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Nose-to-brain delivery of perampanel formulated in a self-microemulsifying drug delivery system improves anticonvulsant and anxiolytic activity in mice

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ABSTRACT

Perampanel (PER) is a potent third-generation antiepileptic drug only available for oral administration. Additionally, PER has shown potential in managing epilepsy comorbidities such as anxiety. Previously, we demonstrated that the intranasal (IN) administration of PER, loaded in a self-microemulsifying drug delivery system (SMEDDS), improved brain-targeting and exposure in mice. Herein, we investigated PER brain biodistribution, its anticonvulsant and anxiolytic effects, and its potential olfactory and neuromotor toxicity after IN administration to mice (1 mg/kg). PER showed a rostral-caudal brain biodistribution pattern when administered intranasally. At short times post-nasal dosing, high PER concentrations were found in olfactory bulbs (olfactory bulbs/plasma ratios of 1.266 ± 0.183 and 0.181 ± 0.027 after IN and intravenous administrations, respectively), suggesting that a fraction of the drug directly reaches brain through the olfactory pathway. In the maximal electroshock seizure test, IN PER protected 60% of mice against seizure development, a substantially higher value than the 20% protected after receiving oral PER. PER also demonstrated anxiolytic effects in open field and elevated plus maze tests. Buried food-seeking test showed no signs of olfactory toxicity. Neuromotor impairment was found in rotarod and open field tests at the times of PER maximum concentrations after IN and oral administrations. Nevertheless, neuromotor performance was improved after repeated administrations. Compared with IN vehicle, PER IN administration decreased brain levels of L-glutamate (0.91 ± 0.13 mg/mL *vs* 0.64 ± 0.12 mg/mL) and nitric oxide (100 ± 15.62% *vs* 56.62 ± 4.95%), without interfering in GABA levels. Altogether, these results suggest that the IN PER delivery through the developed SMEDDS can be a safe and promising alternative to the oral treatment, which supports the design of clinical studies to evaluate the IN PER delivery to treat epilepsy and neurological-related conditions as anxiety.

Abbreviations: AEDs, antiepileptic drugs; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BBB, Blood-brain barrier; C_{max}, maximum concentration; CNS, central nervous system; CSF, cerebrospinal fluid; DTE, drug targeting efficiency; DTOA, distance traveled in open arms; DTP, direct transport percentage; DZP, diazepam; GABA, gamma-aminobutyric acid; HPLC, high-performance liquid chromatography; IN, intranasal; INT, iodonitrotetrazolium chloride; IP, intraperitoneal; IV, intravenous; MES, maximal electroshock seizure; NADH, reduced nicotinamide-adenine dinucleotide; PEOA, percentage of entries in open arms; PER, perampanel; PTOA, percentage of time spent in open arms; SMEDDS, self-microemulsifying drug delivery system; t_{max}, time to reach maximum concentration.

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1. Introduction

Epilepsy is one of the most prevalent neurological diseases worldwide (World Health Organization, 2019). In addition to high mortality, patients with uncontrolled epilepsy experience a decrease in their quality of life and can develop a number of other central nervous system (CNS) disorders (Keezer et al., 2016; Łukawski and Czuczwar, 2021), such as depression and anxiety (Gonzalez-Martinez et al., 2022; Johnstone et al., 2021; Rauh et al., 2022). Therefore, it is imperative to find safer and more effective pharmacological alternatives capable of treating epilepsy as the primary disorder and the associated conditions (Łukawski and Czuczwar, 2021).

Currently, the chronic administration of antiepileptic drugs remains the main therapeutic option for epileptic seizures control (Jacob and Nair, 2016). However, 30% of the epilepsy patients continue to present uncontrolled seizures and/or unacceptable drug side effects (Łukawski and Czuczwar, 2021; Patsalos, 2015). This is mostly associated to the burden of pharmacoresistant epilepsy, in which patients develop recurrent seizures despite the use of the most appropriate currently available antiepileptic drugs (AEDs) (Łukawski and Czuczwar, 2021; Mesraoua et al., 2019). Thus, in order to improve the treatment and prognosis of patients with drug-resistant epilepsy, the investigation of new and more effective drugs is still ongoing (Bialer et al., 2022a, 2022b). In parallel, there is also interest in searching for improved strategies that allow an increase in the brain-targeting of clinically established AEDs. Among these alternative strategies, intranasal (IN) administration stands out (Costa et al., 2019; Kapoor et al., 2016; Pandey et al., 2022).

In addition of being safe, non-invasive, easy to handle and, therefore, more patient-friendly than other administration approaches [e.g., rectal, intravenous (IV)], the IN route also allows drug molecules to bypass the blood–brain barrier (BBB) (Costa et al., 2021; Keller et al., 2021; Pires and Santos, 2018). This is mainly related with the nasal cavity being the only anatomical area that directly connects the brain to the external environment, allowing a partial direct nose-to-brain delivery of drugs (Crowe et al., 2018; Keller et al., 2021; Meirinho et al., 2022b). Additionally, the proportion of a drug that is not directly transported from nose-to-brain and that is instead absorbed through the nasal mucosa into the systemic circulation, also avoids the gastrointestinal and hepatic first-pass effect, increasing the amount of drug systemically available to reach the brain (Crowe et al., 2018; Kapoor et al., 2016; Meirinho et al., 2022b). Hence, the IN doses required to attain therapeutic brain concentrations can be 2- to 10-fold lower than the oral ones, which allows to reach therapeutic effects with less peripheral adverse drug reactions and drug interactions (Bonferoni et al., 2019). Nevertheless, since the capacity of the human nasal cavity is very restricted, it only enables the administration of small dose volumes (25–250 μL) (Bonferoni et al., 2019; Froelich et al., 2021; Kapoor et al., 2016).

To increase drug strength in small volumes, the use of alternative and improved formulation strategies, such as a self-microemulsifying drug delivery system (SMEDDS), can be a promising option (Gupta et al., 2013; Meirinho et al., 2022b; Rajpoot et al., 2019). This type of system is able to maintain highly lipophilic drugs in a solubilized state even after contact with aqueous environments (e.g., nasal mucous). This happens due to the formation of an *in situ* microemulsion, with drugs being immediately entrapped into small lipidic droplets (sizes below 100 nm) (Meirinho et al., 2022b). By encapsulation into these oil droplets, labile molecules can also be protected from chemical and enzymatic degradation. Furthermore, the formed small droplets also generate a large interfacial surface that promotes the partitioning of drug molecules from the oil phase to the cell membranes interface, improving their local absorption and, consequently, their brain bioavailability (Buya et al., 2020; Meirinho et al., 2022b).

Even considering the aforementioned advantages of SMEDDS, IN administration still requires relatively potent drugs to be effective in reaching the intended purposes. In this context, perampanel (PER), a potent third-generation AED (European Medicines Agency, 2022; Patsalos, 2015), stands out as the ideal drug, being for that an attractive choice to be formulated for IN administration. Besides its potency, PER acts through a unique mechanism of action: it is a selective, non-competitive antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors, which enables the reduction of seizures and prevents glutamate-induced neurotoxicity (European Medicines Agency, 2022; Patsalos, 2015). So, PER can be a promising alternative to control other types of seizures beyond those for which it is currently approved. In fact, its therapeutic efficacy has already been demonstrated in cases of pharmacoresistant epilepsy (Chang et al., 2020; Ikemoto et al., 2019) and in *status epilepticus* after PER nasogastric delivery (Newey et al., 2019; Rahbani et al., 2019; Strzelczyk et al., 2019). Additionally, a phase I clinical study has recently demonstrated that an IV solution of PER may be an interchangeable alternative to oral PER in treating status *epilepticus* (Hussein et al., 2022). However, similarly to nasogastric administration, the IV route is also an invasive and difficult approach to be implemented in an emergency condition, a situation that can be overcome by using an IN formulation of PER.

Considering the anxiolytic action of several AEDs (Mula et al., 2007; Van Ameringen et al., 2004), it could also be possible that the PER unique mechanism of action leads to anxiolytic effects. Actually, Bektas *et al.* (2020) study revealed that the intraperitoneal (IP) administration of PER resulted in good pre-clinical evidence in treating anxiety. In fact, the use of PER in anxiety treatment can be of great advantage since PER is not associated with addiction, respiratory depression and hypotension risks, contrary to what occurs with the administration of benzodiazepines, the current gold standard treatment for anxiety and *status epilepticus* (American Epilepsy Society, 2016; Der-Nigoghossian et al., 2019). Additionally, regardless of the clinical conditions, the use of PER might be potentiated by administrating it through the IN route, possibly allowing an increase in PER brain bioavailability using lower doses.

Having this in mind, we previously developed a SMEDDS, loaded with 6 mg/mL of PER, for IN administration (Meirinho et al., 2022a). Before performing the *in vivo* studies, the formulations, containing or not PER, were fully characterized, showing promising features in terms of droplet size upon dilution, pH, viscosity, and *in vitro* PER release. Then, PER was intranasally delivered to mice (1 mg/kg) using a small dose volume of 5 μL/30 g body weight. The maximum concentrations (C_{max}) of PER in plasma and brain were considerably higher and were achieved significantly faster with the IN administration compared with the oral route [i.e., the time to reach maximum concentrations (t_{max}) of PER was 15 min *vs* 120 min, respectively]. After IN administration, total exposure of PER in plasma and brain was also significantly higher, with brain-to-plasma ratios obtained from IN PER being higher at all time points relative to oral dosing. At least until 4 h post-IN dose, plasma concentrations of PER in mice were maintained within the therapeutic range assumed as a reference for humans (0.1 – 1 μg/mL), which did not occur after oral administration (Meirinho et al., 2022a, 2021; Reimers and Berg, 2018). Another important finding of our previous study was the absence of histopathological toxicity of the developed SMEDDS, both systemically and locally in the nasal epithelium (Meirinho et al., 2022a).

Thus, we hypothesized that the previously developed SMEDDS (encoded as FH5) loading PER at 6 mg/mL could be therapeutically effective, which led us to explore this formulation with PER in the current study. Firstly, the biodistribution of PER in different anatomical brain sections was characterized after its IN administration to mice. In a second phase, we investigated the potential of IN PER to cause olfactory and neuromotor toxicity. At a later stage, we explored whether or not IN administration of PER could be superior to the oral route in preventing the occurrence of electrically-induced acute seizures in mice. Additionally, since administration of PER already demonstrated *in vivo* anxiolytic effects, we further tested if the IN administration of PER could also be beneficial for this purpose. A neurochemical evaluation was also performed in order to support the obtained pharmacodynamic results.

