RESEARCH ARTICLE

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Influx of kynurenine into the brain is involved in the reduction of ethanol consumption induced by Ro 61-8048 after chronic intermittent ethanol in mice

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Background and Purpose: The kynurenine pathway has been proposed as a target for modulating drug abuse. We previously demonstrated that inhibition of kynurenine 3-monooxygenase (KMO), using Ro 61-8048, reduces ethanol consumption in a binge drinking model. Here, we investigate the effect of the kynurenine pathway modulation in ethanol-dependent mice.

Experimental Approach: Adult male and female mice were subjected to a Chronic Intermittent Ethanol (CIE) paradigm. On the last day of CIE, mice were treated with Ro 61-8048, Ro 61-8048 + PNU-120596, a positive allosteric modulator of α7nAChR, and Ro 61-8048 + L-leucine or probenecid, which blocks the influx or efflux of kynurenine from the brain, respectively. Ethanol, water consumption and preference were measured and kynurenine levels in plasma and limbic forebrain were determined.

Key Results: Ro 61-8048 decreases consumption and preference for ethanol in both sexes exposed to the CIE model, an effect that was prevented by PNU-120596. The Ro 61-8048-induced decrease in ethanol consumption depends on the influx of kynurenine into the brain.

Conclusion and Implications: Inhibition of KMO reduces ethanol consumption and preference in both male and female mice subjected to CIE model by a mechanism involving α7nAChR. Moreover, this centrally-mediated effect depends on the influx of peripheral kynurenine to the brain and can be prolonged by blocking the efflux of kynurenine from the brain. Here, for the first time, we demonstrate that the modulation of the kynurenine pathway is an effective strategy for the treatment of ethanol dependence in both sexes.

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1 | INTRODUCTION

Alcohol as **ethanol** is the most widely consumed drug in the world. Its harmful use causes up to 5% of the global disease burden, and its consumption is related to more than 200 diseases and injuries (World Health Organization, 2019). Sustained and excessive ethanol intake may lead to dependence, a state characterized by a series of neuroadaptive changes in the brain's reward and stress systems (Koob & Le Moal, 2008). Currently, the therapeutical arsenal for alcohol use disorders (AUD) is quite limited, and these therapeutic drugs show low efficacy and poor patient acceptance leading to an underutilization of such drugs in clinical practice (Heilig et al., 2019). In order to study dependence, we used the chronic intermittent ethanol (CIE) model, a validated model of dependence that uses repeated cycles of vaporization of ethanol, voluntary consumption and withdrawal which subsequently produces an escalation in voluntary ethanol consumption (Becker & Lopez, 2004; Griffin et al., 2009; Lopez et al., 2017; Lopez & Becker, 2005).

The **kynurenine** (KYN) pathway, the main metabolic route of **tryptophan** degradation, has been recently proposed as a new target for modulating drug abuse, seeking, and relapse (Morales-Puerto et al., 2021). Around 40% of KYN is synthesized locally in the mammalian brain, whereas the remaining 60% is captured from the periphery by the large neutral amino acid transporter 1 (LAT-1) (Fukui et al., 1991), whereas efflux of KYN from the brain depends on the organic anion transporter (OAT1) (Wu et al., 2017). From KYN, this pathway separates into two branches: (1) the kynurenine 3-monooxygenase (KMO) branch generates quinolinic acid, a metabolite with neurotoxic actions (Guillemin et al., 2001), and (2) the enzyme **kynurenine aminotransferase** (KAT) branch which transforms KYN into **kynurenic acid** (KYNA), a neuromodulatory metabolite (Han et al., 2010). Inhibition of KMO using the selective inhibitor Ro 61-8048 (a competitive kynurenine 3-monooxygenase inhibitor) shifts the KYN metabolic pathway towards KYNA production in the brain (Röver et al., 1997) and is effective in reducing self-administration and relapse to drug-seeking induced by several drugs (Justinova et al., 2013; Secci et al., 2017; Secci et al., 2019; Vengeliene et al., 2016). Our laboratory has recently shown that Ro 61-8048 can reduce ethanol consumption in the binge drinking model "Drinking in the Dark" (DID) (Giménez-Gómez et al., 2018). Furthermore, this compound prevents ethanol-induced **dopamine** release in the nucleus accumbens (NAc) shell by increasing brain KYNA concentration in the limbic forebrain (Giménez-Gómez et al., 2018).

In the last decades it has been established that KYNA plays a role modulating the mesolimbic reward pathway because it is a negative allosteric modulator of the **α7 nicotinic acetylcholine receptor**

What is already known

- Ro 61-8048 reduces ethanol consumption in a binge drinking model.
- α7nAChR is implicated in the effect of Ro 61-8048 on ethanol consumption.

What does this study add

- Ro 61-8048 reduces ethanol consumption both in male and female ethanol-dependent mice.
- Reduced ethanol consumption is prolonged by blocking the efflux of kynurenine from the brain.

What is the clinical significance

- Modulation of kynurenine pathway is effective in the treatment of ethanol dependence in both sexes.

(α7nAChR) (Hilmas et al., 2001). The activation of this receptor increases extracellular levels of **glutamate** in glutamatergic neuronal afferents in both the ventral tegmental area (VTA) and NAc, which in turn elicits dopamine release in NAc (Jones & Wonnacott, 2004; Liu et al., 2014; Maex et al., 2014; Schilström et al., 2000). Recently it has been characterized that this effect on glutamate transmission involves astrocytes (Secci et al., 2019).

Despite all this evidence there are no data on the effect of the manipulation of this pathway in animals dependent on ethanol, nor on the role played by the blood–brain barrier KYN transporters. Furthermore, with a view to enhancing the translational value of the results inhibiting of KMO as a possible treatment for ethanol dependence and considering there are data that show that KMO knock-out mice show alterations in memory or anxiety (Erhardt et al., 2017), it is necessary to evaluate the effect of Ro 61-8048 on these important functions in mice dependent on ethanol. Furthermore, there is no research conducted on the effect of the manipulation of this pathway in female mice. A growing body of data in the literature shows that sex differences can influence the neural systems implicated in ethanol dependence (Flores-Bonilla & Richardson, 2019). Therefore, it is of the

utmost importance to include females in studies on the effectiveness of ethanol-dependence treatments from the initial preclinical stages.

The aims of this study were (1) to evaluate the effect of the inhibition of KMO on ethanol consumption and preference in mice of both sexes dependent on ethanol, (2) to investigate the involvement of α7nAChRs in the reduction of ethanol consumption, (3) to determine the effect of blocking the influx or efflux of KYN into or from the brain using L-leucine or probenecid to block LAT-1 or OAT respectively, on consumption after treatment with Ro 61-8048 and, finally, (4) to study the effect of Ro 61-8048 on memory and anxiety.

2 | METHODS

2.1 | Materials

Ro 61-8048 (3,4-dimethoxy-[-N-4-(nitrophenyl)thiazol-2-yl]-benzenesulfonamide; PubChem CID: 5282337) and PNU-120596 (PubChem CID: 311434) were purchased from Tocris (USA; Catalogue nos. 3254 and 2498/5, respectively). L-leucine (PubChem CID: 6106) was obtained from Fisher Scientific (USA; Catalogue no. BP385-100). Pyrazole (PubChem CID: 1048), probenecid (PubChem CID: 4911) and L-KYN sulfate salt (PubChem CID: 161165) were purchased from Sigma-Aldrich (Merck, Germany; Catalogue nos. P56607, P8761 and K3750, respectively). Absolute ethanol and HPLC-grade acetonitrile and methanol were obtained from Panreac AppliChem (Panreac Química, Spain, Catalogue nos. 141086, 361881 and 361091, respectively). Other chemicals were of analytical grade and obtained from Sigma-Aldrich (Merck, Germany).

2.2 | Animals

Adult male C57BL/6J mice (Envigo, USA) weighing 20–25 g (8-weeks-old) were used in all the experiments conducted, except in the study to validate the main result of the manuscript in both sexes where adult female C57BL/6J mice (Envigo, USA) weighing 15–20 g (8-weeks-old) were used. For this study, we used a total of 341 male and 45 female mice. For the determinations of the bood ethanol concentrations (BEC) to adjust the model, five male and five female mice were used. Sixteen male (CIE and no-CIE) and eight females (CIE) mice were used for the measure of the ethanol consumption escalation after the CIE model. For the study of the effect of Ro 61-8048 on ethanol consumption, we used 32 male and 32 female mice. For the study of the effect of PNU-120596 on the Ro 61-8048-induced changes, we used 72 male mice. For the experiments regarding L-leucine and probenecid, we used 64 male mice in each experiment. Finally, we used 24 male mice for the locomotor activity experiment, 32 male mice for the EPM maze test and 32 male mice for the NOR recognition test. The C57BL/6J strain was chosen since it consumes greater amounts of ethanol (Belknap et al., 1993) and it has been shown that the ethanol model employed in this study is more effective in this strain than in others (Lopez et al., 2017). Mice were maintained in conditions of constant temperature (21 ± 2°C) and a 12-h reverse lighting cycle (lights on at 8:00 PM). Animals were housed in groups of eight with ad libitum access to food and water for 5 days. Mice were then individually housed, randomly allocated into water or ethanol (CIE) groups and habituated for 7 days to drinking water ad libitum from a 25-ml serological pipette fitted to a drinking spout. All experimental procedures were vetted by the Animal Welfare Committee of the Universidad Complutense de Madrid and approved by the Comunidad de Madrid (ethical approval reference: PROEX 066/17), following the European Union Directive 2010/63/EU. Animal studies are reported in compliance with the ARRIVE guidelines (du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

2.3 | Drug administration

Ethanol for oral consumption was diluted in water to a concentration of 15% v/v and for injection was prepared in 0.9% w/v NaCl (saline) at a concentration of 20% v/v and administered i.p. at a dose of 1.6 g·kg^{-1}. Pyrazole was dissolved in saline and injected i.p. in a volume of 10 ml·kg^{-1} at a dose of 1 mmol·kg^{-1}.

