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Fenfluramine acts as a positive modulator of sigma-1 receptors

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ABSTRACT

Objective: Adjunctive fenfluramine hydrochloride, classically described as acting pharmacologically through a serotonergic mechanism, has demonstrated a unique and robust clinical response profile with regard to its magnitude, consistency, and durability of effect on seizure activity in patients with pharmacoresistant Dravet syndrome. Recent findings also support long-term improvements in executive functions (behavior, emotion, cognition) in these patients. The observed clinical profile is inconsistent with serotonergic activity alone, as other serotonergic medications have not been demonstrated to have these clinical effects. This study investigated a potential role for σ_1 receptor activity in complementing fenfluramine's serotonergic pharmacology.

Methods: Radioligand binding assays tested the affinity of fenfluramine for 47 receptors associated with seizures in the literature, including σ receptors. Cellular function assays tested fenfluramine and norfenfluramine (its major metabolite) activity at various receptors, including adrenergic, muscarinic, and serotonergic receptors. The σ_1 receptor activity was assessed by the mouse vas deferens isometric twitch and by an assay of dissociation of the σ_1 receptor from the endoplasmic reticulum stress protein binding immunoglobulin protein (BiP). In vivo mouse models assessed fenfluramine activity at σ_1 receptors in ameliorating dizocilpine-induced learning deficits in spatial and nonspatial memory tasks, alone or in combination with the reference σ_1 receptor agonist PRE-084.

Results: Fenfluramine and norfenfluramine bound ≥30% to β_2-adrenergic, muscarinic M_1, serotonergic 5-HT_{1A}, and σ receptors, as well as sodium channels, with a K_i between 266 nM (σ receptors) and 17.5 μM (β-adrenergic receptors). However, only σ_1 receptor isometric twitch assays showed a positive functional response, with weak stimulation by fenfluramine and inhibition by norfenfluramine. Fenfluramine, but not the 5-HT_{2C} agonist lorcaserin, showed a positive modulation of the PRE-084-induced dissociation of σ_1 protein from BiP. Fenfluramine also showed dose-dependent antiamnesic effects against dizocilpine-induced learning deficits in spontaneous alternation and passive avoidance responses, which are models of σ_1 activation. Moreover, low doses of fenfluramine synergistically potentiated the low-dose effect of PRE-084, confirming a positive modulatory effect at the σ_1 receptor. Finally, all in vivo effects were blocked by the σ_1 receptor antagonist NE-100.

Significance: Fenfluramine demonstrated modulatory activity at σ_1 receptors in vitro and in vivo in addition to its known serotonergic activity. These studies identify a possible new σ_1 receptor mechanism underpinning fenfluramine's central nervous system effects, which may contribute to its antiseizure activity in Dravet syndrome and positive effects observed on executive functions in clinical studies.

1. Introduction

Racemic fenfluramine has long been considered to act primarily through a serotonergic pharmacological mechanism of action [1–3]. In recent phase 3 randomized controlled clinical trials (NCT02926898 and NCT02682927/NCT02826863), fenfluramine demonstrated robust efficacy as an add-on antiepileptic drug (AED) for treating patients with Dravet syndrome [4,5], the presumed activity of which is mostly due to decreases in neuronal excitability by pharmacological modulation of calcium channels and/or gamma aminobutyric acid-ergic (GABAergic) neurotransmission [6,7]. Further, in an open-label extension study (OLE; NCT02823145), patients with at least 1 year of fenfluramine treatment and meaningful seizure reduction also showed improvements in executive functions (behavior, emotions, and cognition), compared with prerandomization baseline [8]. Animal data also

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support a role for fenfluramine in dose-dependently improving cognitive function via centrally acting mechanisms [9].

Fenfluramine's known pharmacological profile as a potent serotonin (5-HT) releaser with agonist activity at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors [1–3] is likely insufficient to fully explain its clinical efficacy in seizure frequency reduction or its positive cognitive profile. Other predominantly 5-HT-acting agents (e.g., selective serotonin reuptake inhibitors [SSRIs]; lorcaserin), unlike fenfluramine, have inconclusive or inconsistent effects in reducing seizure frequency associated with Dravet syndrome [6,10,11]. Thus, the pharmacological mechanism of fenfluramine is likely to be more complex than a 5-HT-driven response alone, and may include synergy between serotonergic activity and other systems associated with mitigating epileptogenesis and/or seizure activity.

Here, we describe experiments to investigate potential additional mechanisms of action of fenfluramine (Supplemental Table 1) to determine whether fenfluramine affects receptor systems beyond 5-HT, especially σ receptors, as suggested based on prior pharmacological reports [12–14]. A series of experiments was conducted with the intent of answering 3 key research questions:

1. Do fenfluramine and norfenfluramine **bind to receptors** implicated in seizures at clinically relevant doses?
2. What **functional effects** do fenfluramine and norfenfluramine exert in vitro on any receptors identified in (1)?
3. What **physiological effects** do fenfluramine and in vivo metabolism to norfenfluramine exert in mouse models with specificity for these receptors and associated pathways?

The results of these studies have demonstrated that fenfluramine activity at 5-HT receptors is complemented synergistically by functional activation of σ_1 receptors in vitro and ex vivo, as well as in vivo through centrally acting σ_1 receptor-mediated pathways that control behavioral responses in mouse models of spatial and contextual learning.