2. Materials and methods

2.1. Materials

PER (99.9% purity) was a gift sample from MSN Laboratories Ltd. (Hyderabad, India). Fycompa® 0.5 mg/mL oral suspension (Eisai GmbH, Germany) and diazepam (DZP) 10 mg/2 mL injectable solution (Labesfal, Portugal) were commercially obtained from a local pharmacy (Covilhã, Portugal). Terbinafine hydrochloride (99.5% purity), used as internal standard during the bioanalysis, was gently provided by Bluepharma (Coimbra, Portugal). Pentobarbital sodium injection solution (Eutasil®) and the isoflurane solution used for inhaled anaesthesia (Ecuphar IsoFlo®) were purchased from Ceva (Libourne, France). A sample of diethylene glycol monoethyl ether (Transcutol® HP) was kindly supplied by Gattefossé (Saint-Piest, France) and samples of macrogolglycerol hydroxystearate (Kolliphor® RH 40) were donated by BASF Europe. Triglycerides medium-chain (Miglyol® 812) and propylene glycol were obtained from Acofarma (Barcelona, Spain). Acetonitrile (HPLC grade), analytical grade triethylamine, 85% *ortho*-phosphoric acid, sodium hydrogen carbonate and sodium bicarbonate were all acquired from Fisher Scientific (Leicestershire, United Kingdom). Isopropanol (98% purity) and absolute ethanol (99.8% purity) were purchased from Honeywell Riedel-de Haën™ (Seelze, Germany); potassium hydroxide (KOH) from Fisher Scientific (Leicestershire, UK); monobasic and dibasic sodium phosphate from Acros Organics (Geel, Belgium); zinc sulphate ($ZnSO_4$) heptahydrate from VWR (Leuven, Belgium); magnesium sulphate ($MgSO_4$) and trichloroacetic acid from Panreac (Barcelona, Spain); *N*–(1–naphthyl)–ethylenediamine dihydrochloride and sulfanilamide from Alfa Aesar (Karlsruhe, Germany); copper(II) sulphate anhydrous from Scharlau (Istanbul, Turkey); ninhydrin and D-tartaric acid from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride solution (NaCl) 0.9% was obtained from B. Braun Medical (Queluz de Baixo, Portugal). Ultra-pure water was obtained from a Milli-Q water apparatus, 0.22 μm filter, of Merck (Darmstadt, Germany).

2.2. Experimental animals

Healthy adult male CD-1 mice were obtained from local certified facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). All mice aged between 8 and 10 weeks and had weights from 30 to 45 g. Animals were housed under controlled environment conditions (12/12 h light–dark cycles, temperature at 20 ± 2 °C and relative humidity of 50 ± 5%), receiving sterile tap water and standard rodent diet *ad libitum* (4RF21, Mucedola, Italy). The animal experimental procedures were approved by the local Animal Welfare and Ethical Review Body, in agreement with the regulations of the European Directive 2010/63/EU (European Parliament and Council of the European Union, 2010).

2.3. Preparation of intranasal perampanel and comparative treatments

The SMEDDS loading PER, for now on designated as FH5 + PER, was prepared as reported in Meirinho *et al.* (2022a). Miglyol® 812 (10%), Kolliphor® RH40 (40%) and Transcutol HP (40%) were weighted and mixed in the defined proportions. Then, an appropriate amount of PER was dissolved in the anhydrous preconcentrate by vortex-mixing and ultrasonic dissolution. Water (10%) was finally added dropwise, and the formulation was homogenized by gentle inversion and stored at room temperature, protected from light, until used. The final concentration of PER was 6 mg/mL, allowing an IN administration of 1 mg/kg using a volume of 5 μL/30 g of mouse body weight (Meirinho et al., 2022a).

The IV administration of PER was used as comparative treatment in the brain biodistribution study. For that, a clear solution of PER was prepared by dissolving it in a mixture of propylene glycol/NaCl 0.9%/ethanol (50:30:20, v/v/v) to reach a final drug concentration of 0.25 mg/mL. This allowed an IV administration of 0.5 mg/kg by a slow injection (approximately for 1 min) in mice tail veins of a volume of 60 μL/30 g mouse body weight (Meirinho et al., 2022a).

For oral administration of PER, used as comparative treatment during the pharmacodynamic studies, Fycompa® 0.5 mg/mL oral suspension was 5-fold diluted with ultra-pure water to reach a final PER concentration of 0.1 mg/mL. This allowed an administration dose of 1 mg/kg in a volume of 300 μL/30 g mouse body weight using an adequate gavage feeding tube coupled to a syringe (Meirinho et al., 2022a).

IP injection of DZP, a very well-known anxiolytic drug here used as positive control in anxiety behavioural tests, was prepared by diluting 16.7-fold the commercial injectable solution (diazepam Labesfal® 10 mg/2 mL) in NaCl 0.9%. The final concentration was 0.3 mg/mL, allowing to administer 1 mg/kg using a volume of 90 μL/30 g mouse body weight (Bektas et al., 2020; Fan et al., 2019; Lin et al., 2021; Tabari and Tehrani, 2017).

A solution of $ZnSO_4$, used as positive olfactory impairment control in the buried food-seeking test, was prepared at a concentration of 10% (w/v) by diluting an appropriate amount of $ZnSO_4$ heptahydrate in ultra-pure water. The solution was intranasally administered to mice at a volume of 5 μL/30 g mouse body weight.

2.4. In vivo pharmacokinetics of perampanel – A brain biodistribution study

The brain biodistribution study was carried in male CD-1 mice randomly divided into two experimental groups: the first group received a single IN dose of FH5 + PER (1 mg/kg), and the second one received a single dose of a slow IV tail vein injection of PER (0.5 mg/kg) (Fig. 1). Before proceeding to IN or IV administrations, each mouse was anesthetized with pentobarbital (60 mg/kg) through IP injection and kept in a warm environment to avoid hypothermia. At pre-determined times after PER administration (i.e., 5, 15, 30 and 60 min; $n = 4$ per time-point), mice were again subjected to anesthesia and cardiac puncture was used to collect blood into 1 mL tubes containing K_3-EDTA (BD Vacutainer®). Immediately after the blood collection, brain was collected and gently washed using a 0.9% NaCl solution. After it, each excised brain was placed on an ice block to be easily dissected into olfactory bulbs, frontal cortex, cerebellum, and remaining part of the brain (Fig. 1). Blood samples were centrifuged at 3351 *g* for 10 min at 4 °C to obtain plasma samples. The different brain sections were homogenized in a 1 M sodium phosphate buffer pH 5 (4 mL/g tissue) using an Ultra-Turrax tissue homogenizer (Ika Ultra-Turrax® T25 Basic, Staufen, Germany). The homogenates were then centrifuged at 17,350 *g* for 15 min at 4 °C. Both plasma and homogenate supernatants were stored at −20 °C, protected from light, until analysis.

The determination of PER concentrations in plasma and homogenate supernatants of each brain section were performed using a fully validated high-performance liquid chromatography (HPLC) method previously developed by our research group (Meirinho et al., 2020). Briefly, 100 μL of biological sample were spiked with 20 μL of terbinafine (100 μg/mL). Then, 200 μL of isopropanol followed by 100 μL of 1 M $MgSO_4$ were added to induce a salting-out effect. The mixture was then vortex-mixed for 1 min, centrifuged at 12,300 *g* for 3 min, and the upper organic layer was transferred to a glass tube to be further evaporated under gentle nitrogen stream (45 °C). The obtained dried sample was reconstituted using 200 μL of mobile phase, centrifuged for another 3 min at 12,300 *g* and 20 μL of the remaining supernatant was injected into the chromatographic system.

PER analysis was performed using a HPLC system (Shimadzu Corporation, Japan) equipped with a DGU-20A5R automatic degasser, a LC-20AD quaternary solvent pump, a CTO-10AS VP column oven, a SIL-20ACHT refrigerated automatic injector and a RF-20AXS fluorescence detector. Data acquisition and instrumentation were controlled using the LabSolutions software (Shimadzu Corporation, Japan). The separation of PER and internal standard was accomplished using a reversed-

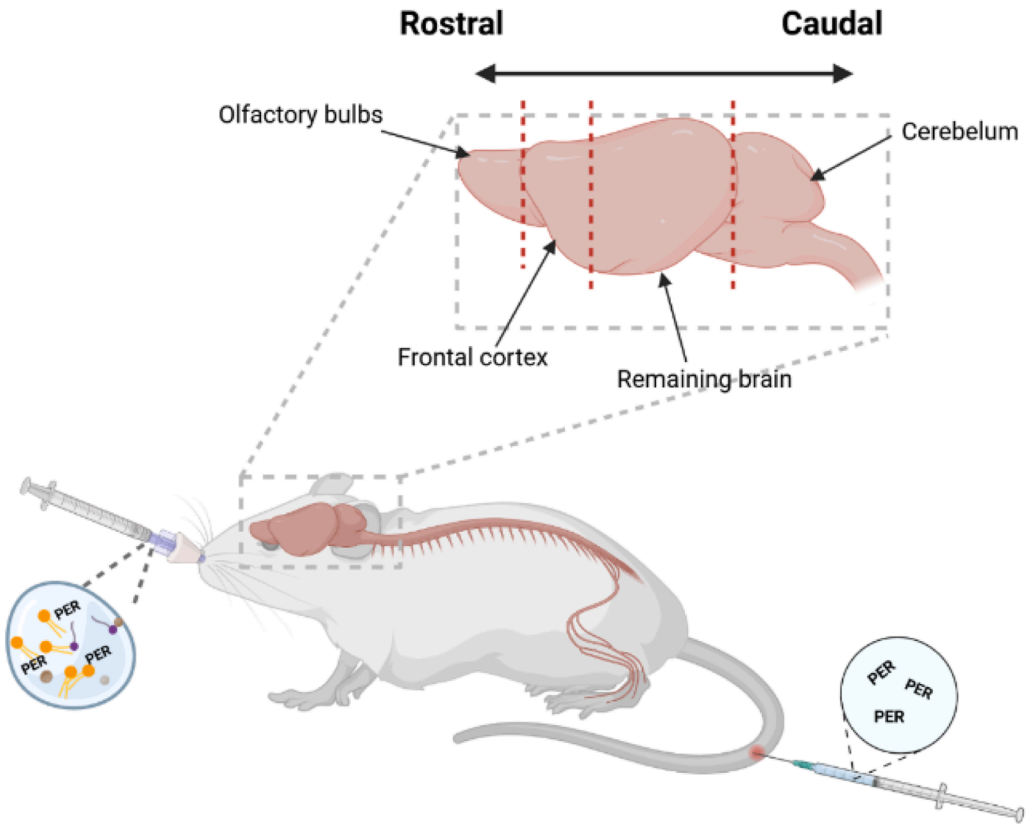

Fig. 1. Schematic representation of *in vivo* perampanel (PER) biodistribution study in different parts of the brain (olfactory bulb, frontal cortex, cerebellum, and remaining part of the brain). The study was performed after a single administration of intranasal (IN) FH5 + PER at a dose of 1 mg/kg, and after the intravenous (IV) administration of a PER solution at a dose of 0.5 mg/kg.

phase LiChroCART® Purospher Star column (C_{18} 55 mm × 4 mm; 3 µm), protected by a LiChroCART® Purospher Star precolumn (C_{18} 4 mm × 4 mm; 5 µm) (Merck, Darmstadt, Germany), both maintained at 35 °C. The mobile phase [ultrapure water containing 1% (v/v) triethylamine at pH 2.5 adjusted with *ortho*-phosphoric acid/acetonitrile (53:47, v/v)] was flushed at 1 mL/min. Terbinafine was detected at 254/372 nm while PER at 275/430 nm, with retention times of 2 and 3 min, respectively.

