# Fenfluramine modulates the anti-amnesic effects induced by sigma-1 receptor agonists and neuro(active)steroids in vivo

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# Fenfluramine modulates the anti-amnesic effects induced by sigma-1 receptor agonists and neuro(active)steroids *in vivo*

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ABSTRACT

Fenfluramine (N-ethyl-α-methl-3-(trifluoromethyl)phenethylamine) is an anti-seizure medication (ASM) particularly effective in patients with Dravet syndrome, a severe treatment-resistant epileptic encephalopathy. Fenfluramine acts not only as neuronal serotonin (5-HT) releaser but also as a positive modulator of the sigma-1 receptor (S1R). We here examined the modulatory activity of Fenfluramine on the S1R-mediated anti-amnesic response in mice using combination analyses. Fenfluramine and Norfenfluramine, racemate and isomers, were combined with either the S1R agonist (PRE-084) or the S1R-acting neuro(active)steroids, pregnenolone sulfate (PREGS), Dehydroepiandrosterone sulfate (DHEAS), or progesterone. We report that Fenfluramine racemate or (+)-Fenfluramine, in the 0.1–1 mg/kg dose range, attenuated the dizocilpine-induced learning deficits in spontaneous alternation and passive avoidance, and showed low-dose synergies in combination with PRE-084. These effects were blocked by the S1R antagonist NE-100. Dehydroepiandrosterone sulfate or PREGS attenuated dizocilpine-induced learning deficits in the 5–20 mg/ kg dose range. Co-treatments at low dose between steroids and Fenfluramine or (+)-Fenfluramine were synergistic. Progesterone blocked Fenfluramine effect. Finally, Fenfluramine and (+)-Fenfluramine effects were prevented by the 5-$HT_{1A}$ receptor antagonist WAY-100635 or 5-$HT_{2A}$ antagonist RS-127445, but not by the 5-$HT_{1B/1D}$ antagonist GR 127935 or the 5-$HT_{2C}$ antagonist SB 242084, confirming a 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor involvement in the drug effect on memory. We therefore confirmed the positive modulation of Fenfluramine racemate or dextroisomer on S1R and showed that, in physiological conditions, the drug potentiated the low dose effects of neuro(active) steroids, endogenous S1R modulators. The latter are potent modulators of the excitatory/inhibitory balance in the brain, and their levels must be considered in the antiepileptic action of Fenfluramine.


## 1. Introduction

Fenfluramine has a unique pharmacological profile that potentially contributes to its marked effectiveness and long-term response profile in treating Dravet syndrome. Initially described by Charlotte Dravet [1], the syndrome is a form of myoclonic type epilepsy in infants, with delayed mental development [2]. The first symptoms appear around the age of one year. It can progress to sudden death in one out of ten cases before the age of seven [3]. A mutation in the *Scn1a* gene, which codes for the alpha-subunit protein in the sodium channel Nav1.1, is found in more than eight out of ten cases [2]. Most antiepileptic drugs (AEDs) used to date in this syndrome decrease neuronal excitability by acting at calcium channels and/or GABAergic neurotransmission [4,5]. Fenfluramine, which has been shown to be an effective add-on therapy in Dravet syndrome [6], acts primarily on the serotonin (5-HT) systems in the brain as a potent 5-HT releaser and 5-HT receptor agonist, particularly at 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors [7,8]. However, other 5-HT-acting ligands including 5-HT reuptake inhibitors (SSRIs) have been studied in Dravet syndrome, but have all led to inconsistent or marginal effects in reducing seizure frequency [4,9–11]. Recent evidence shows that the mode of action of Fenfluramine on several motor and cognitive responses, and putatively also in Dravet syndrome, involves other pharmacological targets that could play in synergy with the 5-HT receptor-mediated activity. In particular, Fenfluramine has sub-micromolar affinity for the sigma-1 ($\sigma_1$) receptor (S1R) [12]. Sigma-1 ($\sigma_1$) receptor is an endoplasmic

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reticulum-associated chaperone [13], expressed in neurons or glia, that modulates intracellular $Ca^{2+}$ exchanges [14], activates intracellular stress pathways [15,16], and modulates neurotransmitter and trophic factors receptors or ion channel permeability at the plasma membrane (for recent reviews, see [17–19]). Drugs acting with similar potency at their main pharmacological target and at the S1R present particular and often synergistic effects. Such effects have been described for the mixed acetylcholinesterase inhibitor/S1R ligand donepezil [20–22], the mixed muscarinic/S1R agonists blarcamesine or AF710B [23,24], or the mixed SSRI/S1R agonist fluvoxamine [15,25], among others.

The antiepileptic activity of Fenfluramine in the *scn1a* mutant zebrafish model of Dravet syndrome was shown to be modulated by co-administration with the S1R agonist PRE-084 [26]. In a N-methyl-D-aspartate acid (NMDA) seizure model in mice [27], Fenfluramine and Norfenfluramine disrupted the regulatory association of S1R with NR1 subunits of NMDA receptors, an effect that was also produced by S1R antagonists such as S1RA and prevented by S1R agonists such as PPCC. The authors concluded that the 5-HT activity of Fenfluramine at $5HT_{2A}$ receptors, and likely also at $5HT_{2C}$ receptors, collaborated with its S1R activity to prevent NMDA receptor overactivation-induced seizures [27].

Using the cellular S1R activity test described by Hayashi and Su [13] and testing several concentrations of the drug, we previously observed that Fenfluramine racemate failed to dissociate S1R from 78 kDa glucose-regulated protein (GRP-78, BiP), as expected for a S1R agonist [28]. However, in combination with the selective S1R agonist PRE-084, Fenfluramine did not prevent the agonist effect, as expected for a S1R antagonist, but increased significantly the dissociation, suggesting a positive allosteric modulatory (PAM) effect. A preliminary *in vivo* investigation of Fenfluramine racemate on learning and memory functions, a well-characterized behavioral effect of S1R agonists [29,30], confirmed that Fenfluramine racemate attenuated dizocilpine-induced learning deficits, when injected alone in a bi-phasic manner, and potentiated PRE-084-induced effect but failed to do so after a pre-treatment with the selective S1R antagonist NE-100 [28]. A similar profile was recently observed with another S1R-positive modulator, OZP002 [31]. Taking into account previous observations in zebrafish or mouse seizure models and the particular bell-shaped effects as a function of concentration systematically described for S1R ligands, agonists and PAMs, that could easily lead to misinterpretations (for a recent review, see [32]), we here systematically examined the anti-amnesic effects of Fenfluramine racemate and isomers and Norfenfluramine racemate and isomers in the dizocilpine model of learning deficits. We used in parallel the spontaneous alternation in the Y-maze and passive avoidance responses. We particularly examined the combination with PRE-084 and with S1R acting neuro(active)steroids, dehydroepiandrosterone sulfate (DHEAS) and pregnenolone sulfate (PREGS). Finally, to relate these observations to the 5-HT component of the pharmacological activity of Fenfluramine, the effects of several 5-HT receptor antagonists were tested. We report that Fenfluramine and particularly its dextrogyre isomer (+)-Fenfluramine, do act as S1R-positive modulators, while Norfenfluramine racemate and both dextrogyre and levogyre isomers rather act as S1R antagonists *in vivo*. Moreover, the steroidal tonus relying on drastic changes in DHEA, PREG, their sulfate esters, and progesterone may impact Fenfluramine activity.

## 2. Materials and methods

### 2.1. Drugs and injections

Fenfluramine and Norfenfluramine, racemate and (+)- and (−)-isomers, were provided by Zogenix Inc. (Emeryville, CA, USA). 2-(4-Morpholinethyl)-1-phenylcyclohexane carboxylate hydrochloride (PRE-084), (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate ((+)-MK-801, dizocilpine), N-(2-(4-(2-Methoxyphenyl)-1-piperazinyl)ethyl)-N-2-pyridinylcyclohexanecarboxamide (WAY-100635), 6-Chloro-2,3-dihydro-5-methyl-N-(6-((2-methyl-3-pyridinyl)oxy)-3-pyridinyl)-1H-indole-1-carboxy-amide (SB 242084), N-(4-Methoxy-3-(4-methyl-1-piperazinyl)phenyl)-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1′-biphenyl-4-carboxamide (GR 127935), 2-Amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine (RS-127445), 4-Methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzene ethanamine hydrochloride (NE-100) dehydroepiandrosterone 3-sulfate sodium salt (DHEAS), pregnenolone sulfate sodium salt (PREGS), and progesterone were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Drugs were solubilized in physiological saline and steroids were solubilized in sesame oil (Sigma-Aldrich). Compounds were injected intraperitoneally (ip) or subcutaneoulsy (sc) in a volume of 100 μL/20 g body weight.