2. Materials and methods

2.1. In vitro radioligand binding assays

Radioligand binding assays for 47 receptors associated with seizures in the literature were conducted by Sekisui Medical Co., Ltd. (Tokyo, Japan), using previously published methods (Supplemental Table 2) [15].

2.2. In vitro receptor functional activity assays

In vitro cellular and nuclear functional assays were conducted by Eurofins Cerep (Celle l'Evescault, France), using published methods [16–21]. Agonist and antagonist activities were assessed using human recombinant receptors expressed in HEK-293 cells (β_1 adrenergic receptors [ARs] and 5-HT_{1A} receptors), SK-N-MC cells (β_3ARs), or Chinese hamster ovary (CHO) cells (M_1 muscarinic acetylcholine receptors [M_1 mAChRs] and β_2ARs), with 3×10^{-9} to 1×10^{-5} M fenfluramine or norfenfluramine. In β_1AR, β_2AR, and β_3AR assays, change in cyclic adenosine monophosphate (cAMP) concentration was measured by homogeneous time-resolved fluorescence after isoproterenol stimulation (antagonist assays: 3 nM, 10 nM, or 5000 nM, respectively) or with isoproterenol controls (agonist assays: 100 nM each), with room temperature incubation for 30 min (β_1AR, β_2AR) or 10 min (β_3AR). For M_1 mAChR assays, intracellular Ca^{2+} concentration was measured by fluorimetry following stimulation with 10 nM acetylcholine (antagonist assays) or 100 nM acetylcholine as a control (agonist assays), with a 30-minute room temperature incubation.

Given that 5-HT_{1A} has been implicated in both antiseizure [10] and learning-and-memory functions [22,23], we tested fenfluramine binding interaction with 5-HT_{1A} receptors. In 5-HT_{1A} assays, cellular dielectric spectroscopy measured impedance at 37 °C after stimulation with 100 nM 8-OH-DPAT (antagonist assays) or 10 μM 8-OH-DPAT as a control (agonist assays). Values for the concentration producing a half-maximal response (EC_{50}) and the concentration that inhibited binding by 50% (IC_{50}) were determined by nonlinear regression analysis of concentration–response curves generated with mean replicate values, using Hill equation curve fitting with Hill software (Cerep), and validated by SigmaPlot (SPSS Inc., Quarry Bay, Hong Kong; v.4.0). σ_1 Receptor activity assays using guinea-pig vas deferens isometric twitch response were conducted by Eurofins Cerep, as described [21]. In the agonist assay, twitch response after fenfluramine or norfenfluramine was compared with the positive control (+)-SKF-10,047. The antagonist assay assessed inhibition of (+)-SKF-10,047 activity by fenfluramine or norfenfluramine (3×10^{-9} to 1×10^{-5} M) on guinea-pig vas deferens tissue by measuring isometric twitch tension with force transducers that recorded isometric tension. Measurements were taken in a 20-mL physiological-salt organ bath (37 °C) with the inhibitors yohimbine, propranolol, (−)sulpiride, cimetidine, atropine, naloxone, and methysergide (1 μM each). Data were expressed as the IC_{50} (μM).

2.3. σ_1 protein-BiP dissociation assays

Using the 5-HT_{2C}-specific agonist and investigational AED lorcaserin [11,24] as a comparator, we performed BiP dissociation assays to test whether fenfluramine (which also has some activity at 5-HT_{2C} receptors [14]) had functional activity at σ_1 receptors. The σ_1 protein-BiP dissociation assays were performed by Amylgen (Montferrier-sur-Lez, France) as described [25]. CHO cells were maintained in minimum essential medium (MEM) culture medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM Glutamax (Invitrogen, Carlsbad, CA). Cells were plated and treated with test compounds dissolved in culture medium for 30 min (37 °C) until reaction termination by replacing culture medium with phosphate buffered saline (3 mL). Cells were harvested and suspended in 50 mM Hepes pH 7.4, then crosslinked with 50 μg/mL of dithiobis succinimidyl propionate (Thermoscientific, Waltham, MA) until reaction termination by adding Tris/HCl 50 mM pH 8.8. After incubating 15 min on ice, cells were lysed in 50 mM Tris pH 7.4 buffer, containing 150 mM NaCl, 1% Triton X-100, 0.3% sodium deoxycholate, 0.1% sodium dodecylsulfate, and protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland). After centrifugation (12,000 ×*g*, 1 min), supernatants were incubated overnight (4 °C) with σ_1 receptor antibody (Abcam, Cambridge, UK). Lysates were incubated with Sepharose Protein-A (Invitrogen) (90 min). After centrifugation (12,000 ×*g*, 1 min), the supernatant was discarded and pellet suspended in 0.5 mL radioimmunoprecipitation assay (RIPA) buffer. The pellet suspension was centrifuged (12,000 ×*g*, 20 min), the supernatant decanted, and the pellet suspended in 0.5 mL 2× sample buffer/bMCE buffer. After centrifugation (12,000 ×*g*, 1 min), supernatants were analyzed for BiP immunoreactivity (SEC343Mu ELISA, USCNK Life Sciences, Wuhan, China).