To compare the brain distribution pattern of PER after IN and IV administrations, the concentration of PER was determined in each section of the brain and in plasma after specific times post-dosing, being then graphically plotted. At each time after administration, comparisons between PER concentrations in plasma and in each section of the brain, and comparisons of PER concentrations between olfactory bulbs, frontal cortex, cerebellum and remaining part of the brain were performed by a two-way ANOVA with Dunnett's post-hoc test. Tissue-to-plasma concentration ratios of PER obtained at each time post-dosing in the groups that received IN and IV administrations were then calculated and compared by a two-way ANOVA analysis with Sidak's post-hoc test. Statistically significant differences were considered for a *p*-value lower than 0.05 ($p < 0.05$).

2.5. *In vivo pharmacodynamic evaluation of perampanel*

After IN administration, several pharmacodynamic effects of PER were evaluated. Since PER is a clinically well-established AED (European Medicines Agency, 2022), the main purpose was to assess if the IN administration of PER resulted in a higher anticonvulsant activity in an animal model of acute seizures – the maximal electroshock seizure (MES) test - than its oral administration, the only pharmaceutical form currently available (European Medicines Agency, 2016). Since the focus of the present work was to evaluate the viability of PER delivery by nasal route, it was also highly important to evaluate if the developed SMEDDS, loading or not PER, could cause any olfactory toxicity. Furthermore, since the most frequently identified adverse effects following PER administration are dizziness, somnolence and fatigue (European Medicines Agency, 2022; Patsalos, 2015), our focus was also to study if intranasally delivered PER could compromise mice neuromotor behaviours such as locomotion and coordination. Finally, since it is already reported that PER can also be beneficial in anxiety treatment (Bektas et al., 2020), we also investigated the anxiolytic potential of IN PER in *in vivo* anxiety models.

A schematic representation of all the performed pharmacodynamic tests is given in Fig. 2. All the IN administrations were performed within 1 min after induction of anaesthesia using isoflurane at 3.5% (v/v) combined with an oxygen flow of 800 mL/min. The defined post-dose assessment times were based on previously determined pharmacokinetics profiles (i.e., 15 min and 2 h, which are the t_{max} of PER after IN and oral administrations, respectively; and 4 h is a time point in which the PER concentrations obtained in mice after IN administration are still within the human therapeutic range) (Meirinho et al., 2022a, 2021; Reimers and Berg, 2018).

2.5.1. *Anticonvulsant activity evaluation*

The anticonvulsant activity of PER was evaluated by the MES test following all procedures of the Anticonvulsant Screening Program approved by the National Institute of Neurological Disorders and Stroke (Rockville, USA) (National Institute of Neurological Disorders and and Stroke, 2016). MES is a model of generalized tonic-clonic seizures and allows to evaluate if a compound is able to prevent seizure spread when all the neurological circuits are maximally active (Barker-Haliski et al., 2018; National Institute of Neurological Disorders and and Stroke, 2016).

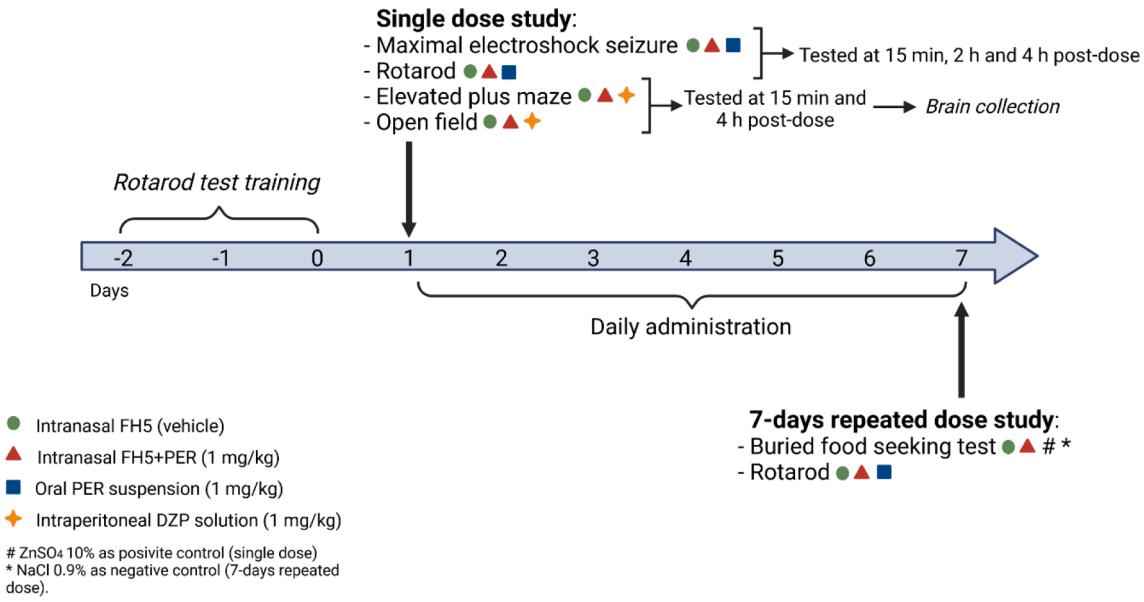

Fig. 2. Schematic representation of *in vivo* pharmacodynamic studies performed in different mice groups after receiving all different treatments under study, in a single dose or in a repeated dose regimen. DZP, diazepam; FH5, self-microemulsifying drug delivery system developed for perampanel intranasal administration; PER, perampanel.

The anticonvulsant activity of PER was evaluated after 15 min, 2 h and 4 h of a single IN and oral administration of PER (1 mg/kg), and in a control group that received the IN vehicle FH5 ($n = 5$ per time point).

To perform the MES test, each animal received an electrical stimulus of 60 Hz (50 mA) delivered during 0.2 s through auricular electrodes connected to an electroconvulsometer (ECT Unit, Ugo Basile, Varese, Italy). Prior to stimulation, electrodes and mice ears were moistened with a NaCl 0.9% solution to improve conductivity. The tonic extension of the hindlimbs after animals receive the electrical stimulus is considered the endpoint of the test. Animals not exhibiting the hindlimb tonic extension component of the seizures are considered protected from MES-induced seizures (Castel-Branco et al., 2009; Matias et al., 2017; National Institute of Neurological Disorders and and Stroke, 2016).

2.5.2. Olfactory toxicity assessment

A possible impairment of mice olfactory sense caused by the IN administration of FH5 or FH5 + PER was assessed following validated protocols of the buried food-seeking test (Fig. 3A) (Lehmkuhl et al.,

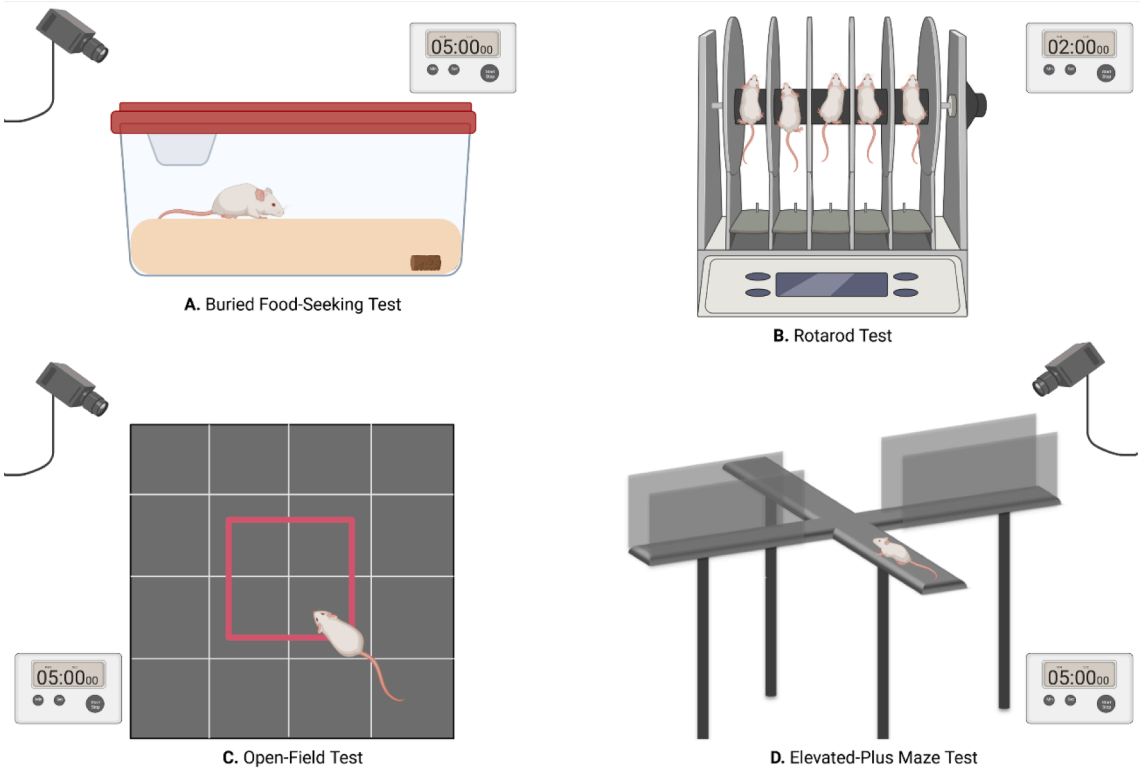

Fig. 3. Schematic illustration of the different behavioural studies conducted in mice groups after receiving the treatments under study.

2014; Yang, 2009).

The buried food-seeking test relies on the natural tendency of mice to use olfactory information for foraging, navigating and other natural activities. Thus, olfactory assessment is critical to proper interpreting numerous mouse behaviours that could further impair other behavioural tests. The assumption of this study is that food-restricted mice, which fail to use odour cues to locate the food, are likely to have deficits in olfactory abilities. In fact, most mice with normal olfaction can find the hidden food piece within a few seconds (Lehmkuhl et al., 2014; Machado et al., 2018).