### 2.2. Animals

Male Swiss OF-1 mice (aged 7–9 weeks, weight 32 ± 2 g), were from Janvier labs. (St Berthevin, France). Mouse housing and experiments took place at University of Montpellier animal facility (CECEMA, registration number D34-172-23). Animals were housed in groups with access to food/water ad libitum in a temperature/humidity-controlled facility ( 12-h/ 12-h light/dark cycle; lights on, 7:00 h; behavioral experiments, between 9:00 h and 17:00 h), in a sound-attenuated and air-regulated experimental room. All animal procedures were conducted in strict adherence to the European Union Directive of September 22, 2010 (2010/63), and the ARRIVE guidelines [33].

### 2.3. Spontaneous alternation in the Y-maze

Animals were tested for spontaneous alternation performance in the Y-maze, an index of spatial working memory [29,34,35]. Made of gray polyvinylchloride, the Y-maze has arms of dimensions 40 cm × 13 cm; 3 cm at bottom, 10 cm at top, converging at equal angle. Each mouse was placed at the end of one arm and allowed to move freely for 8 min. Arm entries, including possible returns into the same arm, were recorded. An alternation was defined as successive entry in the three different arms. The percentage of alternation was calculated as: (actual alternations/maximum alternations) × 100. An exclusion criterion was set: animals not exploring the maze, because of low mobility, aberrant perseverance between two arms or in turning clockwise or counterclockwise when changing arms, were discarded from the calculations. This corresponded to animals showing less than 8 entries in 8 min or alternation percentages >90% or <20%. Attrition was routinely < 5%.

### 2.4. Step-through passive avoidance

The test measures non-spatial/contextual long-term memory [35]. The apparatus was a grid-floor, 2-compartment box separated by a guillotine door: (1) illuminated (60-W lamp 40 cm above) with white polyvinylchloride walls and transparent cover (15 × 20 × 15 cm); (2) black polyvinylchloride walls and cover (15 × 20 × 15 cm). Scrambled foot shocks (0.3 mA, 3 s) were delivered to the grid floor via shock generator scrambler (Lafayette Instruments, Lafayette, MA). During training, the guillotine door was initially closed. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment with all paws on the grid floor, the door was gently closed. A scrambled foot shock was delivered for 3 s. Step-through latency (i.e., the latency spent to enter the dark

compartment) and level of sensitivity to the shock (0 = no sign; 1 = flinching reactions; 2 = flinching and vocalization reactions) were recorded. Twenty-four hours after training, retention tests were performed by placing each mouse into the white compartment for 5 s, raising the door, and recording step-through latency, up to 300 s. Results were expressed as median and interquartile (25–75%) range because the data are non-parametric with an established upper limit. An exclusion criterion was set: animals that failed to realize the task either in terms of configuration of the apparatus (so spending little time in the white compartment before entering the dark one) and by showing either a good memory (=long step-through latency and short escape latency) or bad memory (=short step-through latency and long escape latency) were discarded from the calculations. This corresponded to animals showing latencies during training and retention < 10 s and low shock sensitivity (0 or 1). Attrition was routinely < 5%.

### 2.5. Statistical analyses

The number of animals per group, estimated *a priori* using G*Power Version 3.1.9.2 software (setting effect size at 1.3, alpha at 0.05, and power at 0.8) was routinely 12–18, with the exception of 5-HTR antagonists in Fig. 8 ($n = 6$). Data were analyzed using a one-way analysis of variance (ANOVA, $F$ value) followed by Dunnett's test or a Kruskal–Wallis non-parametric ANOVA ($H$ value) followed by a Dunn's multiple comparison test. The level of statistical significance considered were $p < 0.05$, $p < 0.01$ and $p < 0.001$. For reading clarity, statistical data were included in the legend of the figures.

### 2.6. Combination index calculations

Isobologram analyses were used to evaluate the nature of interaction of two drugs at a given effect level. As expected, dose–response curves followed a bi-phasic effect [32], only the ascending part of the curve was considered in calculations. Isobologram representation followed the concept of Fraser [36] and we previously published such analyses [28,35]. The concentration required to produce a given effect (*e.g.*, where $IC_x = IC_{50}$) is determined for drug A ($IC_{x,A}$) and drug B ($IC_{x,B}$) and indicated on the $x$ and $y$ axes of a two-coordinate plot, forming the two points, ($IC_{x,A}$, 0) and (0, $IC_{x,B}$). The line connecting these two points is the line of additivity. In a mix of drugs A + B, the concentrations of A and B contained in the combination that provide the same effect are represented by the coordinates ($C_{A,x}$, $C_{B,x}$). In the plot, synergy/additivity/antagonism is indicated when ($C_{A,x}$, $C_{B,x}$) is located below/on/above the line, respectively. Operationally, a combination index (CI) is calculated as: $CI = C_{A,x}/IC_{x,A} + C_{B,x}/IC_{x,B}$, where $C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A and B used in a combination that generates $x$% of the maximal combination effect. $IC_{x,A}$ and $IC_{x,B}$ are the concentrations of drugs A and B needed alone to produce $x$% of the maximal effect. A CI less than/equal to/more than 1 indicates synergy/additivity/antagonism, respectively.

To calculate CI based on isobologram representation, alternation percentages and passive avoidance latencies were expressed as percentage of protection (PP) for each treatment group with PP (V/V/V) set as 100% and PP (dizocilpine/V/V) as 0% [28,35]. All calculations are presented in Supplemental Tables annexed to the present manuscript.

## 3. Results

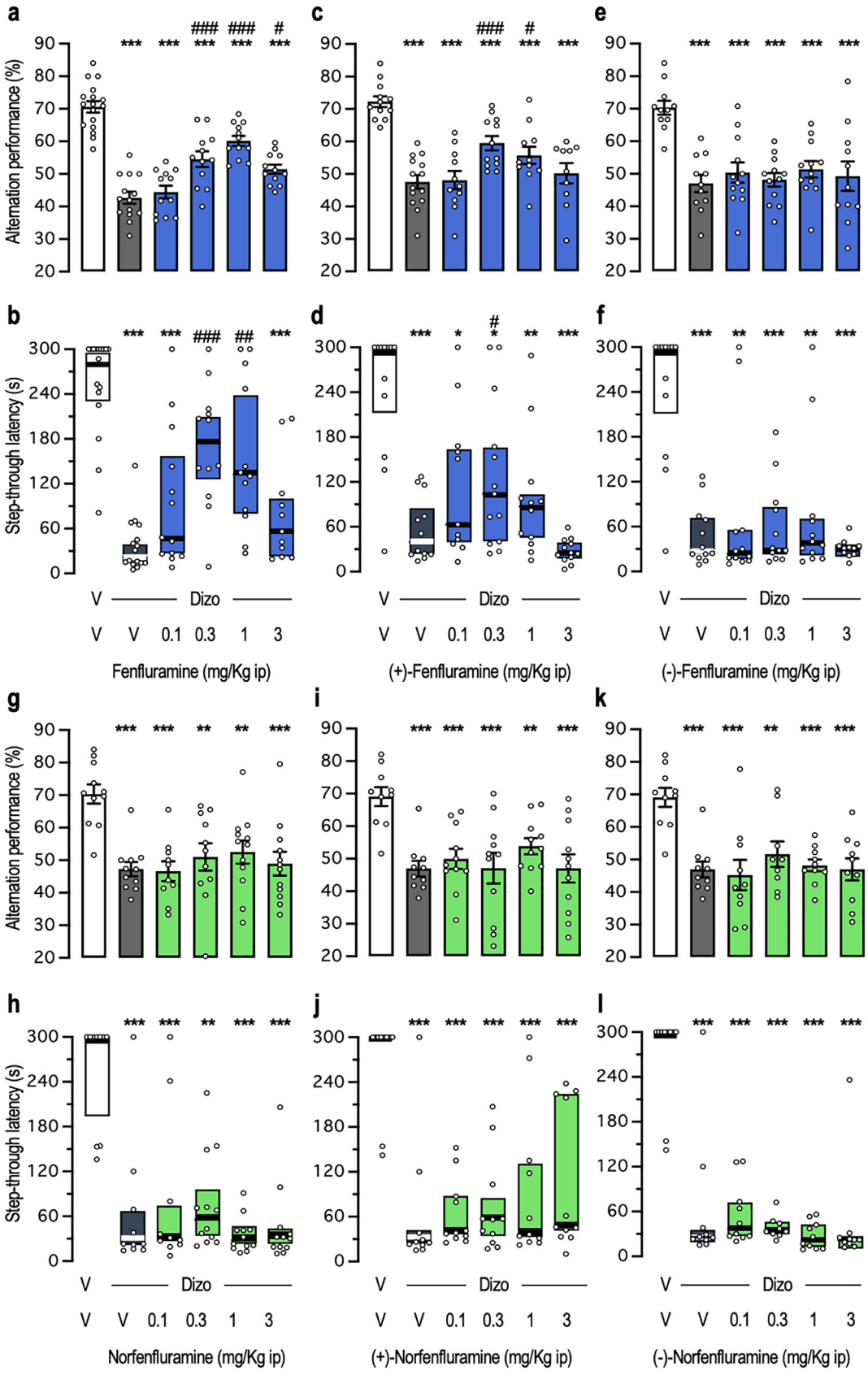
### 3.1. Anti-amnesic effects of Fenfluramine and Norfenfluramine and combinations with the S1R reference agonist PRE-084