2.4. Drugs and injections

Racemic fenfluramine was provided by Zogenix, Inc. (Emeryville, CA). 2-(4-Morpholinethyl)-1-phenylcyclohexanecarboxylate hydrochloride (PRE-084) and (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate ((+)-MK-801, dizocilpine) were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). 4-Methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine hydrochloride (NE-100) was from Tocris Bioscience (Bristol, UK). Drugs were solubilized in physiological saline (vehicle solution). Administrations were intraperitoneal (ip) (100 μL/20 g body weight).

2.5. Behavioral assays

As σ_1 receptor agonists are potent antiamnesic compounds, particularly against learning deficits induced by dizocilpine, a noncompetitive antagonist of the glutamatergic N-methyl-D-aspartate (NMDA) receptor [26–28], fenfluramine activity at the σ_1 receptor was tested using two validated behavior tests: spontaneous alternation in the Y-maze, an index of spatial working memory [27,29–31], and step-through type passive avoidance [30,31], a nonspatial long-term memory assay. Mice were tested on Day 1 for spontaneous alternation, then trained for passive avoidance on Day 2 and retention on Day 3. Animal models were used to test whether fenfluramine has in vivo, centrally acting σ_1 receptor activity. Spontaneous alternation in the Y-maze and step-through passive avoidance are mouse models of spatial and contextual learning, respectively, which are validated metrics for investigating whether a compound acts on neuronal σ_1 receptors to modulate a behavior known to be mediated by σ_1.

2.5.1. Animals

Male Swiss OF-1 mice (aged 7–9 weeks, weight 32 ± 2 g) were from Janvier (St Berthevin, France). Mouse housing and experiments took place at the 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/air-regulated experimental room with 30-minute habituation time). All animal procedures were conducted in strict adherence to the European Union Directive of September 22, 2010 (2010/63), and the ARRIVE guidelines [32].This addition was made per Dr. Schachter's email.

2.5.2. Spontaneous alternation in the Y-maze

Animals were tested for spontaneous alternation performance in the Y-maze, an index of spatial working memory [27,29,31]. 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 consecutive entry into all three arms, with the number of maximum alternations = total arm entries minus 2, and percentage of alternation = (actual alternations / maximum alternations) × 100. Parameters included percentage of alternation (memory index) and total number of arm entries (exploration index).

2.5.3. Step-through passive avoidance

The test assesses nonspatial/contextual long-term memory [30,31]. 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) and (2) having 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 (max, 300 s). Results were expressed as median and interquartile (25%–75%) range because the data are nonparametric with an established upper limit.

2.5.4. Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA, *F* value), followed by Dunnett's test or Kruskal–Wallis nonparametric ANOVA (*H* value), followed by Dunn's multiple comparison tests for passive avoidance latencies (expressed as median and interquartile range; statistical significance, $P < 0.05$).

2.5.5. Combination index calculations

Isobologram analyses, evaluating the nature of interaction of two drugs at a given effect level, were performed according to Fraser's concept [30,33]. Theoretically, the concentrations required to produce the given effect (e.g., where $IC_x = IC_{50}$) are 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, $IC_{x,B}$) (Supplemental Fig. 1). The line connecting these two points is the line of additivity. Concentrations of A and B contained in the combination that provide the same effect, denoted as ($C_{A,x}$, $C_{B,x}$), are placed in the same plot. Synergy, additivity, or antagonism is indicated when ($C_{A,x}$, $C_{B,x}$) is located below, on, or 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 drugs A and B used in a combination that generates x% of the maximal combination effect; CI is the combination index; and $IC_{x,A}$ 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 curves [30,33], alternation percentages and passive avoidance latencies were expressed as percentage of protection (PP) for each treatment group (combinations of vehicle [V] or dizocilpine/PRE-084/fenfluramine), where PP(V/V/V) = 100% and PP (dizocilpine/V/V) = 0%. "Protection" is defined as an intervention's inhibition of dizocilpine-induced memory impairment.

2.5.6. Role of the sponsor

The sponsor provided the study drug (fenfluramine) and participated in the study design; collection, analysis, and interpretation of the data; writing the report; and the decision to submit the article for publication.

3. Results

3.1. Receptor binding affinity studies

A literature search identified 47 receptors associated with seizures in the literature (Supplemental Table 2). Of the 5-HT receptors, only 5-HT_{1A} was examined. Radioligand binding assays were performed to determine binding affinity for fenfluramine or its major metabolite, norfenfluramine. Of the receptor subtypes identified as seizure-associated, fenfluramine and norfenfluramine binding occurred at ≥30% binding at β_2ARs, muscarinic M_1 receptors, sodium channels, 5-HT_{1A} receptors, and nonselective σ receptors, as measured by percent inhibition of radioligand binding (Table 1).

3.2. In vitro functional activity assays

To determine whether the identified receptor binding resulted in functional agonist or antagonist responses at clinically achievable concentrations at the receptor or cellular level, a series of in vitro assays was performed. The assays selected were specific to each receptor subtype (Supplemental Table 1, middle column). Sodium channel profiling, including Nav1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, and 1.8, showed no significant responses, while β_2ARs, muscarinic M_1 receptors, and 5-HT_{1A} receptors

Table 1
In vitro pharmacology of fenfluramine and norfenfluramine.