Mice were randomly divided into four groups ($n = 6$): the negative control group (IN NaCl 0.9%); the IN free-drug FH5 group (5 μL/30 g); the IN FH5 + PER group (1 mg/kg); and the positive control group [IN $ZnSO_4$ 10% (w/v)]. Except for $ZnSO_4$, which was only administered in a single dose, 24 h before the test, the remaining groups were intranasally administered once a day for 7 consecutive days.

Twenty-four hours before the buried food-seeking test, mice were subjected to food deprivation, but with access to water *ad libitum*. To perform the test, each mouse was placed in individual clean cages with a 3 cm-thick corn-cob bedding, being allowed to explore it for 30 min. Then, mice were removed, and a 2–3 g chow pellet was randomly buried approximately 2 cm under the bedding surface. Each mouse was placed back into the cage that had already explored and were video recorded during all experiment. The time between the animal introduction into the cage and the moment at which the animal uncovers the pellet and starts to eat it is scored as the latency to find the food pellet. The latency was measured in seconds up to a maximum of 300 s.

Statistical comparisons between the latency of the negative group (IN NaCl 0.9%) and the latency of the positive control and test groups were performed using a one-way ANOVA analysis with Tukey's post-hoc test. Statistically significance was considered for a *p*-value lower than 0.05 ($p < 0.05$).

2.5.3. Neuromotor impairment evaluation

The neuromotor impairment and coordination of mice were primarily evaluated by the well-established rotarod test (Fig. 3B) using a rotarod apparatus (Ugo Basile, Varese, Italy) (Deacon, 2013).

Before the administrations, all animals were trained on rotarod (4 to 20 rpm for 2 min) two times a day for three consecutive days (Deacon, 2013). Then, the animals were randomly divided into three groups ($n = 6$): a group receiving the IN formulation vehicle FH5 (5 μL/30 g); a group receiving IN FH5 + PER (1 mg/kg); and the third group receiving the oral suspension of PER (1 mg/kg). The test was performed 15 min, 2 h and 4 h after a single dose administration. Then, the administrations continued to be performed once a day for 7 consecutive days, and the test was repeated on the 7th day, 15 min, 2 h and 4 h after the last administration. At the pre-determined time-points after PER single and repeated administrations, the rotarod test was conducted at a constant speed of 20 rpm for 2 min, as described in Food and Drug Administration pharmacology review of PER oral formulations (U.S. Food and Drug Administration, 2012). The latency to fall was recorded, being it indicative of the neuromotor toxicity caused by the treatment.

Statistical comparisons between the time spent in rotarod of negative control group (IN FH5) and the test groups (IN FH5 + PER and oral PER) were determined by a two-way ANOVA with Dunnett's post-hoc test, with statistically significant differences considered for a *p*-value lower than 0.05 ($p < 0.05$).

2.5.4. Locomotion and anxiolytic evaluation

The combined neuromotor and anxiolytic effects of IN PER were evaluated by the open field and the elevated plus maze tests. For that, mice were randomly divided into four different groups ($n = 6$): the negative control group receiving the IN vehicle FH5 (5 μL/30 g); the positive control group of anxiolytic effects receiving IP DZP (1 mg/kg); a group receiving IN FH5 + PER (1 mg/kg) and tested 15 min after administration; and another group receiving IN FH5 + PER (1 mg/kg) and tested 4 h post-administration. Both negative and positive groups were tested 30 min after administration.

2.5.4.1. Open field test.

Open field (Fig. 3C) is a functional test used to evaluate locomotion/exploratory and anxiety stereotypical behaviours, being herein used to evaluate those effects after IN PER administration.

Locomotor and exploratory activities are considered as the general parameters to study the central action of a drug. An increase in these parameters indicates an index of alertness and a decrease is indicative of impaired neurological processes and sedation. Basal locomotion and exploratory activities are measured by the total distance travelled and by the average locomotion speed of mice during the test (Chang et al., 2020; Diniz et al., 2019).

The open field test is also intended for screening anxiolytic or anxiogenic effects of compounds. Less anxious mice tend to spend more time exploring the open central area of the arena. On the contrary, more anxious mice prefer staying close to the walls and travel more on the periphery of the box (Kraeuter et al., 2019a).

Activity in the open field was evaluated using an Actimot device (TSE systems Inc. MI, USA). Each mouse was individually placed in the center of the arena and left to freely explore it for 5 min. The total distance travelled, the average speed, the number of entries in the central and peripheric zones and the number of rearings were automatically recorded using the TSE Phenomaster software (TSE systems Inc. MI, USA). Between animals, the arena was always cleaned with 70% ethanol to eliminate odor disturbance.

Statistically comparisons between all groups were determined by a one-way ANOVA with Tukey's post-hoc test, with significance set for a *p*-value lower than 0.05 ($p < 0.05$).

2.5.4.2. Elevated plus maze test.

The possible anxiolytic effects of IN PER were also evaluated using the elevated plus maze test (Fig. 3D). This test consists of an elevated apparatus standing 60 cm above the ground, with two open arms (36 × 6 cm) and two closed arms (36 × 6 × 15 cm) joined together by a central compartment (6 × 6 cm). This is the gold standard test used for screening anxiety stereotypical behaviours. It is based on the natural tendency of mice to avoid open and/or elevated places balanced with their innate curiosity to explore new areas. So, a less anxious mouse will spend more time exploring the exposed arms of the apparatus, whereas a mouse with high levels of anxiety will have tendency to spend more time in the protected arms (Ari et al., 2019; Kraeuter et al., 2019b).

Each mouse was placed in the central compartment of the apparatus and was video recorded for 5 min. The number of entries, the total distance travelled, and the time spent in open and closed arms, together with the number of rearings and dippings were automatically recorded by the ANY-maze tracking software (Stoelting Europe, Dublin, Ireland).

Statistically comparisons between all groups were determined by a one-way ANOVA with Tukey's post-hoc test, with statistically significant differences considered for a *p*-value lower than 0.05 ($p < 0.05$).

2.6. Neurochemical evaluation in whole brain

After receiving a single dose of each treatment, mice previously subjected to behavioural tests were sacrificed 4 h post-dosing. Brains were collected after decapitation for neurochemical analysis of the levels of nitric oxide, gamma-aminobutyric acid (GABA) and L-glutamate. With that purpose, brain homogenates supernatants were obtained by the homogenization of brains in a 1 M sodium phosphate buffer pH 5 (4 mL/g tissue) using an Ultra-Turrax tissue homogenizer. The homogenates were then centrifuged at 17,350 g for 15 min at 4 °C and the supernatants were subsequently collected for further analysis.

In all cases, statistical comparisons between all groups were determined by a one-way ANOVA with Tukey's post-hoc test, with significance level being set for a *p*-value<0.05 ($p < 0.05$).

2.6.1. Nitric oxide assay

The quantity of nitric oxide levels in brain homogenates supernatants was determined by the reduction of nitrate to nitrite, which then reacts with Griess reagent to form a chromophore azo derivative that can be spectrophotometrically measured (Green et al., 1982; Mustafa et al., 2013).

A volume of 100 μL of brain homogenate supernatant was incubated with 100 μL of Griess reagent (1% sulfanilamide in 5% *ortho*-phosphoric acid and 0.1% N–(1–naphthyl)–ethylenediamine dihydrochloride) for 10 min at room temperature, protected from light. The absorbance of the produced azo derivatives was then measured at 546 nm in a microplate reader (Bio-Rad Laboratories, Hercules, USA).

Nitric oxide levels were expressed as a percentage relatively to the absorbance of the control brain samples (IN FH5). The results correspond to the mean ± standard deviation (SD) of the brain nitric oxide percentages of the six animals ($n = 6$) belonging to each test group used in the behavioural studies.

2.6.2. L-Glutamate assay

A L-glutamate assay kit from Megazyme (Bray Business Park, Bray, Co. Wicklow, Ireland) was used to measure L-glutamate concentrations in brain homogenates supernatants by following the manufacture's protocol (Megazyme, 2018).

A volume of 100 μL of brain homogenate supernatant was deproteinized using an equal volume of 10% trichloroacetic acid. After centrifugation at 12,300 *g* for 10 min at 4 °C, the supernatant was neutralized to pH 7 with 1 M KOH and L-glutamate was quantified by a colorimetric method following the manufacturer's instructions. The principle of the method is based on the L-glutamate oxidation by nicotinamide-adenine dinucleotide, in the presence of glutamate dehydrogenase, leading to the formation of 2-oxoglutarate, reduced nicotinamide-adenine dinucleotide (NADH) and ammonium ions (NH_4^+). A further reaction between NADH and iodonitrotetrazolium chloride (INT), catalyzed by diaphorase, forms an amount of INT-formazan product that is stoichiometric with the amount of L-glutamate. The INT-formazan was measured at 492 nm in a microplate reader (Bio-Rad Laboratories, Hercules, USA) and L-glutamate concentrations were calculated by Equation 1, where ΔA_{sample} is the difference between the blank and the samples absorbance, $\Delta A_{standard}$ is the difference between the blank and L-glutamate standard solution absorbance, the concentration of L-glutamate in standard solution is 0.1 mg/mL, and F is the dilution factor used in sample dilution during preparation:

$$[\mathrm{L-glutamate}] = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times [\mathrm{L-glutamate}]_{standard} \times F \quad \text{(Eq. 1)}.$$

The results correspond to the mean ± SD of L-glutamate concentrations obtained in the brain of the six animals ($n = 6$) belonging to each test group used in the behavioural studies.

2.6.3. GABA assay

The levels of GABA in brain homogenates supernatants were determined by the measurement of the fluorescent products formed when GABA reacts with ninhydrin in an alkaline medium (Kandeda et al., 2022; Lowe et al., 1958).

A volume of 100 μL of brain homogenate supernatant was deproteinized using an equal volume of 10% trichloroacetic acid. After centrifugation at 12,300 *g* for 10 min at 4 °C, the supernatant was collected and its pH was adjusted to 7 with 1 M KOH. 200 μL of 14 mM ninhydrin (in 0.5 M carbonate-bicarbonate buffer pH 9.95) was then added to 100 μL of the deproteinized supernatant. The mixture was incubated at 60 °C for 30 min and then cooled to room temperature, followed by the addition of 5 mL of freshly prepared alkaline copper-tartrate reagent (10 mM sodium carbonate, 1.32 mM copper sulphate and 2.2 mM tartaric acid), vortex and incubation at 25 °C for 15 min. The fluorescence of the formed products was measured at 355/460 nm in a spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, San Jose, CA), being it proportional to the GABA concentrations in brain homogenates.

GABA levels were expressed as a percentage relatively to the absorbance of the control brain samples (IN FH5). The results correspond to the mean ± SD of the brain GABA percentages of the six animals ($n = 6$) belonging to each test group used in the behavioural studies.