We first examined the anti-amnesic effects of Fenfluramine and Norfenfluramine, as racemate or for each isomer, against dizocilpine-induced amnesia in mice. Two behavioral tests were systematically used in parallel which address complementary memory processes. Spontaneous alternation performance in the Y-maze is an index of spatial working memory [34,37,38], while the step-through latency measured in the passive avoidance test is a non-spatial long-term memory parameter [34,37,39]. Drugs were tested in the 0.1–3 mg/kg dose range and we observed that Fenfluramine racemate and (+)-Fenfluramine significantly attenuated dizocilpine-induced learning deficits at the doses of 0.3 and 1 mg/kg (Fig. 1a–d). (−)-Fenfluramine was without effect in both tests (Fig. 1e and f). Moreover, neither the racemate of Norfenfluramine, nor the (+)- or (−)-isomer attenuated dizocilpine-induced deficits in the dose range tested (Fig. 1g–l). As previously described [37], the reference S1R agonist PRE-084 showed an anti-amnesic effect at 0.3 and 1 mg/kg in both tests (Fig. 2a and b). Both (+)- and (−)-Norfenfluramine prevented the anti-amnesic effects of PRE-084 (Fig. 2c and d), and therefore acted likely as S1R antagonists.

It must be noted that Fenfluramine and its isomers did not attenuate dizocilpine-induced hyperlocomotion, measured by the number of arms entered during the Y-maze session, but rather sustained it at the highest doses of Fenflumramine and its dextrogyre isomer (Suppl. Fig. 1a-c). Norfenfluramine and its isomers on the contrary tended to decrease dizocilpine-induced hyperlocomotion (Suppl. Fig. 2a-c). During passive avoidance training, the step-through latency measures exploration and reaction to novelty and shock sensitivity measures changes in nociceptive response. None of these parameters showed significant differences (Suppl. Fig. 1d-i for Fenfluramine and Suppl. Fig. 2d-i for Norfenfluramine), with the exception of (+)-Norfenfluramine that showed a trend to increased latencies (Suppl. Fig. 2e) and decreased sensitivity at the highest dose (Suppl. Fig. 2h) but with non-significant group differences. These data showed that the drug marginally affected locomotion, anxiety and nociception that could interfere with the amnesic responses.

The positive modulatory action of Fenfluramine and (+)-Fenfluramine was tested on PRE-084 effect. The minimal acting doses of each compound, 0.1 and 0.3 mg/kg, were tested in combination, in both tests (Fig. 3). All combinations led to increased and significant anti-amnesic effects for Fenfluramine (Fig. 3a and c) and (+)-Fenfluramine (Fig. 3e and g). Moreover, representation as percentage of protection (Fig. 3b, d, f and h) and calculations of the combination index (Suppl. Tables 1 and 2), showed that most of the combinations led to synergic effects. This was particularly

**Fig. 1.** Effect of Fenfluramine and Norfenfluramine, racemate and isomers, on dizocilpine-induced learning impairments in mice. The anti-amnesic effects of Fenfluramine (a and b), (+)-Fenfluramine (c and d), (−)-Fenfluramine (e and f), Norfenfluramine (g and h), (+)-Norfenfluramine (i and j), and (−)-Norfenfluramine (k and l) were tested in the spontaneous alternation test in the Y-maze (a, c, e, g, i, k) and step-through passive avoidance test (b, d, f, h, j and l). Animals received the drugs (0.1–3 mg/kg) 10 min before dizocilpine (Dizo, 0.15 mg/kg), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a, c, e, g, i and k) and median and interquartile range in (b, d, f, h, j and l). ANOVAs: $F_{(5,73)} = 35.8$, $p < 0.0001$, $n = 12–17$ in (a); $H = 41.8$, $p < 0.0001$, $n = 11–17$ in (b); $F_{(5,64)} = 15.7$, $p < 0.0001$, $n = 10–14$ in (c); $H = 30.3$, $p < 0.0001$, $n = 11–13$ in (d); $F_{(5,63)} = 8.52$, $p < 0.0001$, $n = 11–12$ in (e); $H = 21.7$, $p < 0.001$, $n = 11–12$ in (f); $F_{(5,61)} = 6.86$, $p < 0.001$, $n = 10–12$ in (g); $H = 24.6$, $p < 0.0001$, $n = 10–12$ in (h); $F_{(5,58)} = 5.87$, $p < 0.001$, $n = 10–11$ in (i); $H = 24.1$, $p < 0.0001$, $n = 10–11$ in (j); $F_{(5,53)} = 7.49$, $p < 0.0001$, $n = 9–10$ in (k); $H = 31.1$, $p < 0.0001$, $n = 9–11$ in (l). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* V-treated group; # $p < 0.05$, ### $p < 0.001$ *vs.* Dizo-treated group; Dunnett's test in (a, c, e, g, i and k), Dunn's test in (b, d, f, h, j and l).


a
b
c
d
e
f
g
h
i
j
k
l
Alternation performance (%)
Step-through latency (s)
V
Dizo
V V 0.1 0.3 1 3
Fenfluramine (mg/Kg ip)
(+)-Fenfluramine (mg/Kg ip)
(-)-Fenfluramine (mg/Kg ip)
Norfenfluramine (mg/Kg ip)
(+)-Norfenfluramine (mg/Kg ip)
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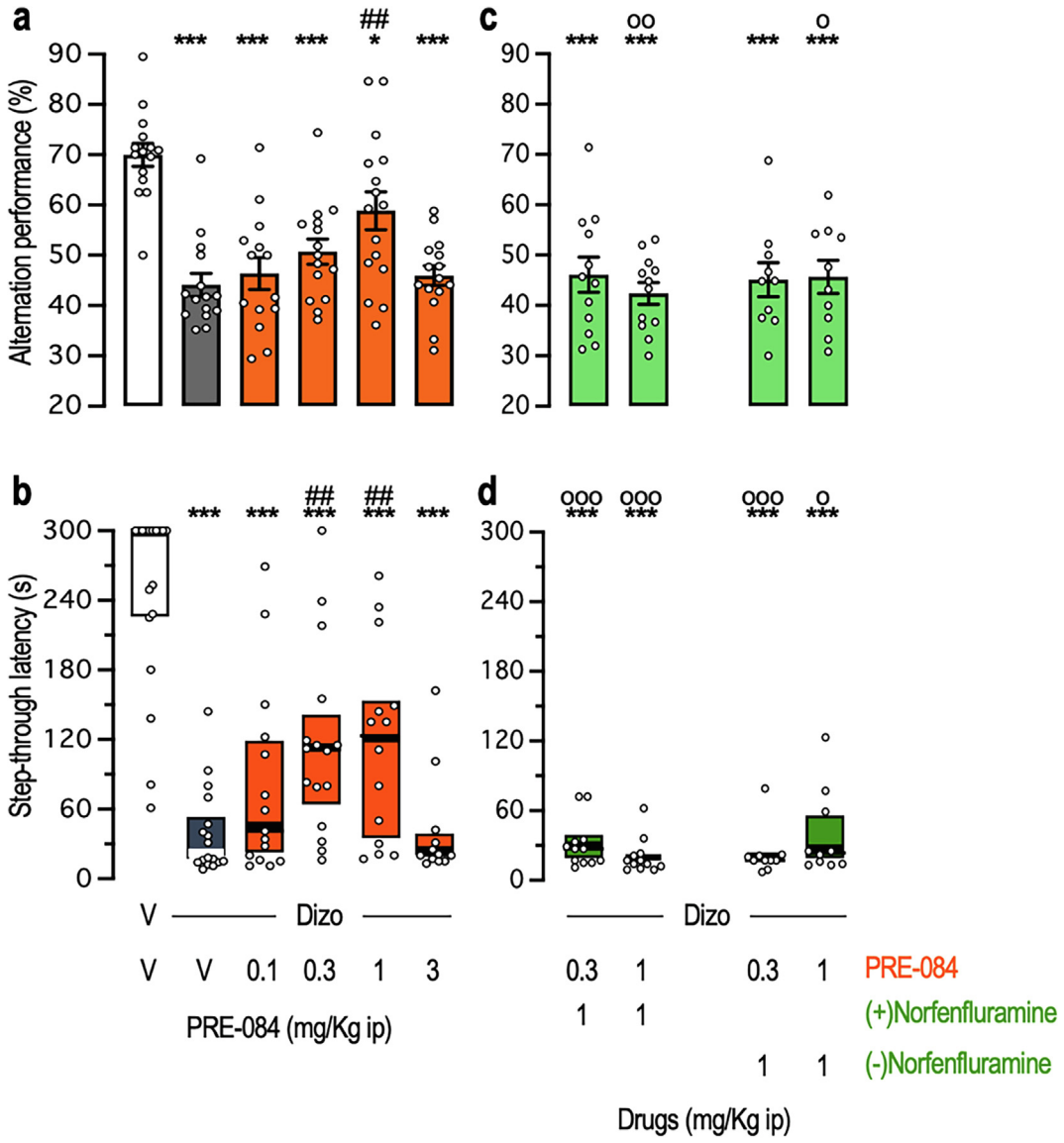