Receptor tested[a]	Source	(±)-Fenfluramine	(±)-Norfenfluramine
K_i values (M) of test compounds on radioligand binding to receptors with ≥30% binding based on percent inhibition			
β-Adrenergic (nonselective)	Rat brain	1.75×10^{-5}	1.20×10^{-5}
β_2-Adrenergic	Human recombinant	1.26×10^{-5}	8.77×10^{-6}
Muscarinic M_1	Rat cerebral cortex	1.13×10^{-5}	3.74×10^{-6}
Sodium channel	Rat brain	4.84×10^{-6}	4.74×10^{-6}
5-HT_{1A} (serotonin)	Rat cerebral cortex	3.27×10^{-7}	6.73×10^{-7}
Sigma σ (nonselective)	Guinea-pig brain	2.66×10^{-7}	2.92×10^{-6}

[a] Assay is radioligand binding with 7 concentrations of fenfluramine/norfenfluramine added to brain extracts or recombinant protein. Ratio of specific radioactivity in the presence of test substance to total bound radioactivity without the test substance was transformed by logit transformation. K_i values were determined from dose–response curves.

did not show functional results within clinically relevant ranges (IC_{50} < 1 μM) (data not shown).

Regarding putative σ_1 activity, the drugs were first tested for the modulation of electrically stimulated contractions of the guinea-pig vas deferens, a classical σ receptor functional assay [34,35]. Fenfluramine failed to affect vas deferens contraction at concentrations up to 10 μM, while norfenfluramine decreased contraction only at the highest concentration tested (Fig. 1A; Supplemental Table 3). When tested in combination with the reference σ_1 receptor agonist (+)-SKF-10,047 (100 μM), fenfluramine moderately increased the agonist-induced response (Fig. 1B; Supplemental Table 3). Norfenfluramine decreased the agonist response, thus appearing to act similarly but less efficiently as rimcazole, a weak σ_1 antagonist, in this test (Fig. 1B). Norfenfluramine could therefore putatively behave as a very weak σ_1 antagonist.

To test whether the fenfluramine-induced increase in (+)-SKF-10,047 response was relevant, we used a more sensitive in vitro assay, first described by Hayashi & Su [25]. Activation of the σ_1 receptor results in its dissociation from the endoplasmic reticulum stress protein BiP. The dissociation can be measured in σ_1-expressing cells by a simple immunoprecipitation/significantly potentiated (ELISA) assay. Fenfluramine, tested at 1 and 10 μM, failed to affect the BiP/σ_1 receptor association, in contrast to the reference σ_1 receptor agonist PRE-084, which dissociates 40% of the complex at 10 μM (Fig. 1C). When fenfluramine was coadministered at 1 and 10 μM with PRE-084, it significantly potentiated the efficacy of the σ_1 agonist. More than 60% of the complex was dissociated with the highest concentrations, 10 μM fenfluramine + 10 μM PRE-084 (Fig. 1C). Lorcaserin, a selective agonist of the 5-HT_{2C} receptor, was tested as a comparator. Lorcaserin did not affect