3. Results

3.1. In vivo brain biodistribution study

To support the previously obtained data regarding a possible direct nose-to-brain delivery of PER (Meirinho et al., 2022a), a brain biodistribution study was performed to understand if PER followed a somewhat rostral-caudal biodistribution after its IN (1 mg/kg) and IV (0.5 mg/kg) administrations.

At specific time-points post-dosing up to 60 min, PER concentrations were quantified in olfactory bulbs, frontal cortex, cerebellum, remaining part of the brain and in the respective plasma samples (Fig. 4). The tissue-to-plasma ratios were also calculated for olfactory bulbs, frontal cortex, cerebellum and remaining part of the brain following IN and IV administrations (Table 1).

By examining Fig. 4B, it is noteworthy that the concentrations of PER in the studied sections of the brain at all times post-IV administration were significantly lower ($p < 0.001$) than the respective concentrations in plasma. However, no statistically significant differences were found between PER concentrations in the different brain sections, being that consistent with the similar tissue-to-plasma ratios obtained at all time points post IV-dose (Table 1). That might demonstrate a somewhat uniform distribution of PER in brain after being intravenously administered. On the other hand, at all times post-IN delivery, the olfactory bulb was the only brain section that did not show statistically significant differences in PER concentrations comparatively with the ones attained in plasma (Fig. 4A). Actually, at 15 min post IN-dosing – the t_{max} of PER in whole brain after IN administration (Meirinho et al., 2022a) – the concentrations of PER in the olfactory bulbs were even higher than in plasma. In contrast, at that same time post-dosing, significantly lower PER concentrations ($p < 0.001$) can be observed in frontal cortex, cerebellum and remaining part of the brain comparatively with the ones in olfactory bulbs, which shows a higher accumulation of PER in the olfactory bulbs at the IN PER t_{max}. This is in line with the olfactory bulb/plasma ratio obtained 15 min after IN administration, with olfactory bulb/plasma ratios after 5 and 15 min of IN PER administration showing to be significantly higher than the corresponding tissue-to-plasma ratios calculated for IV administration (Table 1). It is only after 60 min of IN instillation that the concentrations of PER start to became more uniformly distributed between olfactory bulb, frontal cortex, cerebellum, and remaining part of the brain.

Altogether, the analysis of PER concentrations over time in the different brain parts suggests a direct nose-to-brain drug delivery of IN PER due to an unequal distribution from rostral to more caudal brain areas, contrary to the more homogenous tissue distribution of PER found after IV dosing.

3.2. Anticonvulsant activity evaluation

Since oral PER (Fycompa®) is currently approved for the therapy of generalized tonic-clonic seizures (European Medicines Agency, 2022; Patsalos, 2015), the anticonvulsant activity of IN PER was evaluated using the MES test, a highly representative *in vivo* model of these seizures types (Barker-Haliski et al., 2018; National Institute of Neurological Disorders and and Stroke, 2016). After application of the electric stimulus, the occurrence of hindlimbs tonic extension in each mouse was recorded. If that extension did not occur, the animals were considered protected against MES-induced seizures. This protection was evaluated 15 min, 2 h and 4 h after a single IN and oral dose administration of PER

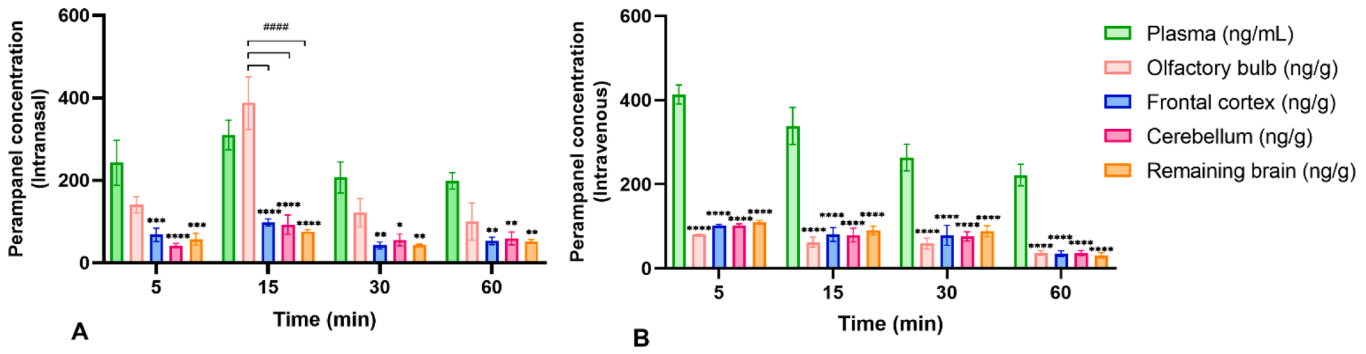

Fig. 4. Perampanel concentration in plasma, olfactory bulbs, frontal cortex, cerebellum and remaining part of the brain up to 60 min after intranasal (1 mg/kg) (A) and intravenous (0.5 mg/kg) (B) administration of PER to mice. Data correspond to mean ± standard error of the mean (SEM) of four mice in each time group ($n = 4$). Statistically significant differences between perampanel concentrations in plasma and different sections of the brain (highlighted by * symbols) and between the different brain sections (highlighted by # symbols) were evaluated by a two-way ANOVA with Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ####/****$p < 0.0001$.

Table 1
Tissue-to-plasma concentration ratios of perampanel after intranasal (1 mg/kg) and intravenous (0.5 mg/kg) administrations to mice. Data expressed as mean ± standard error of the mean (SEM) of four mice in each time group (n = 4). At each time after administration, statistically significant differences (**$p < 0.01$) between intranasal and intravenous groups are marked.

Concentration ratios (mean ± SEM)	Intranasal administration				Intravenous administration			
	5 min	**15 min**	**30 min**	**60 min**	**5 min**	**15 min**	**30 min**	**60 min**
Olfactory bulb/Plasma	0.617 ± 0.067**	1.266 ± 0.183**	0.578 ± 0.142	0.463 ± 0.159	0.198 ± 0.011	0.181 ± 0.027	0.242 ± 0.084	0.176 ± 0.048
Frontal cortex/Plasma	0.292 ± 0.048	0.329 ± 0.039	0.205 ± 0.016	0.268 ± 0.034	0.246 ± 0.006	0.238 ± 0.041	0.329 ± 0.143	0.167 ± 0.044
Cerebellum/Plasma	0.201 ± 0.053	0.294 ± 0.065	0.304 ± 0.100	0.289 ± 0.046	0.249 ± 0.016	0.237 ± 0.042	0.307 ± 0.082	0.166 ± 0.024
Remaining brain/Plasma	0.241 ± 0.049	0.253 ± 0.019	0.223 ± 0.033	0.270 ± 0.038	0.267 ± 0.009	0.277 ± 0.037	0.355 ± 0.093	0.140 ± 0.023

(1 mg/kg). The number of protected animals after each treatment and the respective protection rate (expressed as percentage of the total number of animals tested) are summarized in Table 2. All animals that only received the IN vehicle FH5 experienced MES endpoint, thus ensuring that FH5 was devoid of intrinsic anticonvulsant activity.

After the IN-dose administration of PER, the results indicate a higher protection rate against MES-induced seizures comparatively with the obtained after oral administration (Table 2). A protection rate of 60% was obtained 15 min post IN dosing, which is the t_{max} of PER after a single IN dose (Meirinho et al., 2022a). The same protection rate is maintained at least until 2 h after IN administration, only decreasing for 20% after 4 h. On the contrary, at all times after a single oral dose, the obtained protection rate was only of 20%, a considerably lower value compared with the data obtained after IN administration of PER. These data suggest that the IN administration of PER (1 mg/kg) results in a higher anticonvulsant protection when compared with its oral administration at the same dose.

Table 2
In vivo anticonvulsant protection following intranasal (IN) administration of perampanel (PER) loaded in FH5 (FH5 + PER, 1 mg/kg) and oral administration of a PER suspension (1 mg/kg) to mice ($n = 5$). The anticonvulsant evaluation was performed at different times after a single dose administration of each treatment.

Maximal electroshock seizure (MES) test				
Time post-dosing	**FH5 + PER (1 mg/kg)**		**Oral suspension (1 mg/kg)**	
	$n_{protected}/n_{test}$	% Protection	$n_{protected}/n_{test}$	% Protection
15 min	3/5	60	1/5	20
2 h	3/5	60	1/5	20
4 h	1/5	20	1/5	20

3.3. Olfactory toxicity assessment

Since mice behaviours greatly depend on their olfactory sense, a possible olfactory impairment caused by the IN administration of FH5 and FH5 + PER was assessed by the buried food-seeking test.

As demonstrated in Fig. 5, IN administrations of FH5 and FH5 + PER did not demonstrate any loss of olfactory sense in mice after 7 consecutive days of repeated administrations. The latency to eat food was comparable between animals that were intranasally administered with FH5, FH5 + PER and NaCl 0.9% (Fig. 5), and significantly lower than in the animals treated with 10% $ZnSO_4$ (291.8 ± 11.67 s), a compound known for destroying olfactory epithelium in mice (McBride et al., 2003). These data suggest that the previously developed SMEDDS, loaded or not with PER, is safe for the olfactory epithelium and that the following behavioural assays could be carried out without bias.

3.4. Neuromotor impairment evaluation

3.4.1. Rotarod test

The minimal neuromotor impairment was firstly evaluated by the time spent in the rotarod apparatus after the IN or oral dosing of PER at 1 mg/kg, administered either in a single or after a repeated-dose regimen (Fig. 6A and 6B, respectively).

After a single dose administration, comparisons with the IN vehicle FH5 indicate a significant decrease on the time spent in rotarod, 15 min after the IN administration of PER and 2 h post oral dosing (Fig. 6A). This indicates a slightly neuromotor impairment induced by PER at the corresponding IN and oral t_{max} of PER in brain (Meirinho et al., 2022a). At 4 h post-administrations, the time spent in the rotarod was similar between the animal groups that received the IN vehicle (FH5), IN PER (FH5 + PER) and oral PER. This might indicate that mice fully recover

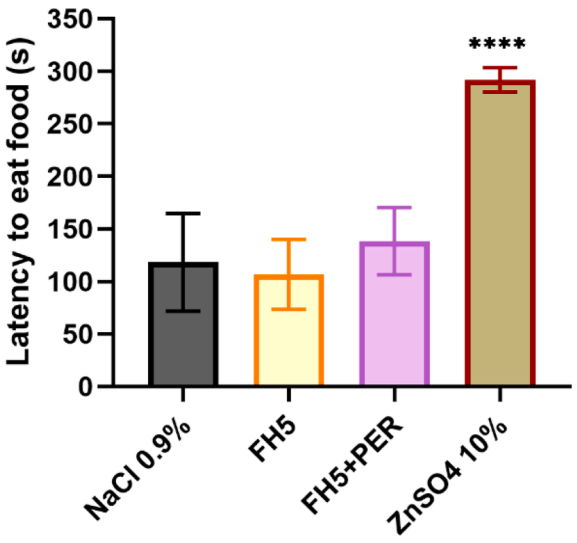

Fig. 5. Latency time (s) evaluated in the buried food-seeking test. Data correspond to mean ± standard deviation (SD, n = 6) of the time that starved animals took to find and start eating the buried food pellet after intranasal administration of the formulation vehicle (FH5) and FH5 loaded with perampanel (FH5 + PER) for 7 consecutive days. A single dose of intranasal $ZnSO_4$ solution at 10% was used as positive olfactory impairment control, and a 7-days repeated intranasal dosing of NaCl 0.9% was used as negative control for olfactory impairment. Statistically significant differences between negative control and test groups were evaluated by a one-way ANOVA with Tukey's post-hoc test. ****$p < 0.0001$.

their neuromotor activity at longer times post-dosing, demonstrating a reduction of minimal neuromotor impairment caused by PER at longer times post-dosing.