**Fig. 2.** Effect of PRE-084 on dizocilpine-induced learning impairments in mice (a and b) and antagonism by (+)-Norfenfluramine and (−)-Norfenfluramine (c and d). Spontaneous alternation performance in the Y-maze (a and c) and step-through latency in the passive avoidance test (b, d). Animals received the drugs (0.1–3 mg/kg ip) 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a and c) and median and interquartile range in (b, d). ANOVAs: $F_{(5,83)} = 14.8$, $p < 0.0001$, $n = 14–15$ in (a); $H = 40.0$, $p < 0.0001$, $n = 12–17$ in (b). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* V-treated group; # $p < 0.05$, ## $p < 0.01$ *vs.* Dizo-treated group; o $p < 0.05$, oo $p < 0.01$ *vs.* same dose PRE-084-treated group; Dunnett's test in (a and c), Dunn's test in (b and d).

observed for low-dose combinations (Fenfluramine/(+)-Fenfluramine + PRE-084: 0.1 + 0.1, 0.1 + 0.3 and 0.3 + 0.1 mg/kg).

Combinations were also tested with (−)-Fenfluramine (Fig. 4). No impact on the anti-amnesic effect of PRE-084 was observed when the drug was combined with either a low dose of (−)-Fenfluramine (0.3 mg/kg; Fig. 4a and c) or with a higher dose (1 mg/kg; Fig. 4e and f). Combination index calculations led to values close to 1 or slightly higher (Suppl. Table 3).

Moreover, a pre-administration of the selective S1R antagonist NE-100 blocked the anti-amnesic effect of PRE-084, Fenfluramine or (+)-Fenfluramine when tested at 0.3 or 1 mg/kg and the potentiation induced by 0.1 mg/kg of Fenfluramine or (+)-Fenfluramine on PRE-084 effect (Fig. 5a and b). The Fenfluramine or (+)-Fenfluramine-induced potentiation of the PRE-084-induced anti-amnesic effect therefore selectively involved modulation of S1R activity.

### 3.2. Modulation of neuro(active)steroids activity

Dehydroepiandrosterone sulfate and PREGS are known to act on learning and memory as S1R agonists [34]. Dehydroepiandrosterone sulfate dose-dependently attenuated dizocilpine-induced learning deficits, in the 5–20 mg/kg dose range in both tests (Fig. 6a and c). The combination of low doses of DHEAS with Fenfluramine at 0.1 mg/kg led to synergic effects for the (5 + 0.1) mix in the Y-maze (Fig. 6b; Suppl. Table 4). The combination of low doses of DHEAS with (+)-Fenfluramine at 0.1 mg/kg led to synergic effects for the (5 + 0.1) mix in both tests (Fig. 6b and d; Suppl. Table 4).

Pregnenolone sulfate dose-dependently attenuated dizocilpine-induced learning deficits, in the 5–20 mg/kg dose range (Fig. 6e and g). The combination of low doses of PREGS with Fenfluramine at 0.1 mg/kg led to synergic effects for the (5 + 0.1) mix in the

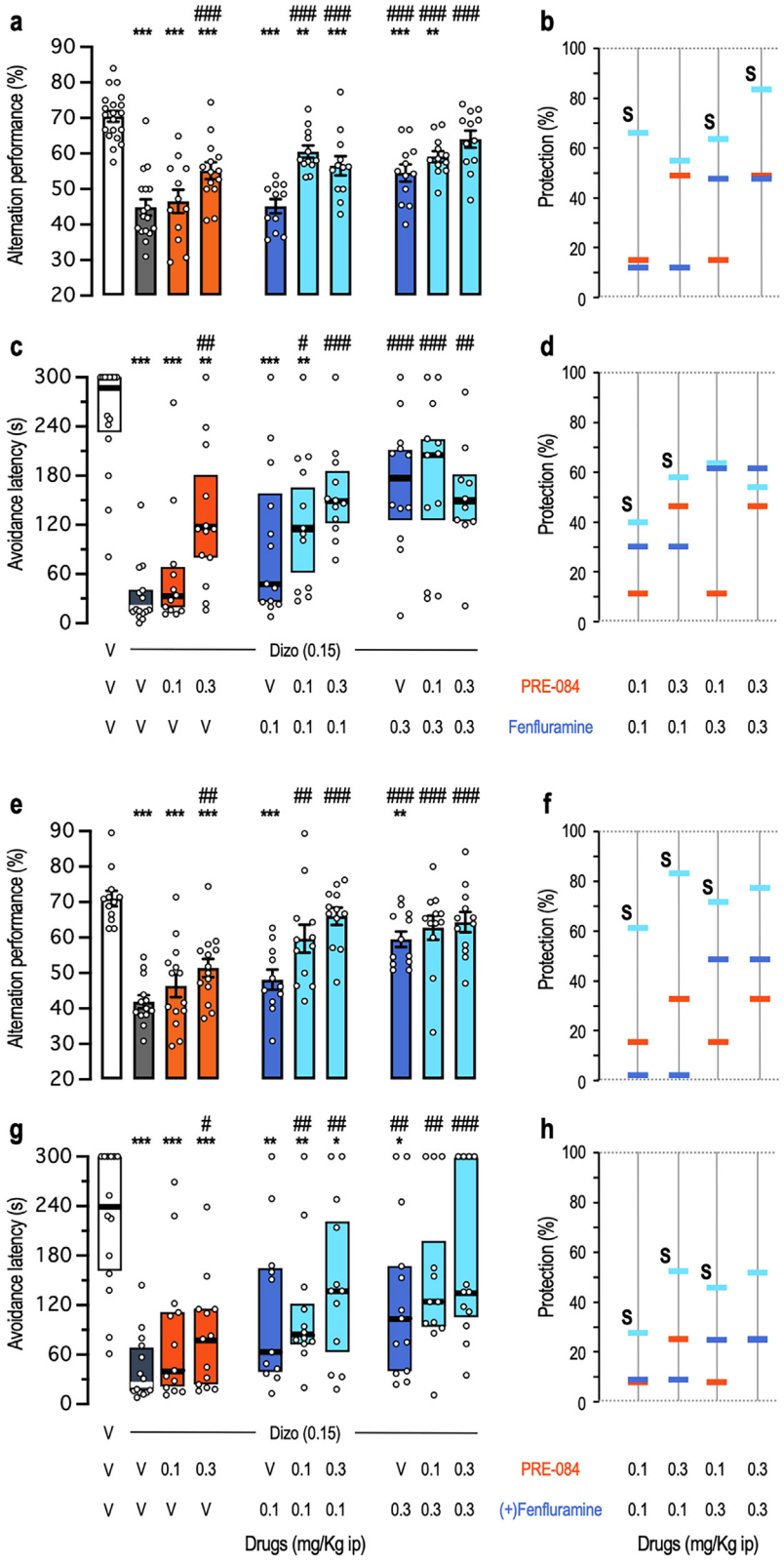


**Fig. 3.** Combination studies between PRE-084 and Fenfluramine (a–d) or (+)-Fenfluramine (e–h) in dizocilpine-treated mice. Spontaneous alternation in the Y-maze (a, b, e and f) and step-through passive avoidance (c, d, g, h). PRE-084 (0.1–0.3 mg/kg ip) and/or Fenfluramine (0.1–0.3 mg/kg ip) and/or (+)-Fenfluramine (0.1–0.3 mg/kg ip) were injected 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a and e) and median and interquartile range in (c and g). In (b, d, f and h), protection is shown using a cursor-on-scale representation, with data from V-treated group as 100% and Dizo-treated group as 0%. ANOVAs: $F_{(9,157)} = 21.2$, $p < 0.0001$, $n = 12–20$ per group, in (a); $H = 66.3$, $p < 0.0001$, $n = 15–18$ per group, in (c); $F_{(9,123)} = 11.7$, $p < 0.0001$, $n = 11–14$ per group, in (e); $H = 37.5$, $p < 0.0001$, $n = 12–14$ per group, in (g). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* V-treated group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* Dizo-treated group; Dunnett's test in (a), Dunn's test in (c). S: synergic effect with combination index (CI) < 1.