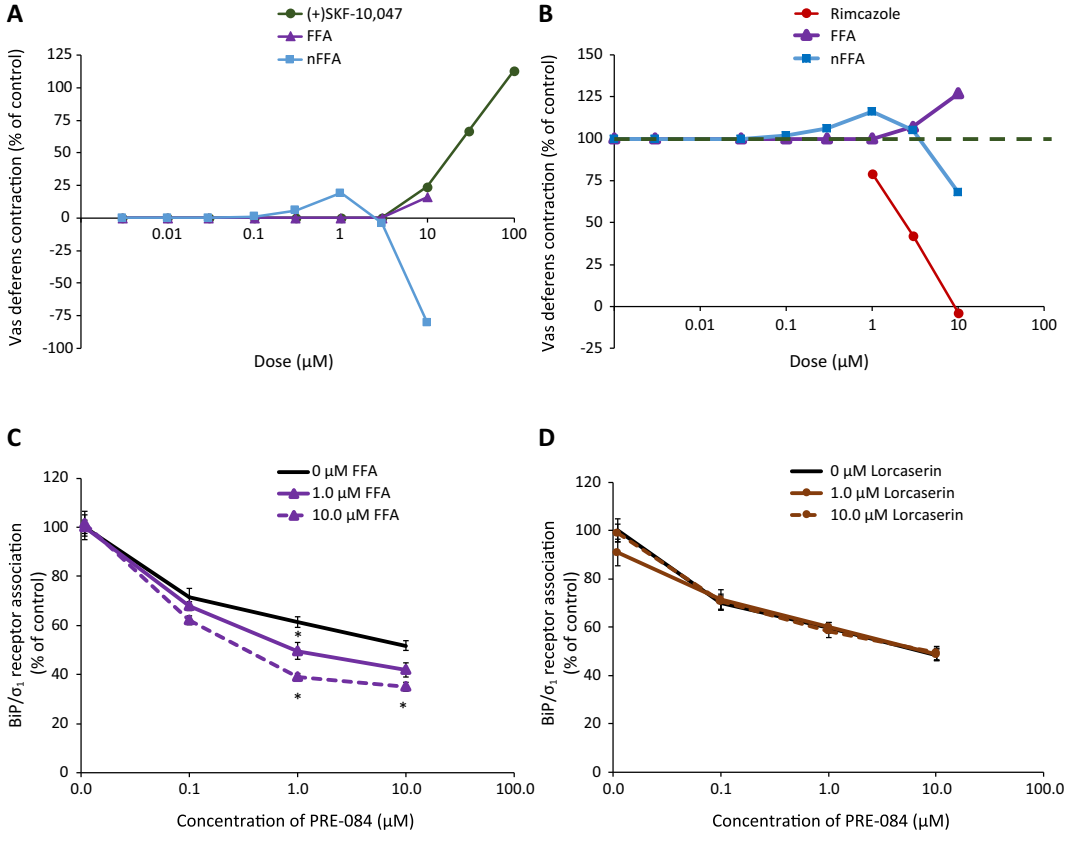

Fig. 1. In vitro pharmacology of fenfluramine (FFA) and norfenfluramine (nFFA) at σ_1 receptors. Concentration–effect curve to determine σ_1 receptor (A) agonist or (B) antagonist activity of fenfluramine, norfenfluramine, or reference (agonist (+)-SKF-10,047 [100 μM]; antagonist rimcazole [10 μM]) on change from control in the amplitude of electrically stimulated contractions in guinea-pig vas deferens (5 Hz, 1 ms at 10-second intervals) in the absence (agonist) or presence (antagonist) of a submaximal dose of σ_1 receptor agonist, (+)-SKF-10,047 (100 μM). (C, D) Positive modulatory effect of fenfluramine, but not lorcaserin, on σ_1 receptors: concentration–response study to characterize the positive modulatory effect of (C) fenfluramine or (D) lorcaserin on PRE-084-induced BiP/σ_1 receptor dissociation after 30 min of incubation in culture medium. *$P < 0.05$ compared with vehicle control by 2-way ANOVA with post hoc Bonferroni analysis; n = 6 per condition.