Ro 61-8048 was dissolved in saline containing 60 mM NaOH, adjusted to pH 7.4 and injected i.p. in a volume of 10 ml·kg^{-1} at a dose of 50 mg·kg^{-1}. This compound has been described in the literature as having no ill-effects on animals at higher doses than the dose used in this study (Clark et al., 2005; Rodgers et al., 2009) and is effective at reducing consumption of ethanol in a binge-drinking paradigm (Giménez-Gómez et al., 2018). A single dose of Ro 61-8048 was injected 30 min before the beginning of the last day of ethanol consumption, under the two-bottle choice limited access procedure in all experiments. We selected this time to minimize stress to mice that might alter drinking behaviour, taking into account that Ro 61-8048 completely inhibits KMO 30 min after administration (Röver et al., 1997).

PNU-120596 was dissolved in a vehicle containing 12% DMSO and 8% Tween 80 in water and injected i.p. in a volume of 10 ml·kg^{-1} at a dose of 3 or 8 mg·kg^{-1} 30 min before Ro 61-8048 injection. PNU-120596 itself does not affect KYN levels and does not modify ethanol consumption in the DID paradigm (Giménez-Gómez et al., 2018) nor modifies self-administration of other drugs of abuse (Justinova et al., 2013; Secci et al., 2017). Moreover, this compound does not affect locomotor activity at the high dose used in this study (Freitas et al., 2013).

L-leucine was dissolved in 6% HCl in PBS, adjusted to pH 7.4 and injected i.p. in a volume of 10 ml·kg^{-1} at a dose of 300 mg·kg^{-1}. A single dose of L-leucine was administered alone (control groups) or 30 min before Ro 61-8048 injection. At this dose L-leucine blocks the influx of KYN into the brain (Walker et al., 2018).

Probenecid was prepared in 0.1-M NaOH, adjusted to pH 7.4 and injected i.p. in a volume of 10 ml·kg^{-1} at a dose of 100 mg·kg^{-1}. A single dose of probenecid was administered alone (control groups) or 30 min before Ro 61-8048 injection. At this dose, probenecid produces an accumulation of KYN in the brain (Moroni et al., 1988; Vécsei et al., 1992).

2.4 | Experimental design

The CIE paradigm is an ethanol dependence and relapse drinking model described by Becker and Lopez (Becker & Lopez, 2004; Griffin et al., 2009; Lopez et al., 2017; Lopez & Becker, 2005), which involves repeated cycles of chronic intermittent exposure to ethanol vapour in inhalation chambers and then periods of withdrawal, generating a progressive escalation in voluntary ethanol consumption and simulating the transition to ethanol dependence (Figure 1a).

First, mice were subjected to a two-bottle choice limited access procedure for 2 weeks to stablish the baseline of ethanol and water intake. They had access to two serological pipettes fitted to a drinking spout, one containing ethanol (15% v/v) and the other water, for 2 h from Monday to Friday. The rest of the time they had access to water ad libitum. Water intake (ml), ethanol intake (ml) and preference for ethanol were measured every day. These data were used to calculate self-administered ethanol dose (i.e., $g \cdot kg^{-1}$), water intake (i.e., $ml \cdot kg^{-1}$) and relative preference for ethanol (i.e., ethanol intake/total fluid intake $\times$ 100). The position of the pipettes in the cages was alternated daily to avoid place preference. Water groups did not have access to ethanol any day. Once stable baseline of ethanol and water intake was established, water (Control) and CIE groups were randomly subdivided into groups ($n = 8$) according to the treatment they would receive on the last day of the experiment. CIE groups received chronic intermittent exposure to ethanol vapour in inhalation chambers (16 h per day for 4 days) whereas water groups inhaled moist air. An additional group, no-CIE group ($n = 8$), which inhaled air (no exposure to ethanol vapour) and then drank ethanol, was included in order to determine the effect of ethanol vapours on ethanol consumption. Air flux and ethanol concentration in the inhalation chambers were adjusted each day to provide a plasmatic ethanol concentration between 150 and 250 $mg \cdot dl^{-1}$ (Becker & Lopez, 2004; Griffin
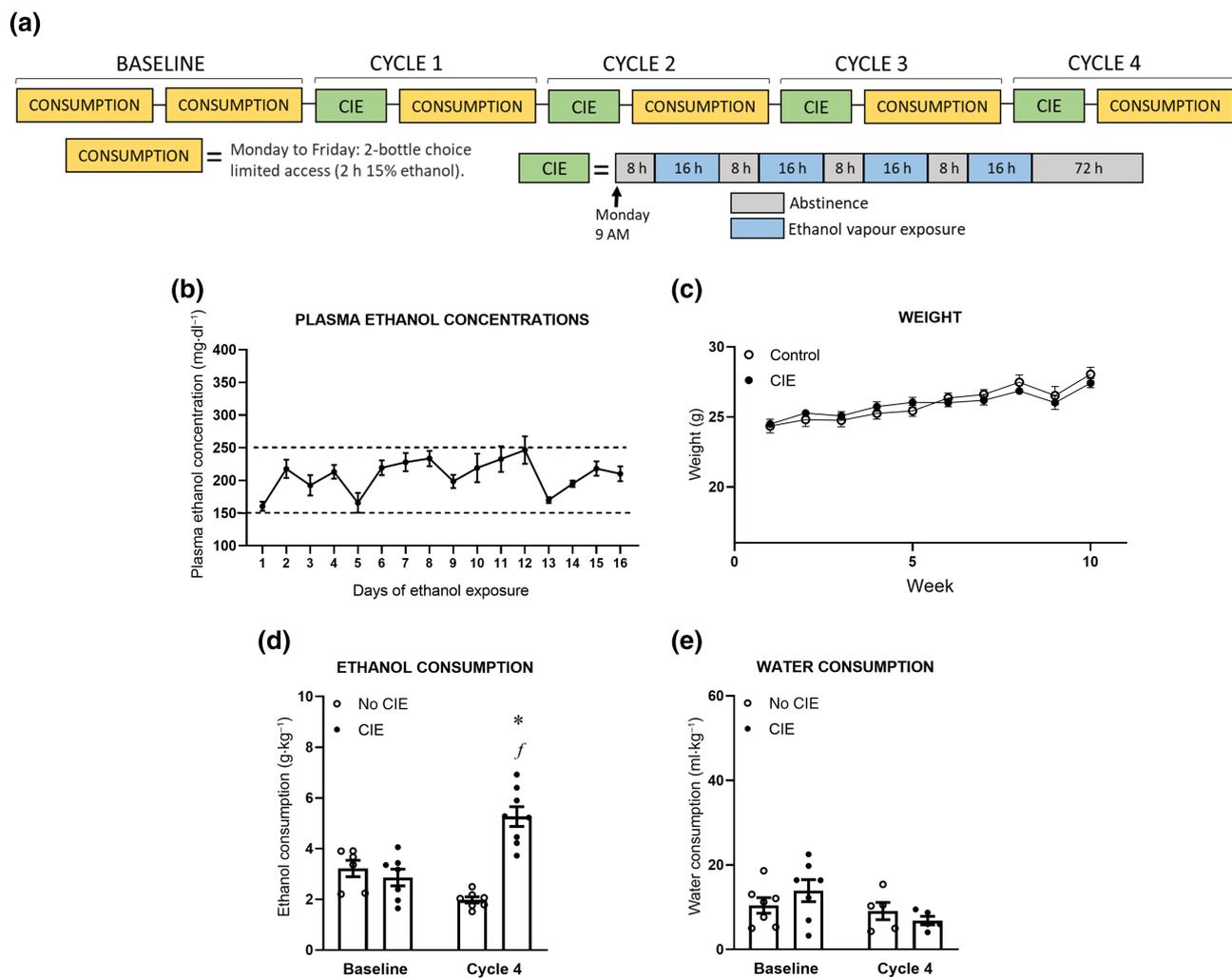
FIGURE 1 Effect of CIE model on male mice. (a) Schedule of the protocol used, (b) plasmatic ethanol concentrations at the end of each day of exposure of ethanol vapour (total of 16 days of ethanol exposure during four cycles), (c) weight of mice submitted to ethanol vapour (CIE groups) or air (water groups), (d) ethanol consumed at the beginning and at the end of CIE model, and (e) water consumed at the beginning and at the end of CIE model. Results are shown as mean ± SEM, $n = 5$ for (b); $n = 8$ for (c); baseline no-CIE = 6, baseline CIE = 7, cycle 4 no-CIE = 7, cycle 4 CIE = 8 for (d); baseline no-CIE = 7, baseline CIE = 7, cycle 4 no-CIE = 5, cycle 4 CIE = 5 for (e). Different from baseline no-CIE: *$P < 0.05$; different from cycle 4 no-CIE: $^{f}P < 0.05$