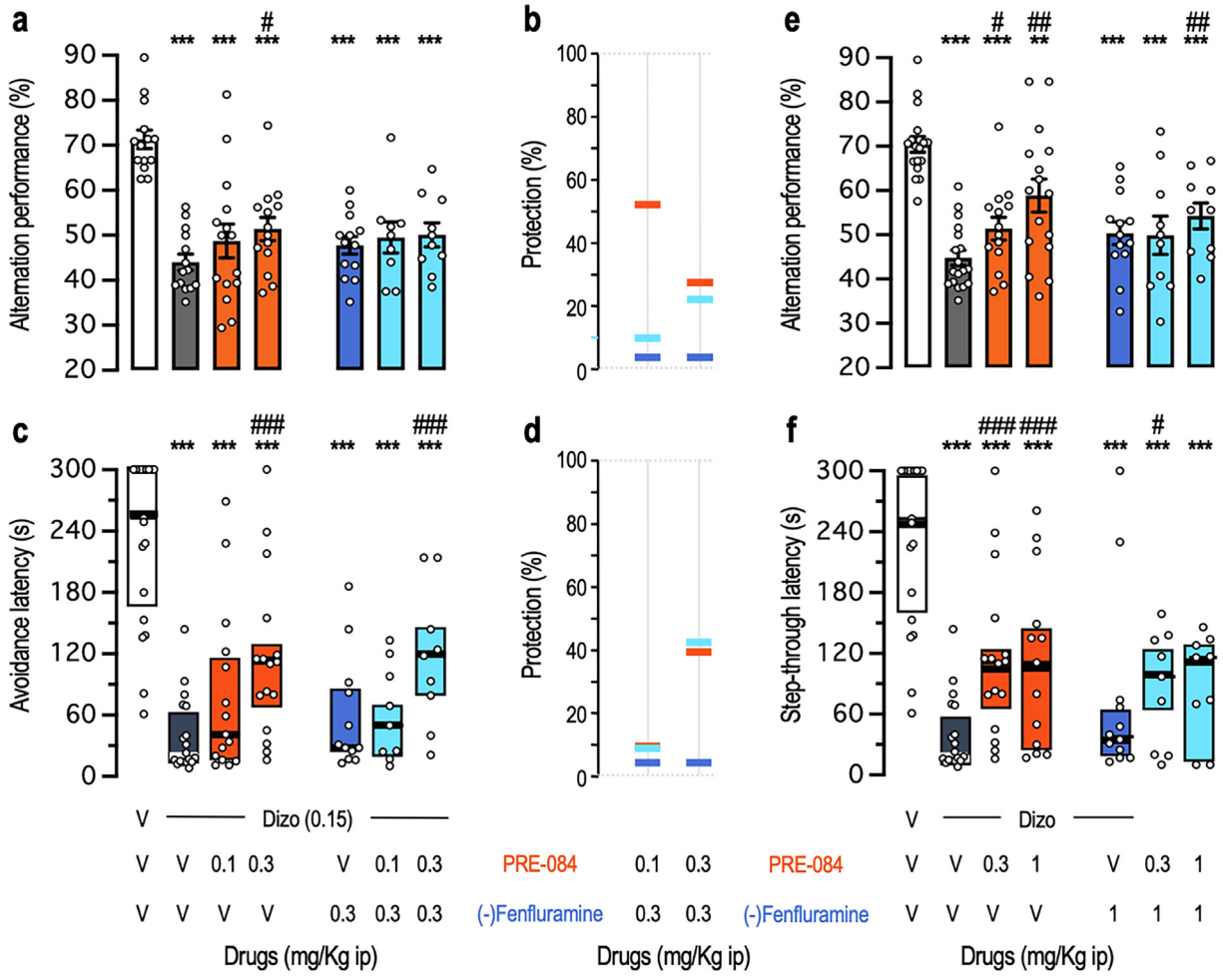


**Fig. 4.** Combination studies between PRE-084 and (−)-Fenfluramine in dizocilpine-treated mice. Spontaneous alternation performance in the Y-maze (a and b) and step-through latency in the passive avoidance test (c and d). PRE-084 (0.1–1 mg/kg ip) and/or (−)-Fenfluramine (0.3, 1 mg/kg ip) were injected 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a and e) and median and interquartile range in (c and f). In (b and d), protection is shown using a cursor-on-scale representation, with data from V-treated group as 100% and Dizo-treated group as 0% in (a and c). ANOVAs: $F_{(9,121)} = 7.07$, $p < 0.0001$, $n = 10–15$ per group, in (a); $H = 47.2$, $p < 0.0001$, $n = 9–19$ per group, in (c); $F_{(6,98)} = 11.0$, $p < 0.0001$, $n = 10–18$ in (e); $H = 40.3$, $p < 0.0001$, $n = 9–19$ per group, in (f). * $p < 0.05$, *** $p < 0.001$ *vs.* V-treated group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* Dizo-treated group; Dunnett's test in (a, e), Dunn's test in (c, f).

Y-maze (Fig. 6f; Suppl. Table 5). The combination of low doses of PREGS with (+)-Fenfluramine at 0.1 mg/kg led to synergic effects for the (5 + 0.1) mix in both tests (Fig. 6f and h; Suppl. Table 5). A synergic effect was also observed in the passive avoidance test for the (10 + 0.1) mix (Fig. 6h; Suppl. Table 5).

Progesterone acts as a S1R antagonist [34]. We observed that pretreatment with the steroid indeed prevented, at the low 5 mg/kg dose, the anti-amnesic effects of Fenfluramine and (+)-Fenfluramine in both behavioral tests (Fig. 7a and b).

### *3.3. Effects of 5-HT receptors antagonists on Fenfluramine and (+)-Fenfluramine effects*

Fenfluramine is a 5-HT releaser and serotoninergic systems are crucially involved in learning and memory processes [40]. In particular, direct administration of 5-$HT_{1A}$, 5-$HT_{2A/2C}$, 5-$HT_3$ or 5-$HT_4$ receptor ligands has a direct impact on recovery from memory impairments. We therefore tested whether Fenfluramine and (+)-Fenfluramine anti-amnesic effects at 0.3 mg/kg, could be prevented by selective antagonists of the 5-HT receptors involved in memory processes. The 5-$HT_{1A}$ receptor antagonist WAY-100635 did not affect dizocilpine-induced deficits but prevented Fenfluramine and (+)-Fenfluramine effect in both tests (Fig. 8a and b). The 5-$HT_{1B/1D}$ antagonist GR 127935 did not affect dizocilpine-induced deficits and failed to affect Fenfluramine and (+)-Fenfluramine effect in both tests (Fig. 8c and d). It marginally decreased (+)-Fenfluramine effect in the passive avoidance test (Fig. 8d). The 5-$HT_{2A}$ antagonist RS-127445 did not affect dizocilpine-induced deficits but prevented Fenfluramine and (+)-Fenfluramine effect in both tests (Fig. 8e and f). The 5-$HT_{2C}$ antagonist SB 242084 did not affect dizocilpine-induced deficits and failed to affect (+)-Fenfluramine effect in both tests (Fig. 8g and h). It marginally decreased Fenfluramine effect in the Y-maze test (Fig. 8d) but significantly decreased its effect in the passive avoidance test (Fig. 8h). These data outlined major involvements of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors in the anti-amnesic effects of Fenfluramine, and particularly (+)-Fenfluramine, against dizocilpine-induced learning deficits, beside the S1R activity.

## 4. Discussion

### *4.1. Fenfluramine modulates S1R activity in vivo*

In the present study, we analyzed the pharmacological action of Fenfluramine and Norfenfluramine, racemate and isomers, at S1R

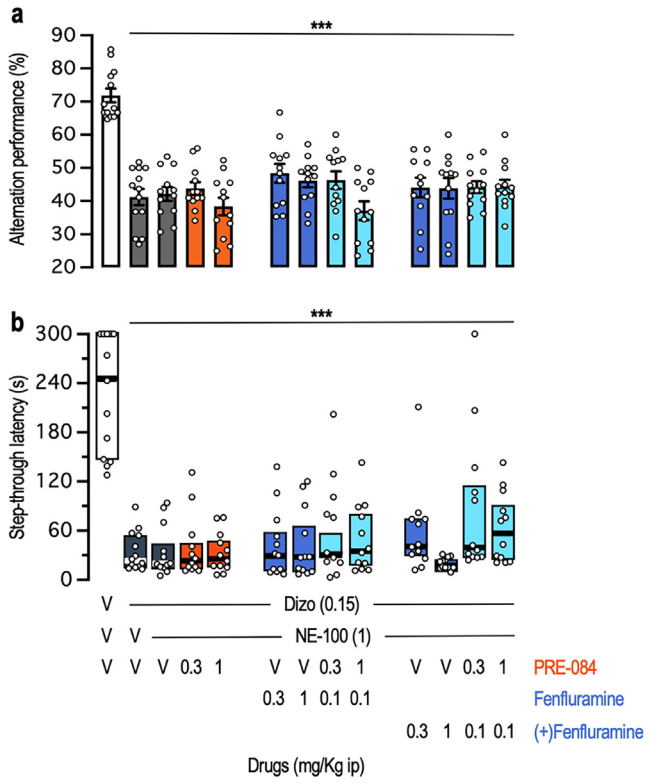


**Fig. 5.** Antagonism by NE-100 of the anti-amnesic effects of Fenfluramine racemate, (+)-Fenfluramine and their combinations with PRE-084 on dizocilpine-induced learning impairments in mice. Spontaneous alternation performance in the Y-maze (a) and step-through latency in the passive avoidance test (b). Animals received NE-100 (1 mg/kg ip), 10 min before the drugs (0.1–1 mg/kg ip), 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a) and median and interquartile range in (b). ANOVAs: $F_{(12,156)} = 12.8$, $p < 0.0001$, $n = 11–13$ in (a); $H = 49.2$, $p < 0.0001$, $n = 12–13$ in (b). *** $p < 0.001$ *vs.* V-treated group; Dunnett's test in (a), Dunn's test in (b).