After 7-days of repeated administrations, no significant differences on the time spent in rotarod were found between groups (Fig. 6B). These findings indicate that a continuous dosing schedule of IN and oral administration can decrease the neuromotor impairment effects of PER found in the rotarod at PER t_{max} values after a single dose administration (i.e., 15 min and 2 h).

3.4.2. Open field test

The effects on locomotion and exploratory behaviours after a single IN administration of PER at 1 mg/kg were evaluated in an open field apparatus by the analysis of the total distance travelled by mice and their respective average speed (Fig. 7).

Before using DZP as a positive anxiolytic control, it was first necessary to confirm that its IP administration at a dose of 1 mg/kg only resulted in anxiolytic effects without compromising the locomotor activity (da Cruz et al., 2019; Fan et al., 2019). Mice treated with IP DZP did not reveal significant differences in the distance traveled and in the average speed compared with mice treated with the IN vehicle (Fig. 7), thus ensuring that DZP will not cause neuromotor impairment during the following anxiety tests. A single dose of IN administration of PER resulted in a trend to decrease locomotor and exploratory behaviours 15 min post IN administration, being this in accordance with the neuromotor impairment effects demonstrated in the rotarod test (Fig. 6). This is confirmed by the reduction in total distance travelled (Fig. 7A) and in the average speed of mice (Fig. 7B) during the open field test, although not statistically different when compared with the same data of the IN FH5 group. After 4 h of IN PER administration, mice fully recover their motor ability. In fact, there is even a statistically significant increase in the total distance traveled and in the average speed 4 h after mice received an IN dose of PER compared to the same data obtained in mice after 15 min of an IN PER administration (Fig. 7A and 7B). This reinforces that mice fully recover their locomotor activity at longer times post-dosing, as already demonstrated by the normalization of the neuromotor impairment effects in rotarod 4 h after PER administration. Interestingly, the total distance travelled and the average speed of the mice 4 h after the IN administration of PER were also significantly greater ($p < 0.05$) than those obtained with IN FH5, but comparable to the data obtained in the mice that received the anxiolytic DZP. This may be a clue as to the existence of similar effects between IP administration of DZP and the effect of IN administration of PER at longer times post-dosing that are not purely influenced by neuromotor activity.

3.5. Anxiolytic evaluation

3.5.1. Open field test

The possible anxiolytic effects of IN PER were firstly evaluated in the open field test by analyzing the number of entries of mice in the central area of the arena and the number of rearings during the test (Fig. 8).

The number of entries in the central area and the number of rearings significantly increased ($p < 0.05$) in mice treated with IP DZP compared with mice treated with IN FH5 (Fig. 8), revealing the already known anxiolytic effects of this drug (i.e., positive control). A single IN administration of PER (1 mg/kg) significantly increased the number of entries in the central area of the open field arena 4 h after administration when compared with the IN FH5 group (Fig. 8A). The number of rearings of the group that received IN PER 4 h after administration was also significantly higher than the number of rearings obtained in the negative control (IN FH5) (Fig. 8B). This might indicate a possible anxiolytic effect of PER at longer times after a single IN dose when PER

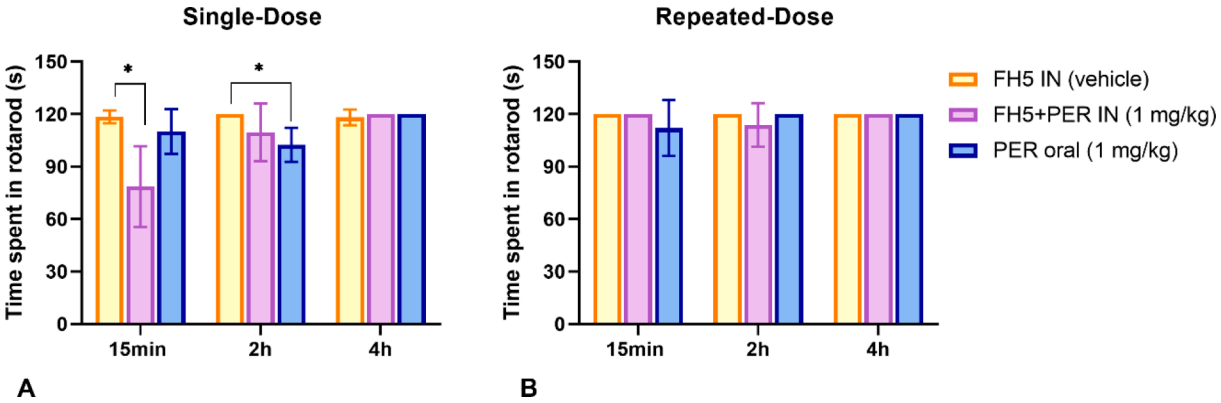

Fig. 6. Neuromotor effects evaluated by the rotarod test in mice subjected to intranasal (IN) or oral administration of perampanel (PER). Data correspond to mean ± standard deviation (SD, n = 6) of the time that mice spent in rod after a single dose administration (A) and a 7-day repeated dose administration (B) of IN or oral PER. IN formulation vehicle (FH5) was used as control. Statistically significant differences between the control and test groups were evaluated by a two-way ANOVA with Dunnett's post-hoc test. *$p < 0.05$.

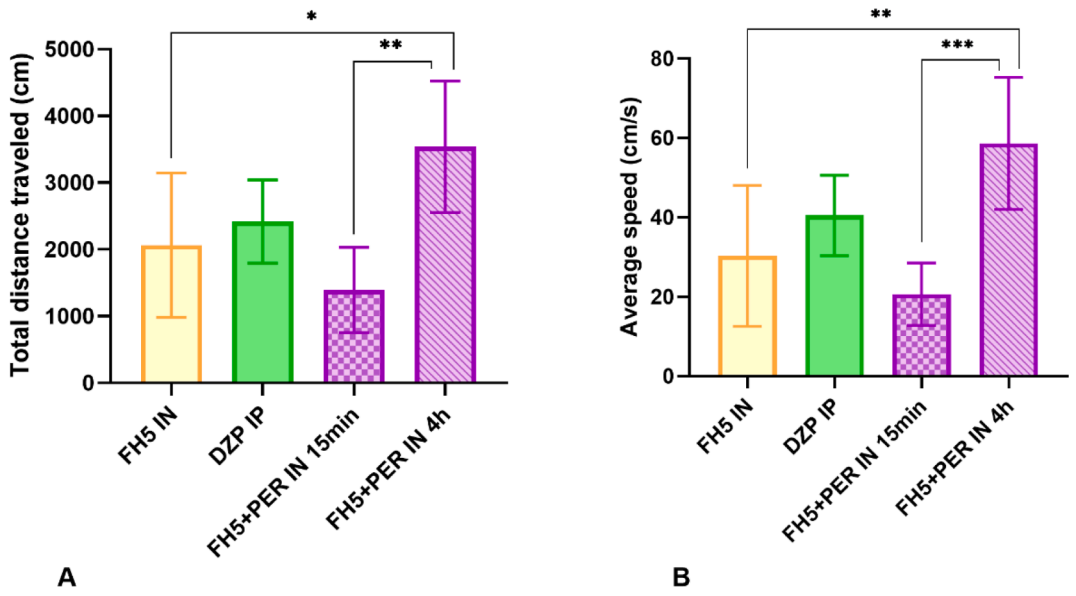

Fig. 7. Locomotor activity evaluated in the open field test. Data correspond to mean ± standard deviation (SD, n = 6) of the total distance travelled (A) and average speed (B) of mice 15 min and 4 h after receiving a single intranasal (IN) administration of FH5 + perampanel (PER) (1 mg/kg), and 30 min after receiving the IN-formulation vehicle (FH5). Intraperitoneal (IP) diazepam (DZP) (1 mg/kg) was also tested in the open field 30 min after administration in order to ensure that its anxiolytic effects were not biased by its effects in locomotor activity. Statistically significant differences were evaluated by a one-way ANOVA with Tukey's post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.

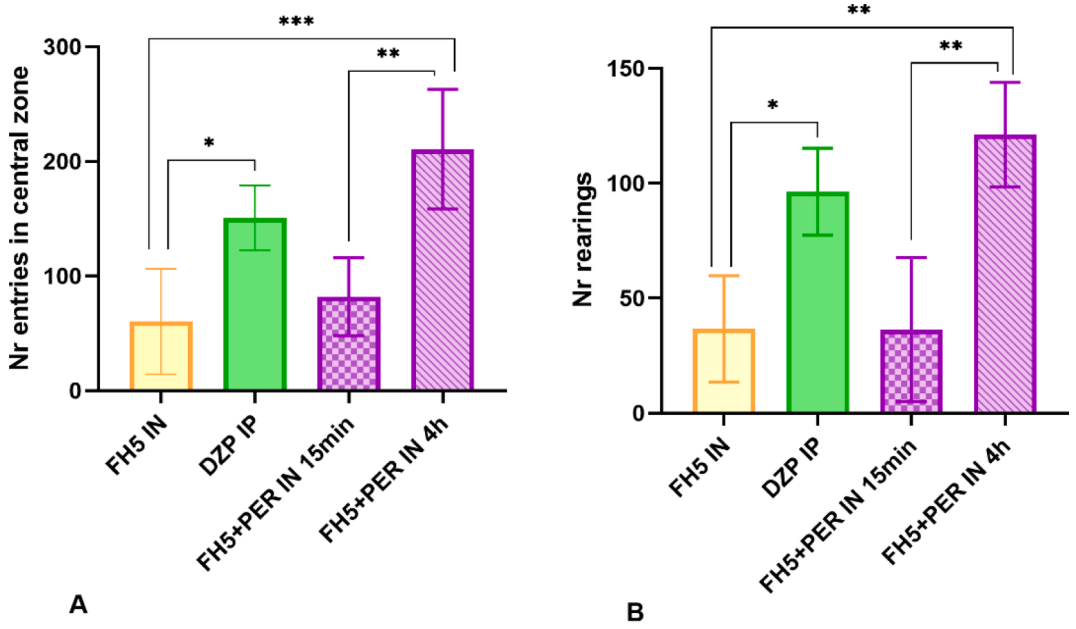

Fig. 8. Anxiolytic effects evaluated in the open field test. Data correspond to mean ± standard deviation (SD, n = 6) of the number of entries in the central area (A) and the number of rearings (B) of mice 15 min and 4 h after receiving a single intranasal (IN) administration of FH5 + perampanel (PER) (1 mg/kg). Mice controls were tested 30 min after receiving intraperitoneal (IP) diazepam (DZP) (1 mg/kg) or IN formulation vehicle (FH5). Statistically significant differences between groups were evaluated by a one-way ANOVA with Tukey's post-hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.

concentrations are lower but still consistent with the reference therapeutic range in humans.