et al., 2009; Lopez et al., 2017; Lopez & Becker, 2005) (Figure 1b). In order to avoid stress to animals or affect the behaviour, we only measured BEC to adjust the model with the first batch of animals. Before introducing mice in the chambers, they were injected i.p. with a dose of ethanol (1.6 g·kg^{-1}) and pyrazole (1 mmol·kg^{-1}; an alcohol dehydrogenase inhibitor), in order to maintain a stable level of intoxication during the 16 h of exposure to ethanol (Becker & Lopez, 2004; Griffin et al., 2009; Lopez et al., 2017; Lopez & Becker, 2005). Water and no-CIE animals received saline and pyrazole. After the fourth inhalation session of 16 h, mice were subjected to a 72-h forced abstinence period after which they were once again exposed for five consecutive days to the same voluntary ethanol consumption model (two-bottle choice limited access procedure) as during baseline. This pattern of chronic exposure to ethanol followed by 5 days of limited access to ethanol was repeated for four consecutive cycles. Mice were weighed once a week. It is interesting to note that animals that underwent the CIE paradigm showed no difference in weight gain when compared with animals who were exposed to air. Two-way ANOVA analysis indicated an effect of time with no effect of treatment and no interaction between factors (Figure 1c). Regarding ethanol consumption after vapour exposure, in line with what is described in the literature, the CIE paradigm produced an escalation in voluntary ethanol consumption. Two-way ANOVA indicated that there was no difference of time, but there was an effect of treatment and an interaction between factors. The Bonferroni post hoc test indicated a significant elevation of ethanol consumption in mice treated with the CIE paradigm on the fourth cycle of ethanol (Figure 1d). As an internal control, we checked the escalation in consumption in all the mice employed in this study, finding the same pattern as we show here. With regard to water consumption, two-way ANOVA showed that there was no significant difference in time nor treatment and no interaction between factors (Figure 1e). In the study involving females, we adapted the air flux to obtain a similar plasmatic concentration of ethanol as that in males (Figure S1A). As occurred in males, females that underwent the CIE paradigm showed no difference in weight gain compared with animals who were exposed to air. Two-way ANOVA analysis indicated an effect of time with no effect of treatment and no interaction between factors (Figure S1B). Regarding ethanol consumption after vapour exposure, as expected, the CIE model produced an increase in ethanol consumption in the fourth cycle versus basal levels (Figure S1C) while water consumption remained stable (Figure S1D).

2.5 | Sample extraction

Immediately after removal of the ethanol in the two-bottle choice limited access procedure, mice were killed by cervical dislocation. Brains were removed and limbic forebrain dissected out over ice using a mouse brain matrix for coronal slices (World Precision Instruments, USA). After removal of the olfactory bulbs, brain tissue anterior to the optic chiasm was collected and referred to as "limbic forebrain" (Wang et al., 2003). This brain region was studied because we had previously demonstrated that a pharmacologically-induced increase in KYN subsequently reduced

ethanol consumption in the DID paradigm (Giménez-Gómez et al., 2018). Samples were stored at $-80°$C. Trunk blood was collected in 10 ml K$_2$-EDTA tubes (BD, USA) and immediately centrifuged twice at $1300 \times g$ ($4°$C) for 10 min to obtain plasma which was stored at $-80°$C. Trunk blood was used for all the experiments except for the determination of plasmatic ethanol concentration. Levels of ethanol in both sexes were determined in blood extracted from the tail of mice immediately after removal from the inhalation chambers to confirm that the dose of vaporized ethanol was correct. A small incision was made in the tail and 25 µl of blood collected with a pipette containing 5 µl of K$_2$-EDTA. Different animals were used for the determination of plasmatic ethanol concentrations and for the rest of the experiments.

2.6 | KYN measurements in plasma and limbic forebrain

Plasma samples were deproteinized by adding 75 µl of 6% perchloric acid to the mixture of 50 µl plasma and 125 µl of water. The acidified plasma was vortexed and kept at room temperature for 10 min, and then centrifuged for 15 min at $16000 \times g$ at $4°$C. Limbic forebrain samples were homogenized in five volumes of deionized water by sonication (Labsonic 2000 U, B. Braun Melsungen AG, Germany) at 30% amplitude during 15 s. Samples were deproteinized by adding 25 µl of 6% perchloric acid per 100 µl of homogenate, vortexed and kept at room temperature for 10 min. Samples were then centrifuged for 15 min at $16000 \times g$ at $4°$C. After centrifugation, supernatants were collected and frozen at $-80°$C until analysis. Sixty (limbic forebrain) or twenty (plasma) microliters of supernatant were applied to a reversed phase column (80 mm $\times$ 4.6 mm, 3 mm; HR-80; Thermo Fisher Scientific, USA), and KYN was isocratically eluted using a mobile phase containing 0.1-M sodium acetate and 4% acetonitrile, pH 4.6, at a flow rate of 1 ml·min^{-1}. KYN was measured by UV detection (360 nm, 2487 UV detector; Waters, USA).

2.7 | Blood ethanol content measurements

Twenty-five microlitres of blood were collected in 1.5-ml Eppendorf tubes with EDTA (final concentration 10%) from the tail of animals anaesthetised with **isoflurane**. The samples were centrifuged at $1300 \times g$ at $4°$C for 10 min, and 5 µl of plasma were injected into an ethanol analyser (AM1, Analox, UK).

2.8 | Locomotor activity

The locomotor activity test was performed after the 2 h of consumption on the last day of the two-bottle choice limited access procedure of the fourth cycle following the previously described protocol (Espejo-Porras et al., 2013). The locomotor activity of the animals was analysed using a computer-assisted actimeter (Actitrack, Panlab, Spain). This apparatus consists of a 45 $\times$ 45 cm enclosure that has infrared rays around it

located 1 cm from the ground and 2.5 cm apart. These are coupled to a control unit that analyses different parameters of the animal's locomotor behaviour. The horizontal activity (distance travelled in cm) of the mice was analysed for 10 min by a blinded observer.

2.9 | Novel object recognition

Novel object recognition test (NOR) was performed to determine whether Ro 61-8048 pretreatment produces alterations in memory. The NOR test was performed after the 2 h of consumption on the last day of the two-bottle choice limited access procedure of the fourth cycle. Mice were transported immediately after the consumption to the dimly lit test room and allow to adapt for 30 min. Next, the mice were placed in an open box (24 × 24 × 15 cm) with two identical objects and were allowed to freely explore it for 3 min. They were then returned to their home cage for 1 min and one of the objects was replaced by a new and different object. The animals were again placed in the box for another 3 min and allowed to explore. The total process was recorded by video camera and later analysed by a blinded observer. Object exploration was defined as intentional contact of the mouse's snout or front paws with the object from a distance of 2 cm or less. The sum of the time exploring the old (familiar) and the new (novel) objects in the second exposure was measured. The discrimination index (DI) was calculated as: $DI = (t_{novel} - t_{familiar})/(t_{novel} + t_{familiar}) \times 100\%$ (Ledesma et al., 2017).

2.10 | Elevated plus maze

The elevated plus maze (EPM) paradigm was performed to determine the effect of Ro 61-8048 pretreatment on anxiety. The apparatus was made of clear Plexiglas and consisted of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm) forming a central platform (5 × 5 cm) at the point of intersection. The entire apparatus was elevated 45 cm above floor level. The EPM test was performed after the 2 h of consumption on the last day of the two-bottle choice limited access procedure of the fourth cycle. Mice were transported immediately after the consumption to the dimly lit test room and allow to adapt for 30 min. Then the animal was placed on the central platform facing an open arm and was allowed to explore the maze freely for 5 min. The maze was cleaned thoroughly between trials with a 10% ethanol solution and dried with a cloth. The behaviour displayed by the mice was video recorded and later analysed by a blinded observer. The number of entries in open arms and the percentage of time in the open arms were used as an indicator of anxiety (Ledesma et al., 2017). In order to avoid interferences between tests, we used different groups of mice for each behavioural test.

2.11 | Data and statistical analyses

The data and statistical analysis comply with the recommendations the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Initial group size ($n = 8$) was selected based on our previous experience to ensure a statistical significance considering the possible loss of mice due to the application of the criteria of humane endpoints if physical signs other than ethanol intoxication were observed, loss of consumption data or outlier values (determined by the extreme studentized deviate method [ROUT test] with significance level of Q = 1%). These three conditions were the only exclusion criteria employed and account for differences in group sizes. Data are presented as mean ± SEM. The threshold for statistical significance was set at $P < 0.05$. Comparisons between two groups were analysed by an unpaired Student's t test. Statistical differences between more than two groups were studied by one-way ANOVA followed by Tukey's post hoc test. Results defined by two factors were evaluated by two-way ANOVA. In the cases of interaction between factors, relevant differences were analysed by post hoc comparison using the Bonferroni test. Post hoc analyses were only carried out when the threshold for statistical significance had been reached and no significant variance in homogeneity was detected. All statistical analyses were performed using GraphPad Prism 8.0 program (GraphPad Software Inc., USA).