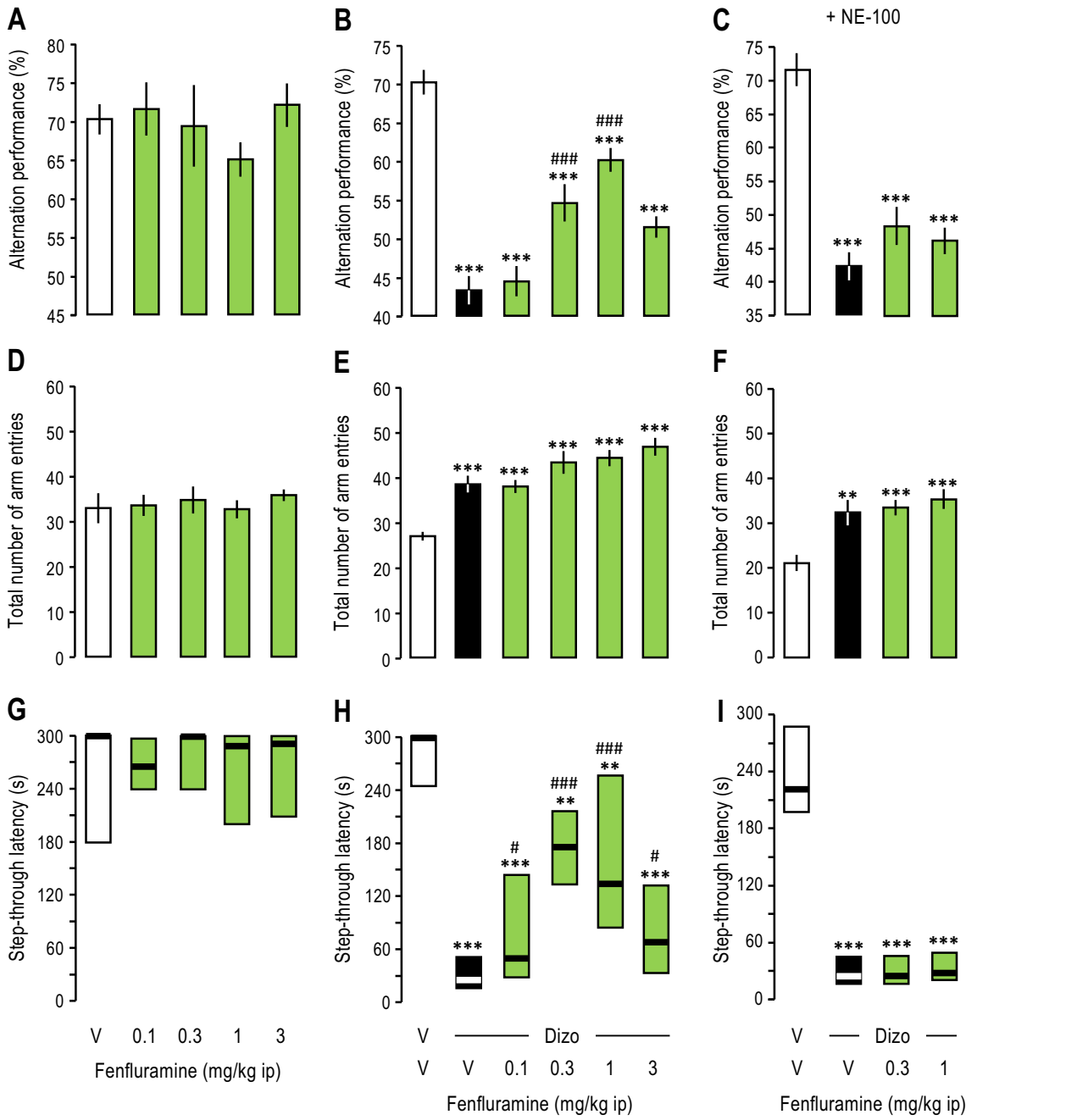

Fig. 2. Dose-dependent attenuation by fenfluramine of dizocilpine-induced learning impairments in mice: dose–response effect of fenfluramine alone (A, D, G), in pretreatment before the amnesic drug dizocilpine (B, E, H), and with blockade of the antiamnesic effect by the σ_1 receptor antagonist NE-100 (C, F, I) for spontaneous alternation performance (A–C), number of arm entries (D–F) in the Y-maze test, and step-through latency (G–I) in the passive avoidance test. Animals received vehicle solution (V) or fenfluramine (0.1–3 mg/kg) ip, 30 min before the Y-maze test session or passive avoidance training or 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the behavioral session. Passive avoidance retention was tested after 24 h, without further drug treatment. In (C, F, I), NE-100 (1 mg/kg) was injected ip immediately before V or fenfluramine. Data show mean ± SEM in (A–F) and median and interquartile range in (G, H, I). ANOVA: $F_{(4,49)} = 0.686$, $P > 0.05$, n = 10 per group in (A); $F_{(5,78)} = 35.8$, $P < 0.0001$, n = 12–17 in (B); $F_{(3,47)} = 30.7$, $P < 0.0001$, n = 12 in (C); $F_{(4,49)} = 0.275$, $P > 0.05$ in (D); $F_{(5,78)} = 16.8$, $P < 0.0001$ in (E); $F_{(3,47)} = 8.55$, $P < 0.0001$ in (F). Kruskal–Wallis ANOVA: $H = 1.43$, $P > 0.05$, n = 10 in (G); $H = 39.9$, $P < 0.0001$, n = 12–17 in (H); $H = 24.8$, $P < 0.0001$, n = 11–12 in (I). $^{**}P < 0.01$, $^{***}P < 0.001$ vs V-treated group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs Dizo-treated group; Dunnett's test in (A–F), Dunn's test in (G–I).

the level of BiP/σ_1 receptor association nor the ability of PRE-084 to dissociate the complex (Fig. 1D).

3.3. In vivo effects of σ_1 receptor positive modulation by fenfluramine

Fenfluramine was tested against learning deficits induced by dizocilpine in the 0.1 to 3 mg/kg dose-range, using spontaneous alternation and the step-through passive avoidance response. The drug failed to affect spontaneous alternation by itself (Fig. 2A) but induced a significant bell-shaped attenuation of the dizocilpine-induced deficit of alternation at the doses of 0.3 and 1 mg/kg ip (Fig. 2B). Cotreatment with the σ_1 receptor antagonist NE-100 (1 mg/kg ip) failed to affect alternation performance by itself or dizocilpine-induced deficits but blocked the attenuation induced by fenfluramine at 0.3 or 1 mg/kg (Fig. 2C). Fenfluramine treatment did not affect the exploratory locomotor activity (measured by the total number of arms entered during the session) by itself (Fig. 2D) nor did it change the increased activity induced by dizocilpine, a well-known psychostimulant (Fig. 2E, F). In the passive avoidance test, fenfluramine, without having an effect alone (Fig. 2G), attenuated dizocilpine-induced deficits in a bell-shaped

manner, with significant attenuation at all doses tested (Fig. 2H). Because fenfluramine is known to produce analgesic effects, shock sensitivity was measured during training. Fenfluramine treatment did not affect shock sensitivity in the dose-range tested (ANOVA for shock sensitivity in Fig. 2G: $H = 0.713$, $P > 0.05$; data not shown). A marked analgesic effect was observed only with some mice tested with the dizocilpine–fenfluramine combination at 10 mg/kg (data not shown), with a shock sensitivity of 0.8 ± 0.5 vs 1.7 ± 0.1 for controls, suggesting that the antiamnesic effect of fenfluramine was disconnected from any analgesic effects. The drug effect was also prevented by NE-100 (Fig. 2I). These observations suggest that, in vivo, fenfluramine may be a positive modulator of σ_1 receptor activity.

Fenfluramine was then tested in combination with PRE-084 (Fig. 3). The σ_1 receptor agonist prevents dizocilpine-induced learning deficits at 0.3 to 1 mg/kg ip [27]. Indeed, we confirmed here that 0.3 mg/kg but not 0.1 mg/kg significantly attenuated the alternation and passive avoidance deficits (Fig. 3A, D). Combinations of fenfluramine and PRE-084 were tested at the nonactive and low active doses, 0.1 mg/kg and 0.3 mg/kg, respectively. All combinations tested resulted in increased, significant attenuations of the deficits in both tests (Fig. 3A, D). PPs for both alternation performance and passive avoidance were greater in combinations of fenfluramine and PRE-084 than with either drug alone, even at subtherapeutic doses of both drugs (Fig. 3B, E). The PPs were calculated for the dizocilpine + PRE-084 (0.1, 0.3 mg/kg) and dizocilpine + fenfluramine (0.1, 0.3 mg/kg) groups and for each combination (cursor positions in Fig. 3B, E). CIs were calculated, as detailed in the Materials and methods section, and results are summarized in Table 2. A CI < 1, indicating a synergy between the two drugs, was measured in 3 combinations out of 4 for each test, most notably with the lowest doses of fenfluramine and PRE-084 (0.1 mg/kg) (Fig. 3B, E). Such synergy is expected for combination between an agonist and a positive modulator [30,36,37]. Finally, we confirmed that the PRE-084 effect and the potentiation by fenfluramine were mediated by σ_1 activity since cotreatment with NE-100 prevented all effects in both tests (Fig. 3C, F). These observations showed that fenfluramine acts as a positive modulator on σ_1 receptor-mediated antiamnesic effects in vivo.