3.5.2. *Elevated plus maze test*

The possible anxiolytic effects of IN PER were also evaluated in the elevated plus maze test. The behavioural responses of mice were quantified in terms of percentage of entries in open arms (PEOA), percentage of time spent in open arms (PTOA) and distance travelled in open arms (DTOA) of the elevated plus maze apparatus. The obtained results are shown in Fig. 9.

The administration of IP DZP resulted in a statistically significant increase ($p < 0.05$) of PEOA, PTOA and DTOA in the elevated plus maze test compared with the IN FH5 control (Fig. 9). Gathering the data obtained in the open field test, this came to reinforce the anxiolytic effects of DZP without compromising the locomotor activity, making it an adequate positive control drug for anxiolytic activity evaluation. At 4 h after a single IN administration of PER, the PEOA was significantly higher ($p < 0.05$) than after the IN administration of FH5, here used as negative control (Fig. 9A). The PTOA and the DTOA were also greater 4 h after a single IN PER treatment, even though no statistical differences

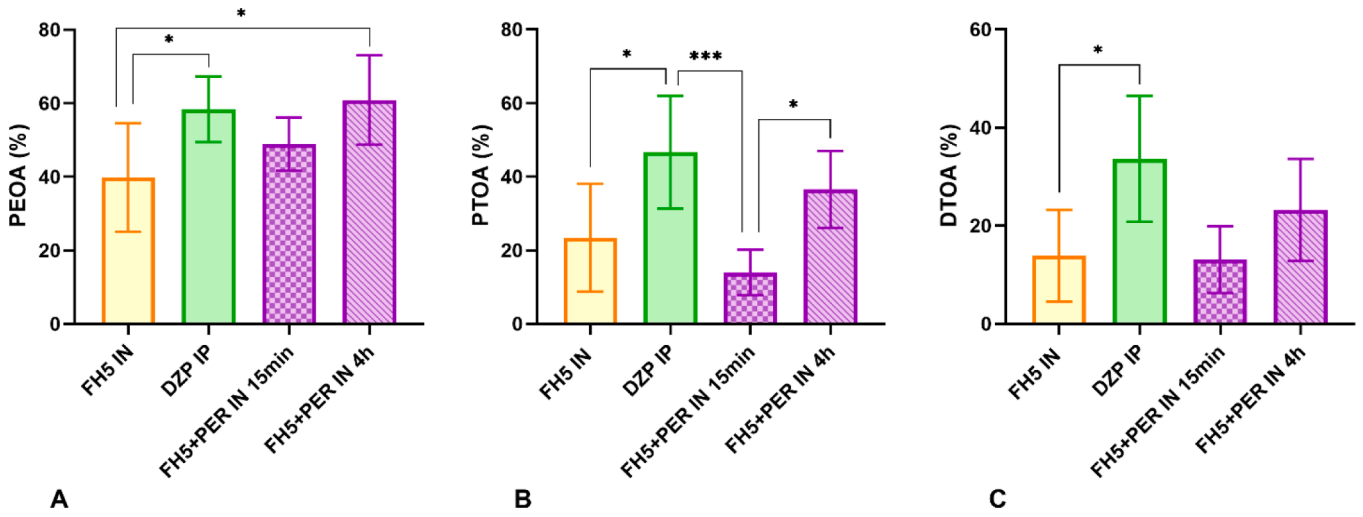

Fig. 9. Anxiolytic effects evaluated in the elevated plus maze test. Data correspond to mean ± standard deviation (SD, n = 6) of percentage of entries in open arms (PEOA) (A), percentage of time spent in open arms (PTOA) (B), and distance travelled in open arms (DTOA) (C) by mice after 15 min and 4 h of receiving a single intranasal (IN) administration of FH5 + perampanel (PER) (1 mg/kg) and 30 min after receiving a single dose of intraperitoneal (IP) diazepam (DZP) (1 mg/kg) and IN formulation vehicle (FH5). Statistically significant differences were evaluated by a one-way ANOVA with Tukey's post-hoc test. *p < 0.05, ***p < 0.001.

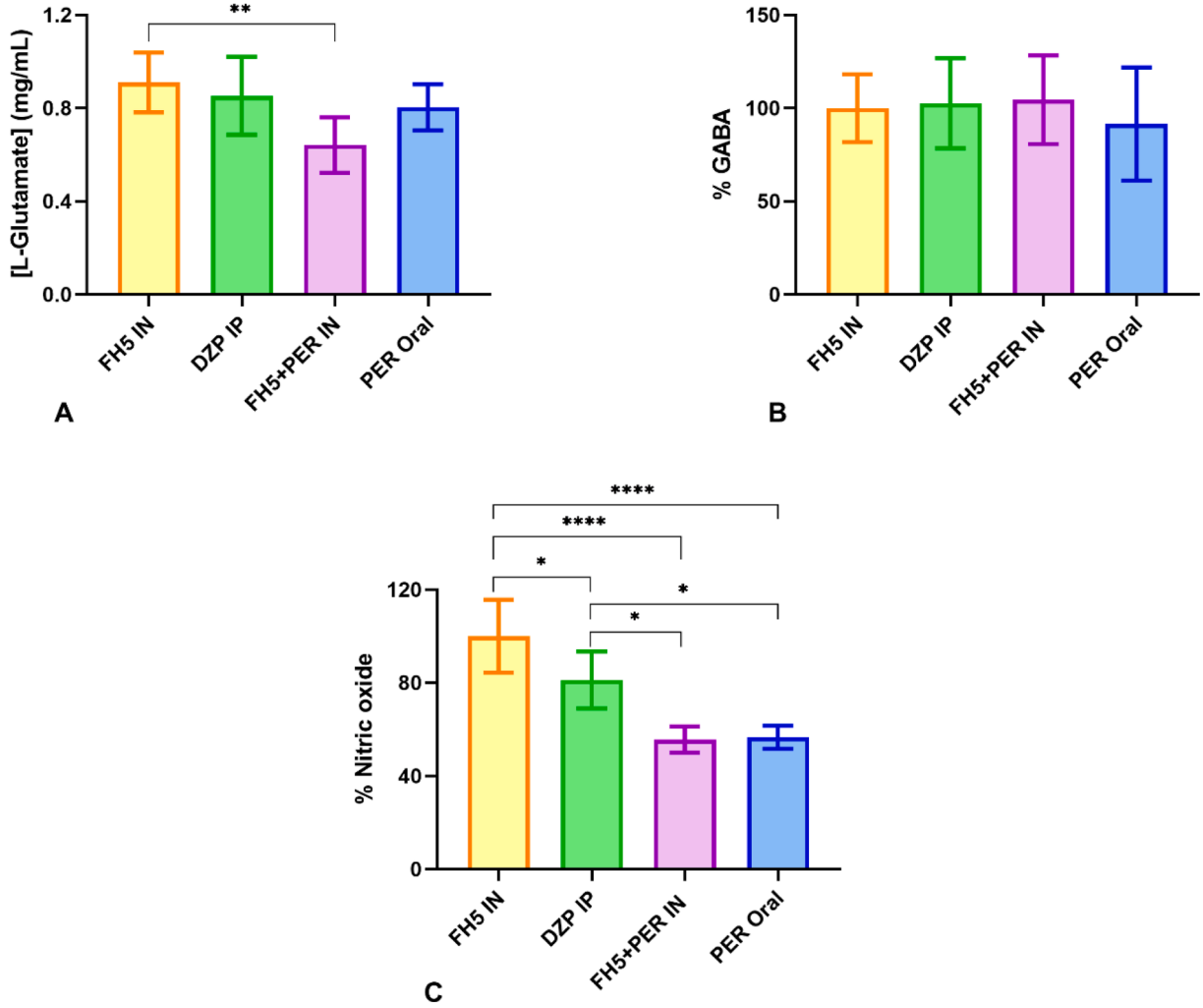

Fig. 10. Brain levels of L-glutamate (mg/mL) (A), GABA (%) (B) and nitric oxide (%) evaluated after a single dose administration of FH5 [intranasal (IN) formulation vehicle], intraperitoneal (IP) diazepam (DZP) at 1 mg/kg, IN perampanel (PER) at 1 mg/kg and an oral suspension of PER at 1 mg/kg to different mice groups. Data correspond to mean ± standard deviation (SD, n = 6) of the neurochemical levels. Statistically significant differences between groups that received dose treatments *vs* dose control (IN FH5) were evaluated by a one-way ANOVA with Tukey's post-hoc test. *p < 0.05, **p < 0.01***p < 0.0001.

were found when compared with negative control (Fig. 9B and 9C, respectively). The values of PEOA, PTOA and DTOA 4 h after IN PER administration were found close to those obtained in animals administered with IP DZP. That might suggest similar anxiolytic effects between DZP and IN PER at longer time post-dosing. Still, when mice were evaluated 15 min after receiving a single dose of IN PER, they displayed a decrease in all the evaluated parameters, with PTOA being significantly lower than the obtained in the IP DZP group ($p < 0.05$), as well as significantly lower than in the group evaluated 4 h after a single IN PER dosing ($p < 0.05$). That can possibly be explained by the prevalence of neuromotor impairment effects over the anxiolytic effects of PER at short times post IN dosing.

3.6. Neurochemical evaluation in whole brain

After being subject to behavioural tests (rotarod, open field and elevated plus maze test), mice were sacrificed and the respective brains were dissected and homogenised to quantify the levels of L-glutamate, GABA and nitric oxide.

A decrease in brain concentrations of L-glutamate was obtained after a single dose administration of IN and oral PER, being it significantly smaller when PER was intranasally administered (0.64 ± 0.12 mg/mL) compared with the concentration obtained after the administration of the IN vehicle FH5 (0.91 ± 0.13 mg/mL) (Fig. 10A). Even without statistical significance, the analysis of Fig. 10A also highlights that L-glutamate levels are lower after nasal administration compared with the obtained after a single oral administration of the same dose of PER.