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Pawson, Southan, et al., 2021; Alexander, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Pawson, Southan, Buneman, et al., 2021; Alexander, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Pawson, Southan, Davies, et al., 2021; Alexander, Mathie, Peters, Veale, Striessnig, Kelly, Armstrong, Faccenda, Harding, Pawson, Southan, et al., 2021).

3 | RESULTS

3.1 | KMO inhibition using Ro 61-8048 decreases consumption of and preference for ethanol in the CIE model

3.1.1 | Effect of Ro 61-8048 in male mice

In the group treated with ethanol vapour, Ro 61-8048 (50 mg·kg⁻¹, i.p.), administered 30 min before the last day of ethanol consumption, markedly decreased ethanol ingestion (Figure 2a) without modifying water consumption (Figure 2b). Ro 61-8048 also reduced ethanol preference (Figure 2c). Ro 61-8048 did not alter water consumption (Figure 2d) in the mice from water groups.

Ro 61-8048 greatly increased KYN concentration in both plasma (Figure 2e) and limbic forebrain (Figure 2f), confirming its
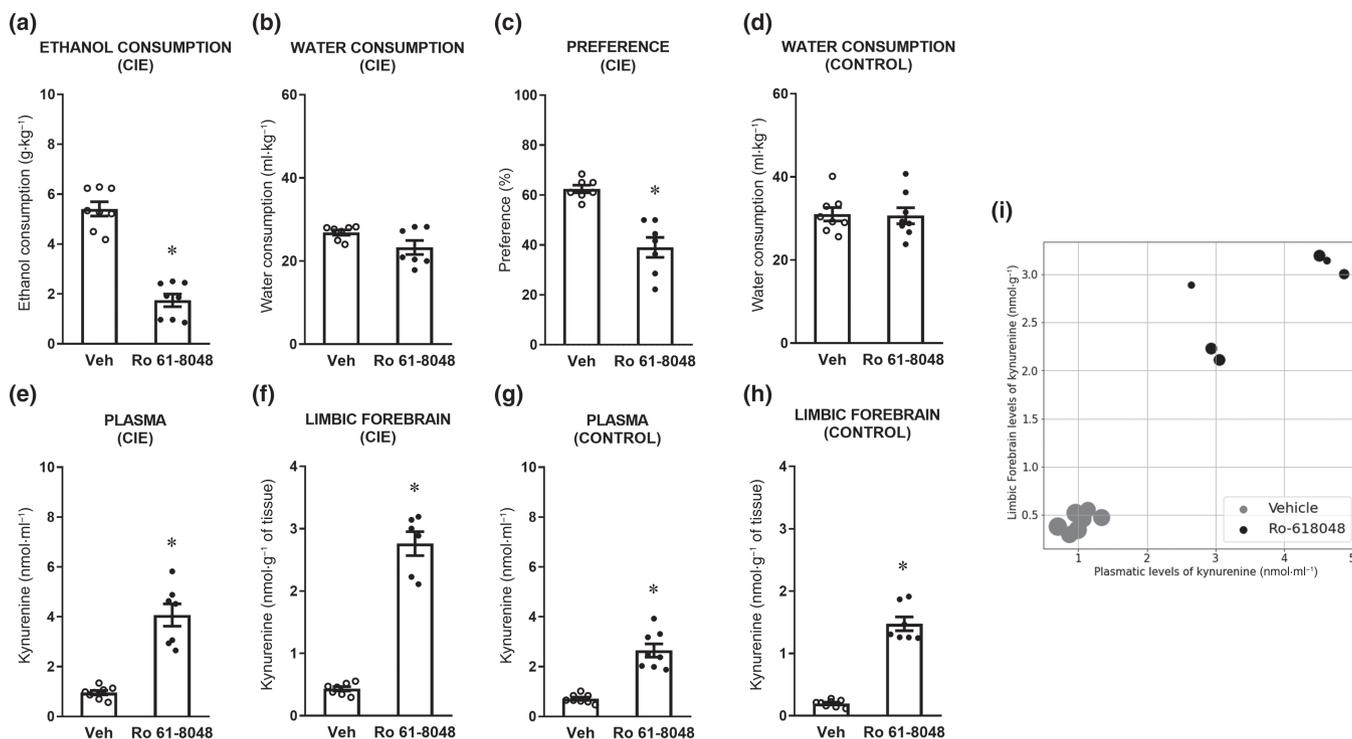
FIGURE 2 Effect of KMO inhibition using Ro 61-8048 (50 mg·kg^{-1}, i.p.) in the CIE model in male mice. Effect of Ro 61-8048 on (a) ethanol consumption, (b) water consumption and (c) preference for ethanol in CIE groups. (d) Effect of Ro 61-8048 on water consumption in water groups. KYN concentrations in (e) plasma and (f) limbic forebrain in CIE groups and (g) plasma and (h) limbic forebrain concentrations of KYN in water groups. (i) Relationship between the consumption of ethanol and KYN concentrations in plasma and in limbic forebrain. Results are shown as mean ± SEM, $n = 8$ for (a), (d) and (g); $n = 7$ for (b) and (c); Vehicle = 8 and Ro = 7 in (e) and (h); Veh = 7 and Ro = 6 in (f). Different from Vehicle (Veh): *$P < 0.05$

inhibition of KMO in the CIE model. Similarly, Ro 61-8048 increased both plasma and limbic forebrain KYN concentrations in the water groups (Figure 2g,h, respectively). Taken together the data demonstrate that mice who received Ro 61-8048 showed higher concentrations of KYN in both plasma and limbic forebrain and consumed less ethanol than mice who received vehicle (Figure 2i).

3.1.2 | Effect of Ro 61-8048 in female mice

Ro 61-8048 also decreased ethanol ingestion (Figure 3a) and preference (Figure 3c) without affecting water consumption (Figure 3b) in female mice previously exposed to ethanol vapours. Regarding water groups, Ro 61-8048 did not alter water consumption (Figure 3d). Ro 61-8048 greatly elevated KYN levels in both plasma (Figure 3e,g) and limbic forebrain (Figure 3f,h) of CIE and water groups, respectively. Our data demonstrate that, as occurs in male mice, female mice receiving Ro 61-8048 showed higher levels of KYN in both plasma and limbic forebrain and consumed less ethanol than mice who received vehicle (Figure 3i).

3.2 | Ro 61-8048 induced decrease in ethanol consumption and preference is prevented by the positive allosteric modulator of α7 nicotinic acetylcholine receptors (α7nAChR) PNU-120596

Ro 61-8048 produces a large elevation in brain KYN which is involved in the reduction of ethanol intake after CIE exposure. It is well established that KYNA concentration also increases after Ro 61-8048 (Giménez-Gómez et al., 2018; Justinova et al., 2013; Secci et al., 2017). Since KYNA is an endogenous negative allosteric modulator of α7nAChR (Hilmas et al., 2001; Stone, 2007), a receptor that can regulate the consumption of ethanol and other drugs of abuse by modulating dopamine release in the VTA and NAc (Giménez-Gómez et al., 2018; Justinova et al., 2013; Secci et al., 2017), the involvement of these receptors in the Ro 61-8048 induced decrease in ethanol consumption in the CIE model was evaluated. One-way ANOVA revealed an overall effect of treatment on ethanol consumption (Figure 4a) and no change in water consumption (Figure 4b). Post hoc analysis revealed that PNU-120596 (3 or 8 mg·kg^{-1}, i.p.), a positive modulator of α7nAChR, administered 30 min before Ro 61-8048 (50 mg·kg^{-1}, i.p.) attenuated or prevented, respectively, the Ro
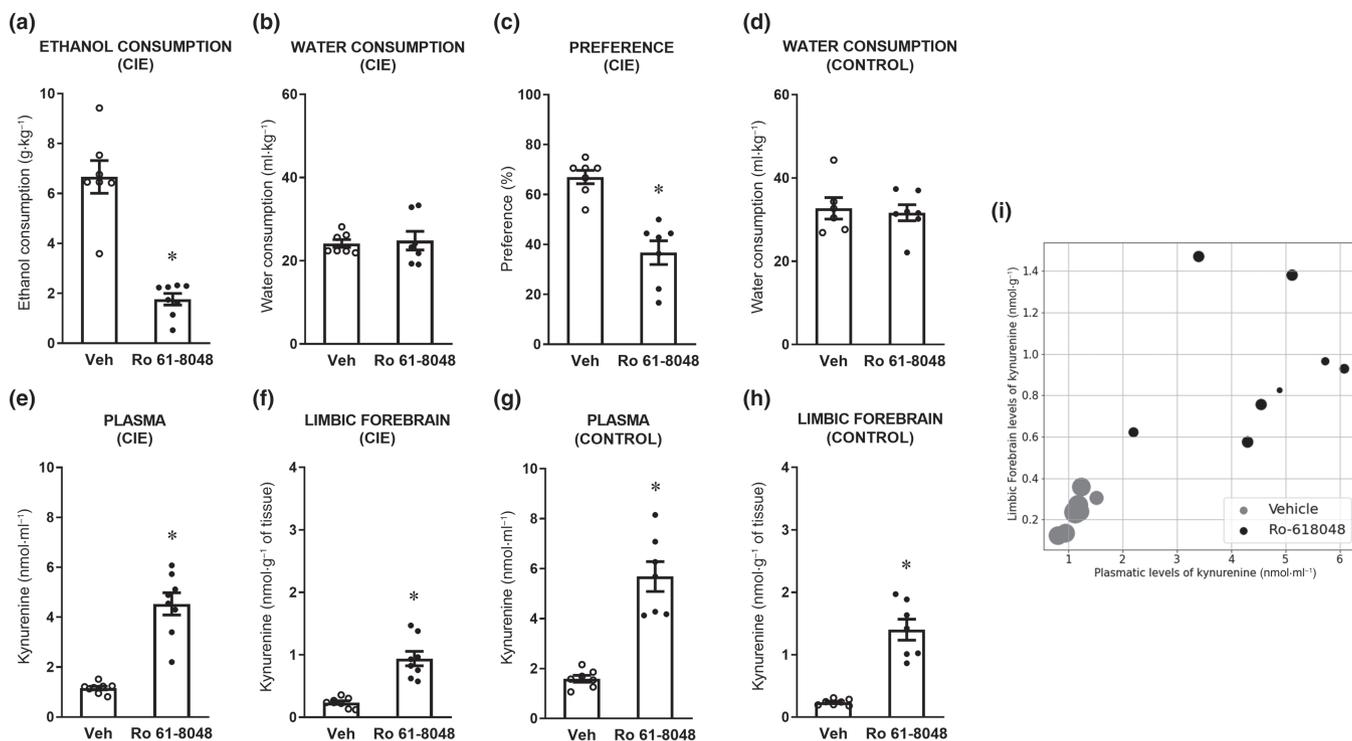
FIGURE 3 Effect of KMO inhibition using Ro 61-8048 (50 mg·kg^{-1}, i.p.) in the CIE model in female mice. Effect of Ro 61-8048 on (a) ethanol consumption, (b) water consumption and (c) preference for ethanol in CIE groups. (d) Effect of Ro 61-8048 on water consumption in water groups. KYN concentrations in (e) plasma and (f) limbic forebrain in CIE groups and (g) plasma and (h) limbic forebrain concentrations of KYN in water groups. (i) Relationship between the consumption of ethanol and KYN concentrations in plasma and in limbic forebrain. Results are shown as mean ± SEM, $n = 8$ for (e) and (f); $n = 7$ for (b), (c), (g) and (h); Vehicle = 7 and Ro = 8 in (a); Veh = 6 and Ro = 7 in (d). Different from Vehicle (Veh): *$P < 0.05$