4. Discussion

This study investigated how fenfluramine might differ mechanistically from other antiepileptic and serotonergic drugs, after such differences were suggested by fenfluramine's proven robust efficacy in treating seizures associated with pharmacoresistant Dravet syndrome, in addition to associated improvements in executive functions [8]. Combined results of our binding studies, receptor functionality assays, and behavioral mouse models suggest that fenfluramine (1) positively modulates σ_1 receptors in vitro and (2) improves cognitive functions of spatial and contextual learning via activity at σ_1 receptors in mouse models.

Our study reports positive modulatory activity of fenfluramine at σ_1 receptors in addition to fenfluramine's well-characterized serotonin agonist properties [1–3]. Fenfluramine, but not the selective 5-HT_{2C} agonist lorcaserin, demonstrated positive modulation at σ_1 in the BiP/σ_1

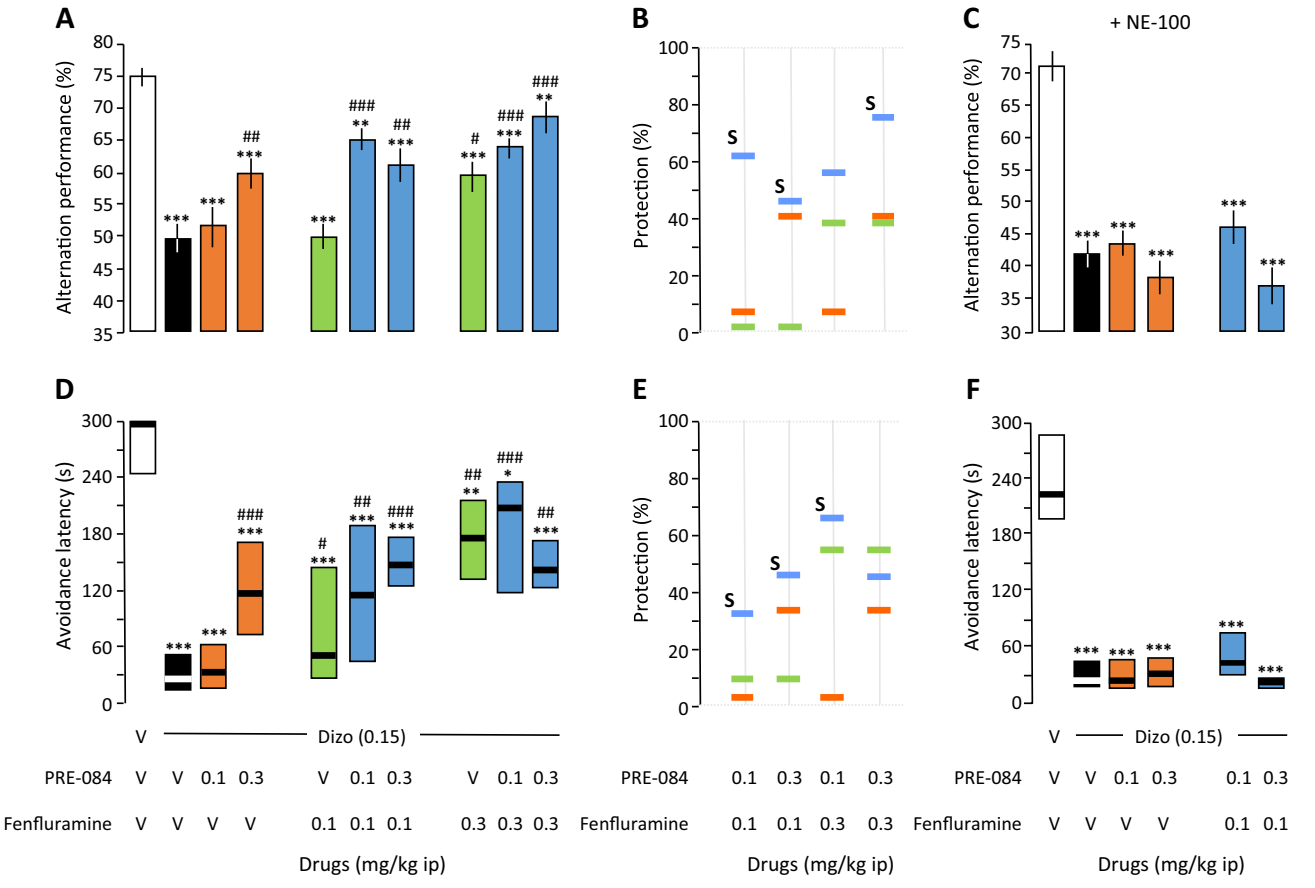

Fig. 3. Dose-dependent positive modulatory activity of fenfluramine on the σ_1 receptor in preventing dizocilpine-induced learning impairment in mice: spontaneous alternation performance in the Y-maze (A–C) and step-through passive avoidance test (D–F). In (A, D), Vehicle solution (V), PRE-084 (0.1–0.3 mg/kg) and/or fenfluramine (0.1–0.3 mg/kg) were injected 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) and median and interquartile range in (D, F). In (B, E), protection is shown using a cursor-on-scale representation, with data from V-treated group as 100% and Dizo-treated group as 0%. In (C, F), NE-100 (1 mg/kg ip) was injected ip immediately before V, PRE-084, and/or fenfluramine. ANOVA: $F_{(9,133)} = 16.1$, $P < 0.0001$, n = 11–20 per group, in (a); $F_{(5,71)} = 26.8$, $P < 0.0001$, n = 12 per group in (c); $H = 58.1$, $P < 0.0001$, n = 11–18 per group, in (d); $H = 32.0$ $P < 0.0001$, n = 11–12 in (f). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs V-treated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Dizo-treated group; Dunnett's test in (A, C), Dunn's test in (D, F). S: synergic effect with combination index CI < 1.

Table 2
Combination index (CI), calculated using spontaneous alternation or passive avoidance results.

Treatment (mg/kg ip)	PP (%)	$C_{x,PRE-084}$	$C_{x,fenfluramine}$	Combination index (CI)*
Spontaneous alternation				
PRE-084 (0)	0.0 ± 9.0			
PRE-084 (0.1)	6.6 ± 12.8			
PRE-084 (0.3)	40.5 ± 9.2[a]			
Fenfluramine (0)	0.0 ± 9.0			
Fenfluramine (0.1)	1.3 ± 7.9			
Fenfluramine (0.3)	37.9 ± 9.4			
Fenfluramine (1)	59.8 ± 6.1[b]			