*in vivo*. We used the anti-amnesic effect on dizocilpine-induced memory impairment in mice as it is historically a very pertinent *in vivo* model for the analysis of drug activity for S1R agonists [20,23,29,30,37,41–43] and S1R-positive modulators [31]. The test is also pertinent for the analysis of serotoninergic drugs [40,44,45]. Pharmacological activities could be differentiated, based on selective antagonists for S1R or subtypes of 5-HT receptors. We first confirmed the anti-amnesic effect of Fenfluramine racemate in both the spontaneous alternation and passive avoidance responses [28] and showed that it was due to the dextrogyre isomer (+)-Fenfluramine. (−)-Fenfluramine indeed was inactive in both responses. The effect involved activity at S1R since it was blocked by the selective S1R antagonist NE-100 *in vivo*. In *in vitro* studies, Fenfluramine racemate shows a moderate affinity for S1R (Ki = 266 nM, determined using $^3$H-ditolylguanidine binding) [28], with a differential binding potency between isomers. (+)-Fenfluramine showed a good affinity (70 nM using $^3$H-(+)-pentazocine binding) while (−)-Fenfluramine was less effective (333 nM) [12]. However, the drugs unlikely acted as S1R agonists, since *in vitro* activity tests failed to confirm the S1R activity of Fenfluramine. In the *vas deferens* contraction assay, historically used as a "sigma activity" test [46,47], Fenfluramine failed to induce contractions, unlike the reference S1R agonist (+)-SKF-10047. Fenfluramine also failed to block the agonist-induced contractions, unlike the reference S1R antagonist Rimcazole, but rather potentiated them moderately [28]. In the S1R/BiP dissociation assay, now widely accepted as a pertinent S1R activity test [13], Fenfluramine failed to dissociate S1R from BiP at doses as high as 10 μM in CHO cells. This assay however revealed that the drug behaved as a S1R-positive modulator, since it potentiated the S1R/BiP dissociation induced by PRE-084 [28]. Moreover, *in vivo*, Fenfluramine potentiated the anti-amnesic effect of the S1R agonist [28]. We started the present study by confirming the latter result and observed that this effect is carried by the (+)-isomer, which appeared particularly active. At 0.3 mg/kg alone or 0.1 mg/kg in combination with PRE-084, it almost fully reversed the learning deficits in both behavioral tests. Combination studies also confirmed that (−)-Fenfluramine has no S1R activity *in vivo*, since it did not potentiate nor block the PRE-084-induced anti-amnesic effect even at the highest dose tested, 1 mg/kg. The effect of (+)-Fenfluramine, alone or in combination with PRE-084, were fully blocked by NE-100 confirming the selectivity of the activity measured. Similar activity has been described for oxazaphosphinane OZP002, a novel phosphorylated derivative of hydroxybupropion [31] and for other S1R positive modulators that that have been identified since the time that S1R modulatory action was first described for the historic drug Phenytoin (for review, see [48]).

We also examined the effects of the metabolite Norfenfluramine racemate and isomers in the same paradigm. The compounds have low micromolar affinity for S1R, with Ki = 2.9 μM, determined using $^3$H-ditolylguandine binding, for the racemate [28] and 1.0 μM, using $^3$H-(+)-pentazocine binding, for (+)-Norfenfluramine [12]. However, norfenfluramine did not behave as a S1R modulator. On the contrary, when co-administered with active doses of PRE-084, both isomers blocked the S1R agonist effect, therefore suggestive of a S1R antagonist activity *in vivo*.

### 4.2. S1R modulation in Fenfluramine's pharmacological profile

Fenfluramine, through its dextrogyre isomer, has therefore a potent S1R-positive modulatory activity that must be taken into account, at doses that are pharmacologically active and therapeutically used. The S1R protein is a membrane-bound protein expressed in neurons and glia [49]. It is involved in signal modulation, chaperoning or interacting with different partners that include for instance inositol 1,4,5-trisphosphate ($IP_3$) receptors, BiP, inositol-requiring enzyme-1 (IRE1), or activating transcription factor-6 (ATF6) [13,14,50–52]. Highly expressed at mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs) [13], it dissociates from $IP_3$ receptors following cellular stress or via agonist stimulation, therefore enhancing calcium entry into the mitochondria [14,51] and stabilizing ER stress responses [15,16]. S1R also directly modulates neurotransmitter receptors, including monoamines or NMDA receptors [27,53], ion channels [18,54] and store-operated $Ca^{2+}$ entry, a mechanism promoted by depletion of intracellular $Ca^{2+}$ stores [55]. S1Rs consequently play an important role in brain plasticity, response to stress, learning and memory, epilepsy, or neuroprotection against neurodegenerative pathologies. More particularly, S1R activity has been shown to modulate 5-HT neurotransmission in different models. For instance, Lucas et al. [56] showed that a 2-day treatment with the selective S1R agonist SA4503 dose-dependently increased the firing rate of dorsal raphe nucleus 5-HT neurons. Remarkably, this 2-day treatment provoked the appearance of a 5-$HT_{1A}$ receptor-mediated tonic inhibitory effect on CA3 pyramidal neurons, and the 5-$HT_{1A}$ receptor antagonist WAY-100635 could thus provoke a significant disinhibition of neuronal activity in SA4503-treated rats [56]. We also observed that a co-treatment with the 5-$HT_{1A}$ receptor agonist 8-OH-DPAT and the S1R agonist igmesine at subactive doses synergistically increased the immobility duration of mice in the forced swim test, an antidepressant-like response (unpublished results, data shown in Suppl. Table 6). Moreover, Fenflu-