61-8048-induced decrease in ethanol consumption. Similarly, there was an overall effect of treatment on preference (Figure 4c). Post hoc analysis revealed that PNU-120596 prevented the decreased preference induced by Ro 61-8048.

There was an overall effect of treatment on KYN levels in both plasma (Figure 4d) and limbic forebrain (Figure 4e). Post hoc analysis revealed that neither dose of PNU-120596 (3 or 8 mg·kg^{-1}, i.p.) altered the Ro 61-8048-induced increase in KYN levels in plasma and limbic forebrain.

PNU-120596 itself did not modify ethanol or water intake and did not alter the concentration of KYN in plasma or limbic forebrain.

Regarding the water groups, there were no changes in water consumption (Figure S2A). Similar to what was observed in CIE groups, there was an overall effect of treatment on KYN concentration in plasma (Figure S2B) and limbic forebrain (Figure S2C). Post hoc analysis showed that both Ro 61-8048 + PNU-120596 treated groups showed elevations in KYN concentrations independent of the dose of PNU-120596 administered (3 or 8 mg·kg^{-1}, i.p.). Again, water intake and KYN levels were not altered by PNU-120596 itself.

3.3 | Ro 61-8048 induced decrease in ethanol consumption and preference depends on the presence of KYN in brain

L-leucine (300 mg·kg^{-1}, i.p.) was administered 30 min before Ro 61-8048 (50 mg·kg^{-1}, i.p.) on the last day of ethanol consumption.

In CIE groups, two-way ANOVA revealed an effect of Ro 61-8048, of L-leucine and an interaction between factors. The Bonferroni post hoc analysis revealed that Ro 61-8048 decreased ethanol consumption and this was prevented by L-leucine pretreatment (Figure 5b). There were no changes in water consumption due to Ro 61-8048 or L-leucine and no interaction between factors (Figure 5c). Regarding ethanol preference, there was an effect of both Ro 61-8048 and L-leucine and an interaction between factors. Post hoc analysis indicated that Ro 61-8048 reduced ethanol preference, an effect that was prevented by pretreatment with L-leucine (Figure 5d).

As expected, Ro 61-8048 increased KYN in plasma but L-leucine did not and there was no interaction between the factors (Figure 5e). Interestingly, in limbic forebrain two-way ANOVA analysis revealed an effect of Ro 61-8048 and of L-leucine and an interaction between the factors. Post hoc analysis indicated that Ro 61-8048 produced an elevation of KYN concentration which was prevented by pretreatment with L-leucine (Figure 5f).

In the water groups, water consumption was not altered by Ro 61-8048 or by L-leucine, and there was no interaction between the factors (Figure S3A). KYN concentrations in plasma were increased by Ro 61-8048 with no effect of L-leucine and no interaction between the factors (Figure S3B). In limbic forebrain, we found an effect of Ro 61-8048 and of L-leucine and an interaction between the factors. Post hoc analysis indicated that pretreatment with L-leucine
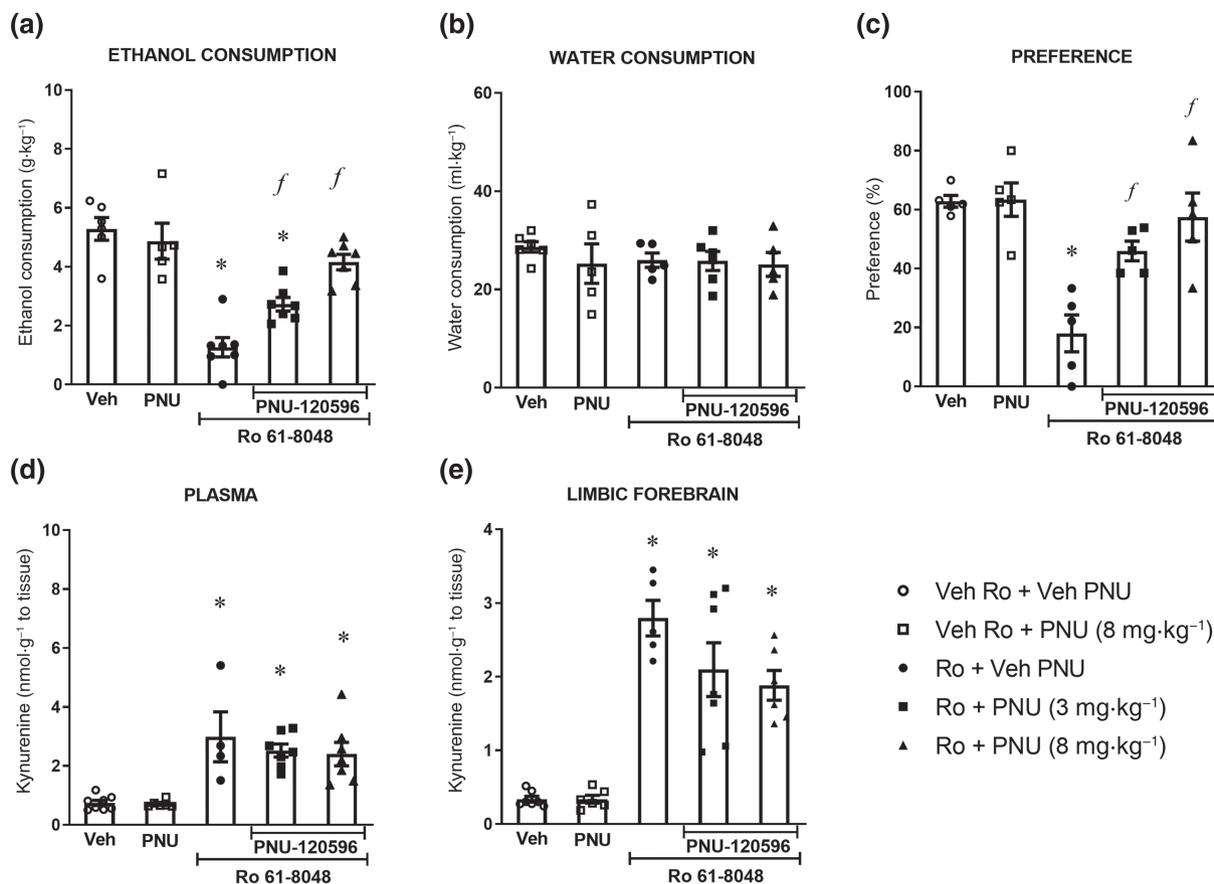
FIGURE 4 Effect of the modulator PNU-120596 (3 and 8 mg·kg^{-1}, i.p.) on the Ro 61-8048 (50 mg·kg^{-1}, i.p.)-induced changes in the CIE model in male mice. Effect of Ro 61-8048 and PNU-120596 on (a) ethanol consumption, (b) water consumption, (c) preference for ethanol, and KYN concentrations in (d) plasma and (e) limbic forebrain. Results are shown as mean ± SEM. Five groups are represented in each graph, the *n* of each group was for (a) 6, 5, 7, 7, 7; for (b) 6, 5, 5, 6, 5; for (c) 5, 5, 5, 5, 5; for (d) 8, 6, 5, 7, 7 and for (e) 7, 6, 5, 7, 6. Different from Vehicle (Veh): *$P < 0.05$. Different from Ro 61-8048: $^{f}P < 0.05$

prevented the elevation of KYN concentrations produced by Ro 61-8048 in the forebrain (Figure S3C).

