only the administration of VLF-loaded NPs was able to significantly reduce the immobility time in the animals submitted to the depressive model.

The absence of antidepressant-like response found after VLF free drug administration in the behavioral assessment was expected. Based on previous studies, the dose selected in our study is too low to produce a solid antidepressant effect (Coutens et al., 2022). As observed in clinical settings, it typically takes over two weeks of antidepressant administration to achieve a significant effect capable of reversing the depressive phenotype induced by an animal model (David et al., 2009; Coutens et al., 2022) . However, our results show that the same dose and regimen of administration (1 week) of intranasal VLF-loaded NPs can produce a clear antidepressant-like response. Thus, our results suggest that the capsulation of VLF in PLGA NPs potentiates the antidepressant-like effect of this drug and may also be a promising strategy to reduce the onset of action of this drug, although further research is needed.

Although we cannot determine the precise mechanism by which NPs improve the pharmacological profile of VLF *in vivo*, one possibility is that this formulation offers a long-acting controlled release. We propose that NPs-based drug delivery systems can potentially overcome the effects of P-glycoprotein (P-gP) in the BBB by achieving a slow release of VLF. P-gP, an ATP dependent drug transport protein, acts as an active efflux pump that removes substances from the brain. VLF has been shown to induce P-gP at certain concentrations (1–50 μM) (Bachmeier et al., 2011). In a previous study conducted by our team, we compared the effects of increasing concentrations of free VLF versus VLF-NPs at same dose on the basolateral side in an *in vitro* BBB model. Our results indicated that the gradual release of VLF from NPs to the basolateral side (drug concentration < 0.25 μM) did not reach levels sufficient to induce P-gP (Cayero-Otero et al., 2019). This observation aligns with previous reports suggesting that brain concentrations and the behavioral effects induced by chronic VLF administration are influenced by the activity of P-gP (Karlsson et al., 2011). Therefore, these data highlight the potential of NPs-based drug delivery systems to mitigate the impact of P-gP on VLF pharmacokinetics at the BBB. By achieving a controlled and gradual release of VLF, these NPs effectively maintain drug concentration below the threshold known to induce P-gP activity, as demonstrated in our *in vitro* BBB model. Additionally, nanoparticles cause not only extension in the retention time it also increases the cellular uptake. Furthermore, in addition to its effects at the BBB level, it is conceivable that VLF encapsulated in PLGA NPs alters its pharmacological activity at the site of action. Recent studies suggest that the onset of the antidepressant effect of monoaminergic reuptake inhibitors is mediated by their affinity for the transmembrane domains of TrkB receptors (Casarotto et al., 2021). TrkB is a receptor tyrosine kinase for brain-derived neurotrophic factor (BDNF), and the role of BDNF in mediating antidepressant-like behavioral responses has been well-established (Castrén and Monteggia, 2021; Neto et al., 2011). Recent evidence indicates that direct binding of antidepressant drugs to TrkB receptors promotes BDNF signaling, potentially mediating the onset of antidepressant responses to these compounds (Enkavi et al., 2024). This concept may explain why typical antidepressants act slowly; they gradually accumulate in the brain, achieving a plateau after several weeks of treatment (Karson et al., 2006; Kornhuber et al., 1995). This suggests that clinical response occurs only when drug concentrations in the brain are sufficiently high to interact with low-affinity binding targets such as TrkB. Therefore, the enhanced cellular uptake of VLF facilitated by NPs could be crucial in increasing its binding to TrkB transmembrane domains, potentially explaining the enhanced potency of the antidepressant effect observed with VLF-loaded NPs *in vivo*.

Overall, we hypothesized that the non-invasive nose-to-brain delivery of our formulation would improve the antidepressant action of VLF and be as effective as a chronic treatment of a higher dose of VLF.

6. Conclusions

PLGA NPs containing VLF could be a potential platform for the treatment of neurological disorders, specifically for the depression. A previous biodistribution study developed by authors, established the presence of this type of plain-PLGA NPs in the brain after intranasal administration and, in this work, *in vivo* behavioural assays have confirmed the access of NPs to brain. The NPs prepared in the experiments showed a size which not exceeding 200 nm, an essential requirement to reach the brain by olfactory region according to the literature. Moreover, in accordance with ISO guidelines, the prepared PLGA-NPs nanoparticles did not exhibit cytotoxicity at the concentrations tested in ONE cells.

From the *in vivo* studies, we can conclude that VLF-loaded NPs evoke very fast (1 week) and robust antidepressant-like effects in CORT-treated mice. Thus, the use of nanoparticle formulation could overcome some of the limitations of antidepressant treatments such as the induction of adverse effects of high doses of VLF or the slow onset of action, improving compliance and the outcome.

Further *in vivo* studies in animal models are necessary to validate these findings and confirm their real therapeutic potential, as well as the mechanisms of the NPs that enabled a successful access to the brain, are warranted to provide additional information for further potential optimization of the formulation, as well as for the development of alternative related neurological therapies. Also, preclinical testing to assess the safety of these NPs, including their long-term effects and cytotoxicity, immunogenicity, and potential for bioaccumulation will be necessary.

Overall, our results highlight the potential use of plain PLGA NPs as a novel therapy and, particularly, point to nose-to-brain administration of plain PLGA NPs formulations as feasible and promising antidepressant non-invasive therapy.

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CRediT authorship contribution statement

M. Dolores Cayero-Otero: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Laura Perez-Caballero:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Irene Suarez-Pereira:** Writing – original draft, Investigation, Formal analysis, Data curation. **María Hidalgo-Figueroa:** Methodology, Investigation, Formal analysis, Data curation. **Alejandra Delgado-Sequera:** Investigation, Formal analysis, Data curation. **Juan Manuel Montesinos:** Methodology, Investigation. **Esther Berrocoso:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lucía Martín-Banderas:** Writing – review & editing, Writing – original draft, Visualization, Validation,

Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2025.125692.

Data availability

Data will be made available on request.

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