GABA levels remained similar between the different animal groups after a single dose treatment with IN vehicle, IP DZP, IN FH5 + PER (1 mg/kg) and oral PER (1 mg/kg) (Fig. 10B).

Regarding brain levels of nitric oxide (Fig. 10C), a statistically significant decrease in nitric oxide levels occurred in the groups intranasally (55.6 ± 5.68%) and orally (56.62 ± 4.95%) administered with a single dose of PER compared with mice that received a single dose of IN FH5 (100 ± 15.62%).

4. Discussion

PER is an antiepileptic drug with highly attractive pharmacological properties (Patsalos, 2015), which means that its therapeutic potential can be extended to other epilepsy types and CNS disorders. However, PER is only available as oral tablets and suspension (European Medicines Agency, 2022). This limits its brain concentration and bioavailability, since PER needs to cross several absorptive membranes and pass through the complex BBB until reach the brain parenchyma (Meirinho et al., 2022b).

Once PER is a highly potent lipophilic drug, in an early study we have developed a non-toxic SMEDDS - FH5 - for the IN administration of PER. This allowed us to reach our goals of increasing PER brain exposure and bioavailability when compared with a single oral administration of the same dose (1 mg/kg) (Meirinho et al., 2022a). In fact, by presenting several advantages in being used as vehicles for IN administration, the IN delivery with SMEDDS formulations has proven to enhance absorption and bioavailability of different CNS-active drugs (Meirinho et al., 2022b). They are able to solubilize high concentrations of lipophilic drugs, which are then entrapped in the microemulsion oil droplets formed after contact with nasal mucous (Buya et al., 2020; Meirinho et al., 2022b). In this way, drugs are maintained stable and can be absorbed through nasal epithelium entrapped in nanometric droplets (<100 nm). Furthermore, since droplet sizes <200 nm potentiate delivery through olfactory and trigeminal nerves (Nguyen et al., 2022), the direct nose-to-brain transport can be enhanced, leading to higher drug concentrations in the brain. In fact, our previous study demonstrated that a percentage of the PER intranasally administered reached brain by a direct pathway [drug targeting efficiency (DTE) of 116.3% and direct transport percentage (DTP) of 14.03%] (Meirinho et al., 2022a). Based on PER quantification up to 12 h post-dosing, we also hypothesized that the percentage of PER that directly reached the brain made it through the olfactory pathway (Meirinho et al., 2022a). Unlike the trigeminal pathway, the uptake through the olfactory pathway affords a preferential drug delivery to olfactory bulb, which are located in the rostral portion of the brain (Fig. 1) (Serralheiro et al., 2014).

To explore our previous hypothesis, after IN administration of PER, we characterized its biodistribution in olfactory bulbs, frontal cortex, cerebellum and remaining part of the brain (Fig. 4 and Table 1). With that, we confirmed a higher delivery efficiency of PER to olfactory bulbs via the nasal route. Therefore, it is probable that the percentage of PER that quickly reaches the brain through direct nose-to-brain transport made it preferentially through the olfactory pathway. There is also evidence that drugs administered intranasally can be directly transported from olfactory mucosa to the cerebrospinal fluid (CSF), and then delivered into the brain parenchyma by the CSF circulation (Colombo et al., 2011; Inoue et al., 2020; Yu et al., 2011; Yu and Kim, 2009). So, it is also possible that CSF might have contributed to the distribution of PER into the brain tissue after IN dosing. Thus, to better understand the nose-to-brain delivery of PER, in future studies the sampling and analysis of CSF should be performed. Nevertheless, among other factors, the direct nose-to-brain transport of PER could be explained by the droplet size upon dilution (20.07 ± 0.03 nm), homogeneous distribution (PDI of 0.06 ± 0.001) and composition of the previously developed FH5 (Meirinho et al., 2022a) able to promote an enhancement of the endothelial permeability and the olfactory passage of PER after its IN administration.

The high and fast brain exposure of PER after its IN single dosing could be of great advantage concerning its potential therapeutic applications. So, we tested the IN delivery of PER in an animal model of generalized tonic-clonic seizures (MES test) and compared the IN PER anticonvulsant efficacy with the obtained after oral administration of PER at the same dose. In agreement with the possible efficiency of the IN route in delivering PER to brain and with the previous brain pharmacokinetic results (Meirinho et al., 2022a), when compared with a single oral administration of PER, an improved anticonvulsant activity was obtained after a single IN dose. The protection rate against MES-induced seizures was particularly higher at shorter times post IN single dosing, being it maintained until 2 h of that dosing (Table 2). This is consistent with brain C_{max} being reached 15 min post single IN dosing. So, considering these results, the possible use of PER nose-to-brain delivery in *status epilepticus* treatment might be here reinforced, as already proposed in our previous study (Meirinho et al., 2022a) and by several authors after PER nasogastric (Newey et al., 2019; Rahbani et al., 2019; Strzelczyk et al., 2019) and IV administrations.

The anxiolytic potential of a single IP dose of PER was previously demonstrated in a non-clinical study performed by Bektas *et al.* (2020). So, once anxiety is a common comorbidity in patients with epilepsy (Gonzalez-Martinez et al., 2022; Johnstone et al., 2021; Rauh et al., 2022), we intended to study if nasal PER delivery to the brain will also present anxiolytic effects, which would possibly be translated into great clinical advantages and as an alternative to the benzodiazepines use. The obtained results in open field and elevated plus maze tests demonstrated a lack of anxiolytic effects 15 min post IN single PER administration to mice. However, in both anxiety models, PER showed anxiolytic effects in mice 4 h post single IN administration, a post-dosing time in which mice plasmatic concentrations were expectably within the reference therapeutic range in humans (Meirinho et al., 2022a). The lack of anxiolytic effects 15 min after IN PER dosing could be explained by the possible neuromotor impairment caused by PER. That was revealed by a reduction in motor coordination, exploratory behaviours and locomotion activity of mice in rotarod and open field tests 15 min after IN single PER delivery, being it consistent with the rotarod test results 2 h after a single oral administration of PER. Similar to benzodiazepines, where sedative effects prevail when higher doses are administered but anxiolytic effects are more prevalent after the administration of lower doses, these results

may reveal that a single dose of 1 mg/kg, either administered intranasally or orally, can be associated with some neuromotor deficits when PER concentrations in mice plasma and brain are higher - at PER t_{max}. However, when the plasmatic and brain levels of PER are lower at later phases post dosing (e.g., after 4 h of IN dosing), anxiolytic effects may prevail. A normalization of the motor impairment effects was found in the rotarod test after administering PER to mice once a day for 7 consecutive days (Fig. 6). Even though the pharmacokinetic parameters may be different between mice and humans (Meirinho et al., 2022a; U.S. Food and Drug Administration, 2012), these findings are consistent with the clinical data describing that most adverse effects of PER occur during the titration phase and tend to subside within a few weeks (Bonanni et al., 2021).

The neurochemical findings in whole brain after the different treatments might support the obtained non-clinical pharmacodynamic results. A decrease in L-glutamate concentrations occurred after IN and oral administration of PER, being significantly lower when PER was intranasally administered compared with the IN administration of the vehicle. PER is known to pharmacologically act as a selective non-competitive antagonist of AMPA receptors, thereby reducing cerebral glutamatergic neurotransmission, consequently decreasing neuronal excitability (European Medicines Agency, 2022; Patsalos, 2015). However, that does not explain what could lead to the decrease of L-glutamate levels observed in this study. Still, an *in vitro* study that used glioblastoma cells demonstrated that PER led to an increase in the expression of *GLUL*, a gene that encodes a glutamine synthetase protein, further leading to an increase of the reconversion of glutamate into glutamine, decreasing by this way the cytosolic levels of glutamate (Damavandi et al., 2023). Even though that could explain the results here obtained, this cannot be interpreted with certainty without further studies being carried out. The obtained GABA levels in mice dosed with IP DZP were similar to those obtained in mice receiving the IN vehicle (FH5). That is not surprising since DZP only increases the efficiency of synaptic transmission of GABA without increasing GABA levels (U.S. Food and Drug Administration, 2002). Also, the GABA levels in mice brain dosed with IN and oral PER did not reveal any difference comparatively with IN FH5 and IP DZP. Even though most anxiolytic effects are related with an upregulation of the GABAergic system, the here obtained results might reveal that the possible anxiolytic effects of PER did not occur by modulation on GABA neurotransmission, as it occurs with the anxiolytic effects mediated by DZP. In general, the mice groups that demonstrated a decrease of L-glutamate levels also revealed a decrease in cerebral nitric oxide levels. In fact, it is reported that a stimulation of the AMPA receptors by L-glutamate can increase the influx of calcium, causing an increase of nitric oxide levels that consequently leads to oxidative stress. Additionally, it is also reported that this increase in nitric oxide production is consistent in experimental epileptogenic and anxiety attacks, with elevated nitric oxide being particularly related to both the cause and consequence of convulsions (Łukawski and Czuczwar, 2021; Walia et al., 2018). In fact, animal models of acquired epilepsy have shown an increase in nitric oxide levels and in mitochondrial oxidative stress, which can possibly be one of the causes of the subsequent cell damage after persistent seizures (Łukawski and Czuczwar, 2021).

5. Conclusions

The present study reveals that the IN route can promote a somewhat rostral-caudal distribution of the high potent antiepileptic drug PER when formulated in the safe developed SMEDDS – FH5. Additionally, it is suggested that intranasally administrated PER was delivered to brain by a combination of both systemic transport and a direct olfactory neuronal pathway. This work also came to establish a possible relationship between the previous pharmacokinetic data and the superior pharmacodynamic effects resulting from the nose-to-brain delivery of PER. In fact, the obtained data demonstrate a greater anticonvulsant and anxiolytic activities after a single IN administration of PER, only causing a slight neuromotor impairment when PER attains its maximal plasma and brain concentrations. Thus, the IN administration of PER formulated in the previously developed SMEDDS could be very promising alternative to the oral and IV formulations in managing acute seizure clusters and *status epilepticus* emergencies. So, all the set of evidence resulting from our work can support the design of future clinical trials to demonstrate the usefulness of the IN administration of PER to humans so that its delivery to the brain can be improved with less peripheral systemic exposure, possibly resulting in an improved clinical efficacy/safety binomial in epilepsy treatment and in related anxiety disorders.

CRediT authorship contribution statement

Sara Meirinho: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing – original draft. **Márcio Rodrigues:** Supervision, Methodology, Conceptualization, Writing – review & editing. **Adriana O. Santos:** Writing – review & editing. **Amílcar Falcão:** Supervision, Funding acquisition. **Gilberto Alves:** Supervision, Methodology, Conceptualization, Resources, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Sara Meirinho reports financial support was provided by Foundation for Science and Technology.].

Data availability

No data was used for the research described in the article.

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