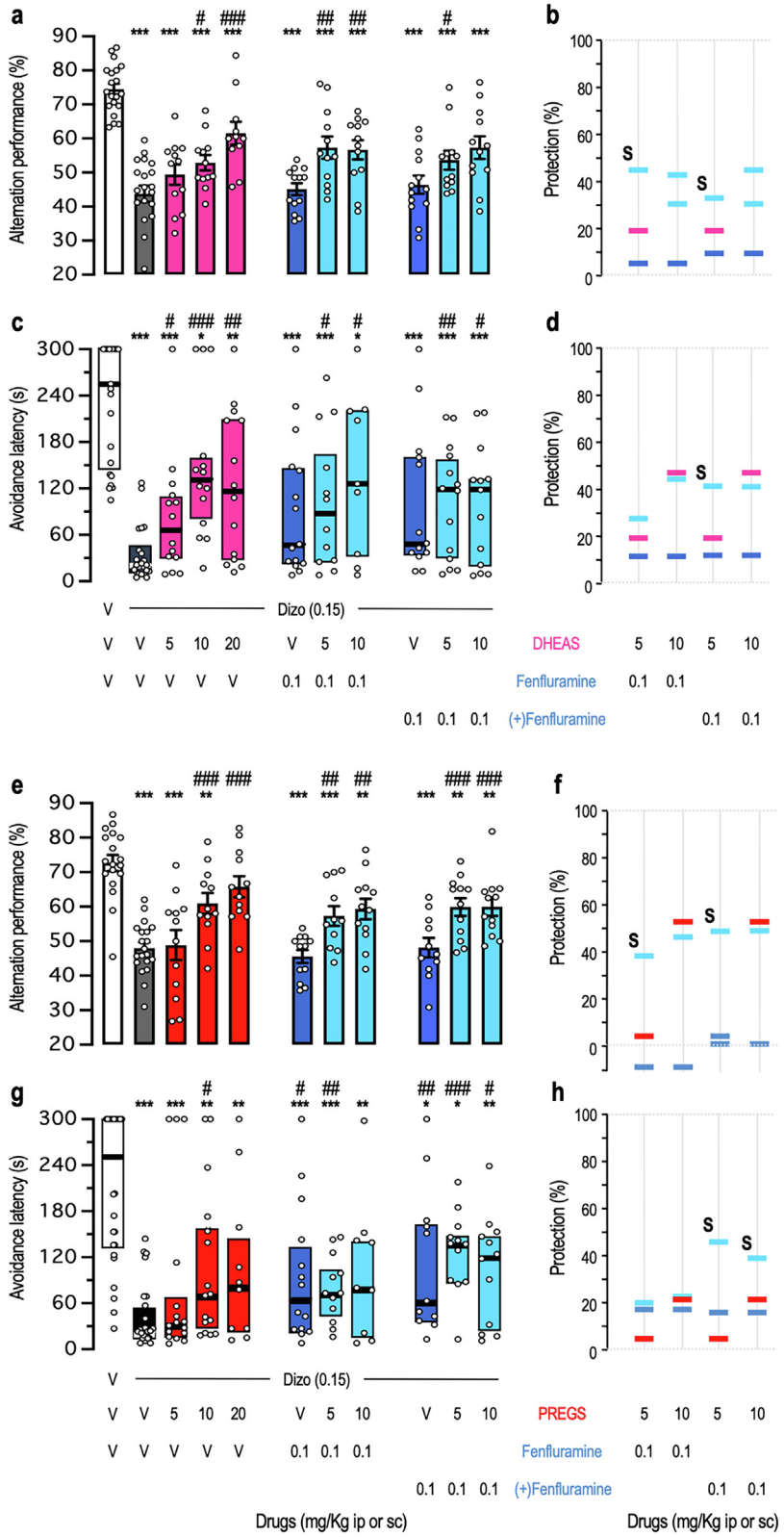


**Fig. 6.** Combination studies between DHEAS or PREGS and Fenfluramine or (+)-Fenfluramine in dizocilpine-treated mice. Spontaneous alternation in the Y-maze (a, b, e, and f) and step-through passive avoidance (c, d, g and h). DHEAS or PREGS (5–20 mg/kg sc) and/or Fenfluramine or (+)-Fenfluramine (0.1–0.3 mg/kg ip) were injected 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a and e) and median and interquartile range in (c and g). In (b, d, f and h), protection is shown using a cursor-on-scale representation, with data from V-treated group as 100% and Dizo-treated group as 0%. V: vehicle solution (saline solution or sesame oil for DHEAS). ANOVAs: $F_{(10,148)} = 14.5$, $p < 0.0001$, $n = 11–20$ per group, in (a); $H = 50.6$, $p < 0.0001$, $n = 12–24$ per group, in (c); $F_{(10,143)} = 11.0$, $p < 0.0001$, $n = 11–20$ per group, in (e); $H = 44.3$, $p < 0.0001$, $n = 10–26$ per group, in (g). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* V-treated group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* Dizo-treated group; Dunnett's test in (a, e), Dunn's test in (c, g). S: synergic effect with combination index (CI) < 1.

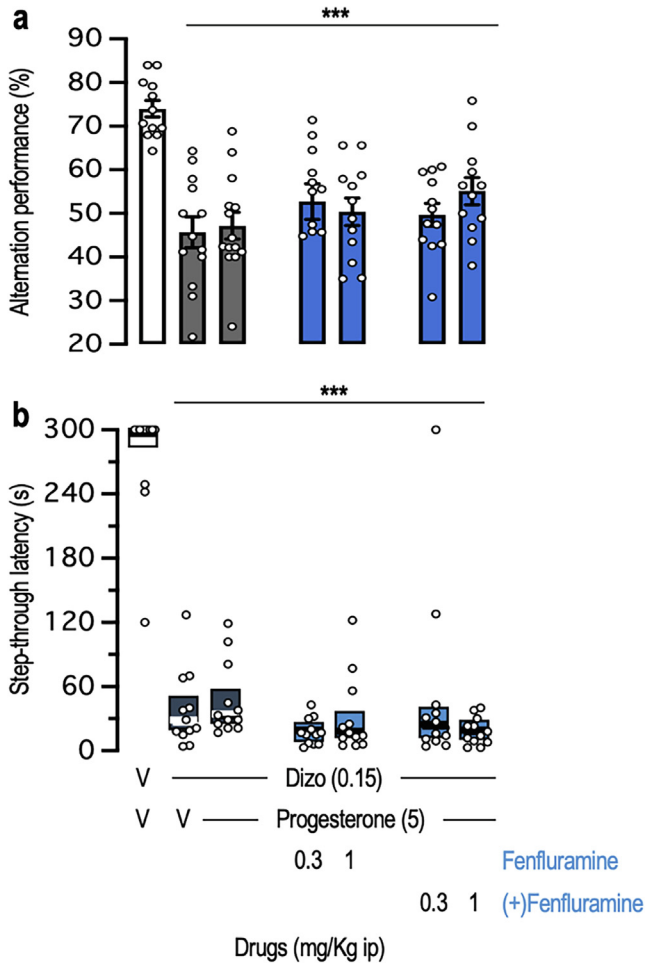


**Fig. 7.** Antagonism by Progesterone of the anti-amnesic effects of Fenfluramine racemate and (+)-Fenfluramine on dizocilpine-induced learning impairments in mice. Spontaneous alternation in the Y-maze (a) and step-through passive avoidance (b). Animals received Progesterone (5 mg/kg sc), 10 min before the drugs (0.1–0.3 mg/kg ip), 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a) and median and interquartile range in (b). ANOVAs: $F_{(6,86)} = 9.22$, $p < 0.0001$, $n = 12–14$ in (a); $H = 36.4$, $p < 0.0001$, $n = 12$ in (b). *** $p < 0.001$ *vs.* V-treated group; Dunnett's test in (a), Dunn's test in (b).

ramine racemate and (+)-isomer effects on Dizocilpine-induced learning impairments involved both 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors, as they were sensitive to a co-treatment with WAY-100635 or RS-127445. This observation confirmed that Fenfluramine mode of action involved an interplay between 5-HT receptors and S1R, the latter allowing a synergistic modulation of serotoninergic neurotransmission. Moreover, Fenfluramine is known to act as a potent serotonin releaser with agonist activities at 5-$HT_{1A}$, 5-$HT_{2A}$, 5-$HT_{2B}$, and 5-$HT_{2C}$ receptors [7,8,57–59]. Noteworthily, a very recent study pointed out a crucial role of 5-$HT_4$ receptors in the action of Fenfluramine to block seizure-induced sudden death in a mouse model of epilepsy [60]. This subtype was not addressed in the present study although 5-$HT_4$ receptors are involved in memory processing and future studies must include this putative mechanism. Our experimental paradigm based on memory-related responses pointed a particular involvement of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors. However, previous studies using experimental models of epilepsy, based on pharmacologically or genetically induced seizures, pointed out effects of Fenfluramine on 5-$HT_{1A}$, 5-$HT_{1D}$, 5-$HT_{2B}$, and 5-$HT_{2C}$ receptors, in NMDA receptor overactivation-induced seizures in mice [27] or in *scn1a* or *scn1ab* mutants zebrafish larvae [26,61]. The interplay with S1R was observed in these studies on different readouts, related to different receptors in different symptoms.

### *4.3. How S1R activity impacts Fenfluramine's mechanism of action in vivo: the example of neuro(active)steroids*

An important aspect of the present study was to examine the relation of Fenfluramine, racemate and (+)-isomer, with neuro(active)steroids. The S1R is an ER chaperone protein and its activation is mainly triggered by physiological signals, such as local increase in oxidative stress or $Ca^{2+}$ mobilization through the MAMs. However, several endogenous ligands have been proven to activate S1R directly, thus acting as putative "endogenous S1R ligands", and these included neuropeptides related to Neuropeptide Y or Calcitonin gene-related peptide [62,63], the hallucinogenic trace amine N,N-Dimethyltryptamine (DMT) [64], Choline [65], or neuro(active)steroids. The latter refer to cholesterol derivatives like pregnenolone (PREG), dehydroepiandrosterone (DHEA), their sulfate esters, progesterone, or allopregnanolone (3α-hydroxy-5α-pregnan-20-one), whose levels were higher in the brain than in plasma, and unrelated to peripheral sources [66,67]. They are named "neurosteroids", and "neuroactive steroids", generated in endocrine glands and transported into the brain from peripheral circulation to modify nervous cells activity and regulate gene expression locally (for reviews, see [68–70]). Steroids from both sources, thus designed globally as "neuro(active)steroids", directly interact with neurotransmitter receptors to impact neuronal excitability and signaling and are involved in different physiopathological conditions. In particular, and although fine neuroanatomical considerations must be considered to draw a precise landscape of their neuromodulatory actions, DHEAS and PREGS are negative modulators of $GABA_A$ receptors, enhancers of glutamate release, positive modulators of NMDA receptors and S1R agonists [71–73]. Progesterone can bind to multiple receptors including the classical intracellular receptors (PR), the membrane receptors (mPRs), and the membrane-binding sites (PGRMC1), but it also acts as a S1R antagonist and NMDA receptor inhibitor and NMDA receptor gene regulator [72,74–79]. Finally, some effects of progesterone are mediated by its neuroactive metabolites and particularly 3α,5α-tetrahydroprogesterone (allopregnanolone, ALLO). ALLO has no affinity for the intracellular PR or S1R but is a potent allosteric modulator of $GABA_A$ receptors [75]. Through these dual actions at NMDA and $GABA_A$ receptors, neuro(active)steroids play a major role in controlling the balance between brain excitation and inhibition and are endogenous regulators of seizure susceptibility [72]. Steroidal deregulation is observed in several epileptic conditions, with direct consequences on glutamatergic or GABAergic neurotransmission: alterations in glutamate or GABA release, deregulation of neuronal and glial transporters expression, or even alterations in related gene expressions, that are differentially affected in brain regions including the frontal or temporal cortex, amygdala, hippocampus, and hypothalamus. Fine control of glutamate and GABA systems is permitted through local effects of neuro(active)steroids such as DHEAS or Progesterone. Moreover, DHEAS and PREGS could be proconvulsants at high doses, partly because they directly block $GABA_A$ receptors and facilitate NMDA receptors. For instance, acute intracerebral or chronic systemic injection of DHEAS and PREGS reduced the threshold of pentylenetetrazole-induced seizures and induced status epilepticus [80]. At these doses of steroids, the S1R may not be involved, particularly since S1R activity dose–response is bell-shaped for all drugs and blocked at high doses [32]. Moreover, in the present study, we observed that Fenfluramine and (+)-Fenfluramine synergistically potentiated only subactive doses of PREGS and DHEAS,