3.4 | Ro 61-8048 induced decrease in ethanol consumption and preference can be prolonged by maintaining KYN in the brain

To elucidate if the effect of Ro 61-8048 (50 mg·kg^{-1}, i.p.) in the CIE model could be prolonged and thus maintain mice consuming small amounts of ethanol for longer, probenecid (100 mg·kg^{-1}, i.p.) was administered 30 min before Ro 61-8048 (50 mg·kg^{-1}, i.p.) on the last day of ethanol consumption. After 2 h of consumption, there was an effect of Ro 61-8048 on ethanol consumption but no effect of probenecid nor an interaction between factors (Figure S4A). There were no differences in water consumption due to Ro 61-8048 nor due to probenecid and no interaction between factors (Figure S4B). Finally, similar to what was observed in consumption, Ro 61-8048 reduced the preference for ethanol with no effect of probenecid and no interaction between factors (Figure S4C).

Interestingly, the results obtained after 12 h of consumption revealed an effect of Ro 61-8048 and probenecid on ethanol consumption and an interaction between factors. The Bonferroni post hoc test showed that probenecid reduced ethanol consumption in Ro 61-8048-treated mice at 12 h (Figure 6b). Thus, although probenecid did not alter the effect of Ro 61-8048 at an early time-point (2 h), it did prolong the effect of Ro 61-8048 on ethanol consumption. There were no changes in water consumption due to Ro 61-8048 or probenecid and no interaction between factors (Figure 6c). Finally, as with ethanol consumption, preference was affected by Ro 61-8048 and by probenecid and there was an interaction between factors. Post hoc analysis showed that probenecid reduced ethanol preference in Ro 61-8048-treated mice at 12 h (Figure 6d).

No differences in water consumption were detected at 2 h (Figure S4D) nor at 12 h (Figure S4E).

In the CIE group, KYN levels in plasma were modified by Ro 61-8048 but not by probenecid, and there was no interaction between factors (Figure 6e). In limbic forebrain, there was an effect of Ro 61-8048 and of probenecid and an interaction between the factors. The Bonferroni post hoc test indicated that probenecid in combination
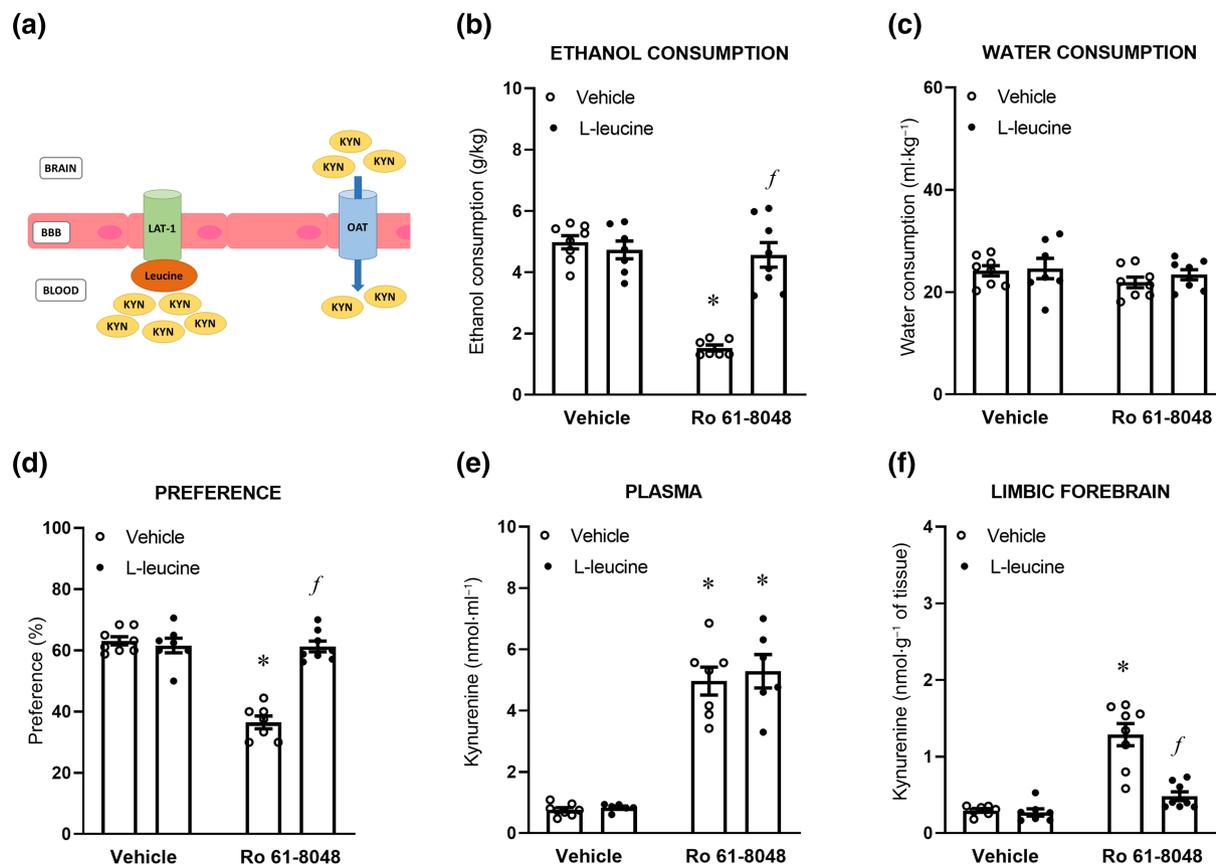
FIGURE 5 Effect of the blockade of the influx of KYN into the brain using L-leucine (300 mg·kg^{-1}, i.p.) on the Ro 61-8048 (50 mg·kg^{-1}, i.p.)-induced changes in the CIE model in male mice. (a) Schematic representation of the action of L-leucine in the brain. Effect of Ro 61-8048 and L-leucine on (b) ethanol consumption, (c) water consumption, (d) preference for ethanol, and KYN concentrations in (e) plasma and (f) limbic forebrain. Results are shown as mean ± SEM. Four groups are represented on each graph, the n of each group was for (b) 8, 7, 7, 8; for (c) 8, 7, 8, 8; for (d) 8, 7, 7, 8; for (e) 8, 6, 7, 6 and for (f) 7, 7, 8, 8. Different from Vehicle (Veh): *$P < 0.05$. Different from Ro 61-8048: $^f P < 0.05$

with Ro 61-8048 maintains the levels of KYN in the limbic forebrain elevated for longer than if Ro 61-8048 were administered alone (Figure 6f). Similarly in the water groups, in plasma, there was an effect of Ro 61-8048 but no effect of probenecid nor an interaction between factors (Figure S4F). In limbic forebrain, there was an effect of Ro 61-8048 and of probenecid and an interaction between factors. The Bonferroni post hoc test indicated that probenecid in combination with Ro 61-8048 maintains the limbic forebrain KYN levels elevated for longer than if Ro 61-8048 were administered alone (Figure S4G).

3.5 | KMO inhibition by Ro 61-8048 does not alter locomotor activity, memory or anxiety in mice exposed to the CIE model

To rule out that Ro 61-8048 administration and the subsequent elevation of KYN might produce alterations in locomotor activity, anxiety or memory, parameters which, on the other hand, are closely associated with ethanol dependence, the locomotor activity test, the NOR test or the EPM test was performed immediately after the consumption period on the last day of ethanol consumption in different groups

of mice to avoid possible contamination between tests. We did not observe changes in the locomotor activity, measured as the distance travelled, due to ethanol, Ro 61-8048 nor an interaction between factors (Figure 7a). In the EPM we found no changes due to ethanol, Ro 61-8048 nor an interaction between factors in number of entries to open arms nor in the time spent in open arms (%) (Figure 7b,c). Similarly, in NOR, there were no significant differences in the discrimination index (Figure 7d).

4 | DISCUSSION

This research study demonstrates, for the first time, that KMO inhibition and the consequent elevation in KYN levels reduces both ethanol consumption and preference for ethanol in a model of ethanol dependence using C57/BL6J mice. Moreover, this is the first evidence that indicates Ro 61-8048 also is effective in ethanol dependent females. The decrease in the amount of ethanol consumed is due to the inhibition of KMO and the accumulation of KYN, and possibly KYNA which acts by stimulating the α7nAChR to produce its effect. In addition, we show that the cerebral accumulation of KYN is necessary to produce
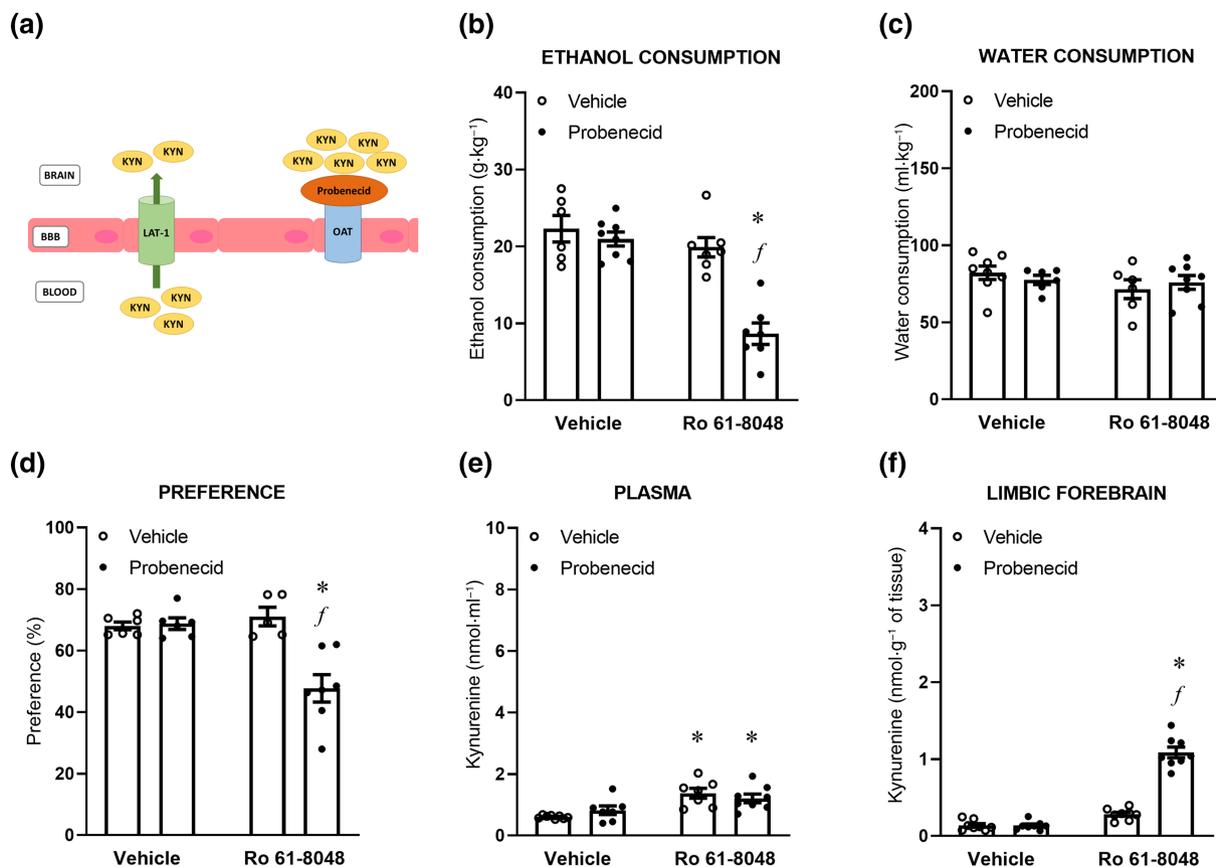
FIGURE 6 Effect of the blockade of the efflux of KYN from the brain using probenecid (100 mg·kg^{-1}, i.p.) on the Ro 61-8048 (50 mg·kg^{-1}, i. p.)-induced changes in the CIE model after 12 h of consumption in male mice. (a) Schematic representation of the action of probenecid in the brain. Effect of Ro 61-8048 and probenecid on (b) ethanol consumption, (c) water consumption, (d) preference for ethanol, and KYN concentrations in (e) plasma and (f) limbic forebrain after 12 h of consumption (f). Results are shown as mean ± SEM. Four groups are represented on each graph, the *n* of each group was for (b) 6, 8, 7, 7; for (c) 8, 6, 6, 8; for (d) 6, 6, 5, 7; for (e) 8, 7, 7, 8 and for (f) 8, 8, 8, 8. Different from Vehicle (Veh): *$P < 0.05$. Different from Ro 61-8048: $^f P < 0.05$
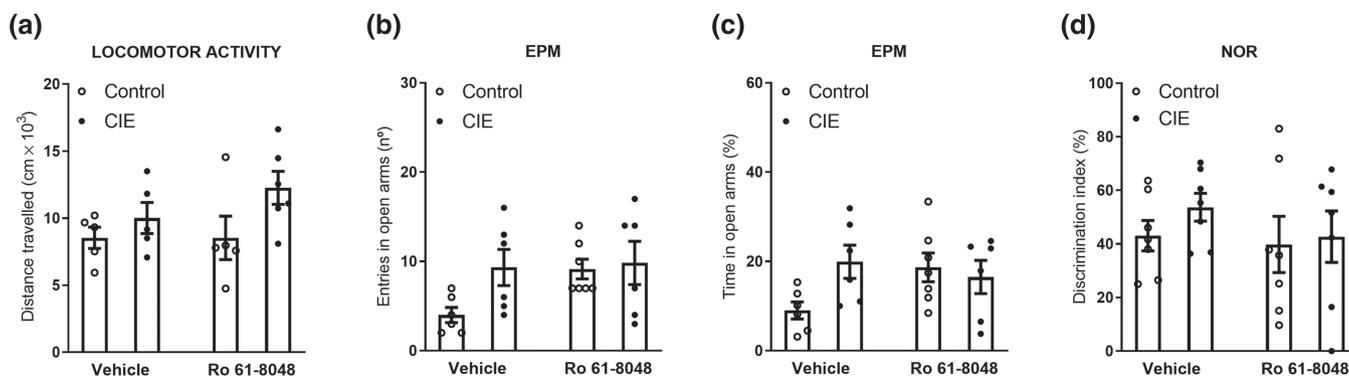
FIGURE 7 Effect of the CIE model of ethanol consumption and KMO inhibition using Ro 61-8048 (50 mg·kg^{-1}, i.p.) on (a) locomotor activity, (b) number of entries into open arms in the elevated plus-maze test, (c) time spent in open arms (%) in the elevated plus-maze test and (d) discrimination index (%) in the novel object recognition in male mice. Results are shown as mean ± SEM. Four groups are represented on each graph, the *n* of each group in (a) was 5, 5, 5, 6, in (b) was 6, 6, 7, 6; for (c) was 6, 6, 7, 6 and for (d) was 7, 7, 7, 7

the observed modification in the consumption of ethanol, since blockade of KYN influx into the brain prevents the effect of Ro 61-8048 on ethanol consumption. Furthermore, maintaining KYN in the brain via the blockade of its efflux from the brain results in Ro 61-8048 remaining effective after 12 h of ethanol consumption.

Recently, our group has shown that modifications in the kynurenine pathway are involved in the decrease of ethanol intake in the DID voluntary binge model of consumption (Giménez-Gómez et al., 2018). However, to date, there are no data on the effect of manipulating the kynurenine pathway in a model of ethanol

dependence. We therefore studied the effect of Ro 61-8048 in the CIE model, which produces an escalation in the consumption of ethanol after four cycles of ethanol vapour exposure and generates a pattern of excessive ethanol consumption (Griffin, 2014) and handling-induced convulsions mimicking the transition from voluntary consumption to dependence (Riegle et al., 2014). Our data show that Ro 61-8048 produces a large decrease in the consumption and preference of ethanol without affecting water consumption, thus broadening the range of opportunities to treat ethanol abuse. Keeping in mind that Ro 61-8048 did not alter the consumption of natural reinforcers such as sucrose or saccharin which provide similar calories or taste to ethanol (Giménez-Gómez et al., 2018), the data indicate that KMO inhibition by Ro 61-8048 selectively reduces ethanol intake.

Importantly, our study shows for the first time that KMO inhibition by Ro 61-8048 also reduces ethanol consumption and preference in females. Typically, females have been underrepresented in preclinical and clinical studies of drugs (Agabio et al., 2016). However, current knowledge points to numerous differences between male and female physiopathology, drawing attention to the importance of carrying out studies in females. Regarding substances of abuse, it is well established that sex differences play a key role in the development of AUD (Flores-Bonilla & Richardson, 2019). Despite this, several medications approved for the treatment of ethanol dependence have underrepresented females in their clinical trials. In this respect, the rates of women recruited for studies evaluating the efficacy of disulfiram (1%), benzodiazepines (3%), and anticonvulsants (13%) were too low to establish possible gender differences (Agabio et al., 2016). This suggests that women may receive medications for treatment of ethanol dependence for which efficacy in females has not been well established, making the current study particularly relevant. It is important to point out here that females have a different response to ethanol than male mice, although there are no evidence using the CIE paradigm, other models such as DID, to show that females drink more ethanol than males (Sneddon et al., 2019) and to show a higher escalation in consumption (Hwa et al., 2011). This result has been reported using operant boxes (Sneddon et al., 2019), in continuous paradigms (Jury et al., 2017) and in intermittent consumption (Li et al., 2019), although other models do not describe this difference in consumption (Radke et al., 2020). In the same line, females show more compulsion-like drinking than male, using models that associate consumption with foot-shock or quinine, females show a greater resistance to punishment by tolerating higher concentrations of quinine (Sneddon et al., 2020). Moreover, not only is the sensibility to ethanol different between males and females, the consequences of consumption also are different, with different affectation in the brain and other systems (Radke et al., 2021). Our results show that Ro 61-8048 decreases the consumption of ethanol similarly in both males and females and produces a similar elevation of KYN in both sexes, leading us to tentatively conclude that the main pharmacological effect of Ro 61-8048 is similar between the sexes. It would be interesting to carry out further studies to extend the other aspects of this study also to female mice. In addition, although we did not find a differential effect of Ro 61-8048, it could be that the different sensibility

and the differences in compulsion-like behaviour exhibited by females may require an adjustment of dosage in other models that measure these parameters in order to reach the same decrease in consumption as male mice.