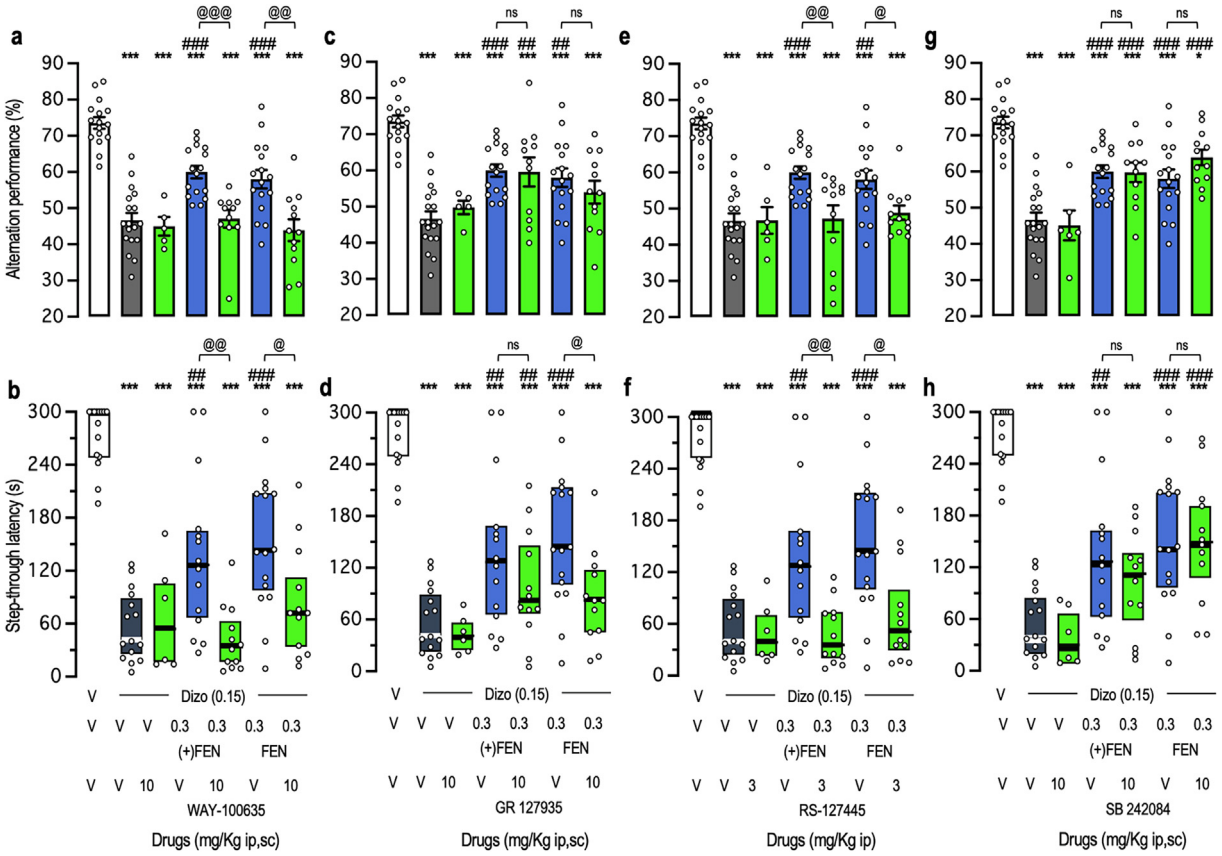


**Fig. 8.** Antagonism by 5-HT receptor antagonists of the anti-amnesic effects of Fenfluramine racemate (FEN) and (+)-Fenfluramine ((+)FEN) on dizocilpine-induced learning impairments in mice. Spontaneous alternation in the Y-maze (a, c, e and g) and step-through passive avoidance (b, d, f and h). Animals received WAY-100635 (10 mg/kg sc) (a and b), GR 127935 (3 mg/kg ip) (c and d), RS-127445 (10 mg/kg sc) (e and f), or SB 242084 (10 mg/kg sc) (g and h), 10 min before the drugs (0.3 mg/kg ip), 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session or the passive avoidance training session, retention being tested after 24 h, without further drug treatment. Data show mean ± SEM in (a, c, e and g) and median and interquartile range in (b, d, f and h). ANOVAs: $F_{(6,93)} = 23.6$, $p < 0.0001$, $n = 5–18$ in (a); $H = 48.9$, $p < 0.0001$, $n = 6–16$ in (b); $F_{(6,93)} = 13.5$, $p < 0.0001$, $n = 5–18$ in (c); $H = 46.7$, $p < 0.0001$, $n = 6–16$ in (d); $F_{(6,95)} = 19.2$, $p < 0.0001$, $n = 6–18$ in (e); $H = 49.8$, $p < 0.0001$, $n = 6–16$ in (f); $F_{(6,94)} = 18.35$, $p < 0.0001$, $n = 6–18$ in (g); $H = 47.9$, $p < 0.0001$, $n = 6–16$ in (h). *** $p < 0.001$ *vs*. V-treated group; ## $p < 0.01$, ### $p < 0.001$ *vs*. Dizo-treated group; @ $p < 0.05$, @@ $p < 0.01$, @@@ $p < 0.001$ *vs*. respective (+)FEN- or FEN-treated group; ns, not significant; Dunnett's test in (a, c, e and g), Dunn's test in (b, d, f and h).

suggesting that: (i) the S1R modulatory effect of the drugs is observed on S1R-acting endogenous agonists, and (ii) the modulatory effect on S1R activity will likely not result in an amplification of the proconvulsant effect of the steroids. Indeed, we previously reported, in a coherent manner, that DHEAS is neuroprotective but not neurotoxic, in a mouse model of excitotoxicity [32]. Finally, as neuroactive steroid levels, and particularly the physiological fluctuations of progesterone level in female patients, will directly impact the S1R activity, it pointed out that the S1R pharmacological component of Fenfluramine action may be more relevant in infants, when steroidal tonus is low, than in adults. This must be particularly considered in Dravet syndrome, a pathological condition appearing very early in lifespan.

### *4.4. Conclusions*

We used, in the present study, isobologram calculations of combination indexes to show that Fenfluramine, and particularly (+)-Fenfluramine, act as effective S1R-positive modulators *in vivo* in mice. The metabolite Norfenfluramine rather acts as a S1R antagonist. This pharmacological activity impacts the serotoninergic activity of the drug at several 5-HT receptors. It also applies to endogenous S1R modulators, *e.g.*, neuro(active)steroids, without amplifying their putative excitatory/proconvulsant effects. Fenfluramine provides a sustained, long-term treatment effect of seizures associated with Dravet syndrome and Lennox–Gastaut syndrome. Positive efficacy has also been reported on seizures in a small cohort of patients with CDKL5 deficiency disorder [81]. In addition to its effectiveness as an ASM, patients taking fenfluramine also showed significant and clinically meaningful improvements on the Behavior Rating Inventory of Executive Function (BRIEF®2), a measure of emotional and cognitive functioning [82]. The data presented within this review suggest that fenfluramine's profound clinical effectiveness is due to mechanisms that likely include the release of serotonin, agonist activity at certain serotonin receptors and activity at S1R. The positive modulation of S1R by fenfluramine may occur through amplification of an endogenous S1R agonist such as a neuro(active) steroid. As noted above, the metabolite norfenfluramine acts as an antagonist at S1R. This complex multifaceted mechanism of fenfluramine could account for its profound activity as an ASM and helps point to potential new targets for the treatment of developmental and epileptic encephalopathies such as Dravet syndrome.

## Declaration of Competing Interest

P. Martin, A. Gammaitoni, B. Boyd, G. Farfel, and B. Galer are employees of, and/or own stock in, Zogenix, Inc. T. Maurice received consultancy fees from Zogenix.

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