Concomitant with the decrease in ethanol consumption, we found a marked elevation in KYN concentration both in plasma and in limbic forebrain of male and female mice. In the CNS, only 40% of KYN is formed locally, while 60% is captured from the periphery (Gál & Sherman, 1978). In this sense, changes in brain levels of the KYN metabolites may be affected by the changes in circulating levels especially considering that Ro 61-8048 does not effectively cross the blood–brain barrier (Zwilling et al., 2011). In line with this, we and others have shown that peripheral administration of Ro 61-8048 increases not only KYN but also KYNA concentration in plasma and in brain areas such as limbic forebrain and NAc (Giménez-Gómez et al., 2018; Justinova et al., 2013; Vengeliene et al., 2016). KYNA is a ligand of several receptors including the α7nAChR, NMDA receptors and AhR. Its marked increase, both peripherally and centrally following KMO blockade, suggests it could be a metabolite of key importance in mediating the effects of KMO blockade on drug consumption. Supporting this, evidence shows that Ro 61-8048 exerts its effects on drugs of abuse via the α7nAChR (reviewed in Morales-Puerto et al., 2021) without the involvement of AhR for which both KYN and KYNA are agonists (Giménez-Gómez et al., 2018). Our present data indicate that cotreatment with PNU-120596, a positive allosteric modulator of α7nAChR, can prevent the effects of Ro 61-8048 on ethanol consumption and preference. On the other hand, our results demonstrate that PNU-120596 itself has no effect on EtOH consumption when administered alone. This result is consistent with the literature showing that PNU-120596 does not alter the self-administration of other drugs of abuse (Justinova et al., 2013; Secci et al., 2017). Thus, central KYNA may act by antagonizing α7nAChR (Justinova et al., 2013; Secci et al., 2017) and decreasing extracellular glutamate levels (Carpenedo et al., 2001) which also occurs in α7nAChR knock-out mice (Bowers et al., 2005). Moreover, in the reward system, α7nAChR controls glutamate release from cortical afferents which subsequently modulates dopamine release in NAc (Maex et al., 2014). In this sense, it is well established that Ro 61-8048 reduces self-administration of THC (Justinova et al., 2013), nicotine (Secci et al., 2017) and ethanol consumption (Giménez-Gómez et al., 2018) by reducing the ability of the drugs to stimulate dopamine release in NAc.

As mentioned above, under physiological conditions at least 60% of brain KYN is transported from the periphery to the brain (Fukui et al., 1991). The influx of KYN into the brain is mediated by LAT-1 which also transports several branched-chain amino acids and aromatic compounds that compete for transport (Sekine et al., 2015). In order to determine the differential role played by the increase in peripheral and central KYN in the effects observed, we used L-leucine, an amino acid which exhibits high affinity for LAT-1 (Boado et al., 1999) and can block the transport of KYN into the brain. Our results show that the influx of KYN into the brain is necessary to disrupt ethanol consumption and preference. We also found that

L-leucine disrupts the influx of KYN into the brain only in animals treated with Ro 61-8048 and not in those treated with vehicle, an effect previously described (Walker et al., 2018). Recent evidence shows that L-leucine inhibits rat cortical KYNA production in vitro (Sekine et al., 2015) and, more interestingly, that in vivo it is a therapeutic tool for reducing inflammation-induced depression-like behaviour caused by LPS administration (Walker et al., 2018). Our results demonstrate that the reduction of ethanol consumption produced by Ro 61-8048 is dependent on the influx of KYN into the brain. In addition, in line with the lack of effect of Ro 61-8048 on plasma, previously we found that Ro 61-8048 does not alter acetaldehyde concentration in the DID paradigm (Giménez-Gómez et al., 2018), these results indicate that the effect of Ro 61-8048 on ethanol consumption in the CIE model is not due to the conversion of the accumulated peripheral KYN to metabolites such as KYNA, 3-hydroxykynurenine or 3-hydroxyanthranilic acid which can produce aversion to ethanol by inhibiting aldehyde dehydrogenase activity and consequently elevating blood acetaldehyde concentration (Badawy et al., 2011).

As we have demonstrated that the accumulation of KYN in brain is necessary to reduce the consumption of ethanol, we wanted to test if a maintained increase in KYN in the brain would prolong the effect of Ro 61-8048. For that purpose we used probenecid, a known inhibitor of OAT, a transporter which mediates the transport of several active compounds including KYN (Wu et al., 2017) producing an accumulation of KYN and KYNA in brain (Moroni et al., 1988; Vécsei et al., 1992). Probenecid has been recently described as able to modulate ethanol intake without affecting water or saccharin consumption (Tunstall et al., 2019). However, the authors demonstrated this effect only at elevated doses (200 mg·kg^{-1}) and not with the dose used in this study (100 mg·kg^{-1}), a result that has been validated in the present manuscript. Our results show that the combination of Ro 61-8048 with probenecid is effective at maintaining increased KYN in the brain for at least 12 h and at reducing ethanol consumption up to this time, an effect that was no longer observed with Ro 61-8048 only treatment. Interestingly, we also demonstrated that 2 h after starting the consumption, probenecid did not modify the effect of Ro 61-8048 on consumption indicating that probenecid acts only by maintaining the effect of Ro 61-8048 over time. These results indicate that it is possible to prolong the therapeutic effect of Ro 61-8048. Regarding L-leucine and probenecid, it is interesting that they do not exert their effect under physiological conditions, since we did not find changes in the groups that received vehicle. Although this is a result previously found with L-leucine (Walker et al., 2018) more research is necessary to fully characterize the differential role that the transporters of KYN play under physiological conditions or under conditions that involve increases in KYN. Moreover, we did not determine the role that the modulation of the influx or efflux of KYN into the brain can play on downstream metabolites of the kynurenine pathway and if this can alter the equilibrium between neurotoxic and neuroprotective metabolites.

Finally, we studied the effect that Ro 61-8048 can exert in memory and anxiety having first ruled out effects of the drug on spontaneous activity. We tested these parameters because there is evidence in the literature that showed that KMO inhibition can alter memory and anxiety affecting the behaviour of the animals (Erhardt et al., 2017). These authors demonstrate that KMO knock-out mice showed alterations in the same parameters as we measured here. Our results showed that Ro 61-8048 did not produce alterations in these parameters in the same way as other authors previously demonstrated (Justinova et al., 2013). This reflects that, although KMO knock-out mice show several alterations, one single dose of Ro 61-8048 (50 mg·kg^{-1}, i.p.) seems to do not alter behavioural parameters of mice. Moreover, we previously demonstrated that Ro 61-8048 did not produce alterations in locomotor activity (Giménez-Gómez et al., 2018). It is interesting to note that the CIE model did not itself produce alterations in these parameters. This is likely due to the fact that the CIE has been reported to produce anxiety-like behaviour during withdrawal (Pati et al., 2020) a situation that our mice were not in when tested since they had just undergone voluntary drinking. In future studies, it would be interesting to study the effect of Ro 61-8048 on CIE-induced alterations in anxiety or memory.

Our results are in line with emerging evidence in the last decade indicating that the manipulation of KYN and KYNA is a novel target for the treatment of acute consumption of ethanol. This manipulation could be done via different strategies, from direct injections of KYN or of inhibitors such as Ro 61-8048 in order to achieve a short term reduction in consumption, to more sophisticated approaches that involve the temporal and spatial inhibition of KMO using viruses that can temporarily disrupt the enzyme, or the application of systems such as Crispr-cas9 in order to block the expression of the gene that codifies the enzyme thus producing increases in KYN levels specifically in the brain areas of interest. All this accumulated evidence regarding the relationship between the kynurenine pathway and addiction can also have implications in the study of the variability of ethanol consumption in humans. It is necessary to gather data in humans to study if changes in different enzymes of the kynurenine pathway correlate with susceptibilities or resistances to consumption. Regarding this, we can hypothesize that individuals who show differences in the expression of KMO or of the transporters LAT and OAT might show different patterns of consumption, drink differently or have different sensibility to the effects of ethanol.

In conclusion, our results indicate that the inhibition of KMO and the consequent elevation in KYN reduces ethanol consumption and preference in male and female mice in a model of dependence. This mechanism is brain-dependent and is prolonged by blocking the efflux of KYN from the brain.

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AUTHOR CONTRIBUTIONS

LGBE and PGG carried out the experimental procedures, analysis of data and wrote the original draft of the manuscript. NMP, RV and CNC carried out the experimental procedures and analysis of data, MDGL supervised experimental procedures, carried out the formal analysis of data and reviewed and edited the manuscript and EOS and MIC conceived and supervised the study, acquired funding for and administered the project, managed resources, and reviewed and edited the manuscript. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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