PRE-084 (0.1) + Fenfluramine (0.1)	61.5 ± 6.8	0.46 ± 0.05	0.96 ± 0.08	**0.32 ± 0.03**
PRE-084 (0.1) + Fenfluramine (0.3)	55.9 ± 6.1	0.42 ± 0.04	0.87 ± 0.07	**0.58 ± 0.05**
PRE-084 (0.3) + Fenfluramine (0.1)	45.6 ± 10.5	0.35 ± 0.04	0.70 ± 0.06	1.01 ± 0.09
PRE-084 (0.3) + Fenfluramine (0.3)	75.1 ± 9.5	0.56 ± 0.06	1.19 ± 0.10	**0.79 ± 0.07**
Passive avoidance				
PRE-084 (0)	0.0 ± 3.8			
PRE-084 (0.1)	4.9 ± 9.9			
PRE-084 (0.3)	34.6 ± 10.5[c]			
Fenfluramine (0)	0.0 ± 3.8			
Fenfluramine (0.1)	11.0 ± 11.6			
Fenfluramine (0.3)	55.7 ± 10.5			
Fenfluramine (1)	40.8 ± 13.0[d]			
PRE-084 (0.1) + Fenfluramine (0.1)	33.7 ± 11.0	0.30 ± 0.03	0.55 ± 0.05	**0.51 ± 0.06**
PRE-084 (0.1) + Fenfluramine (0.3)	66.8 ± 12.9	0.58 ± 0.05	1.51 ± 0.12	**0.58 ± 0.08**
PRE-084 (0.3) + Fenfluramine (0.1)	46.3 ± 7.6	0.41 ± 0.04	0.91 ± 0.09	**0.84 ± 0.09**
PRE-084 (0.3) + Fenfluramine (0.3)	43.8 ± 10.5	0.39 ± 0.04	0.84 ± 0.07	1.13 ± 0.15

Percent protection was calculated using 100% for vehicle-treated animals and 0% for dizocilpine-treated animals. CI, combination index; C_x, drug was calculated using the linear regression from responses with the drug alone; ip, intraperitoneal; PP, percent protection. *CI in bold shows synergy.
[a] $y = 139.89x - 2.938$.
[b] $y = 60.06x + 3.734$.
[c] $y = 120.14x - 2.827$.
[d] $y = 34.44x + 14.8$.

receptor dissociation assay, suggesting that fenfluramine could act as a positive modulator of the σ_1 receptor, devoid of activity when administered alone but potentiating the response of σ_1 receptor agonists (Fig. 1). The σ_1 receptor is a unique protein, chaperoning numerous targets, and there is some evidence for σ_1 receptor interaction with 5-HT to facilitate intracellular heteromeric protein–protein interactions [12] in normal physiological conditions that can be activated by physiological cellular triggers (e.g., endoplasmic reticulum stress, oxidative stress) [26,36]. Maurice et al. [26] originally reported behavioral correlates of this activity by describing dose-dependent, bell-shaped attenuation of dizocilpine-induced learning deficits by σ_1 agonists. Results from our studies show a similar effect (Figs. 2 and 3), suggesting that positive modulators can potentiate the physiological activity of the σ_1 chaperone, which can be demonstrated in a behavioral assay.

The concentrations for σ_1 receptor binding and functional activity reported in this study are clinically achievable at therapeutic doses. After a single dose of fenfluramine, plasma C_{max} values range from 62 to 76 ng/mL (0.27–0.33 μM) for fenfluramine and 10 to 16 ng/mL (0.049–0.079 μM) for norfenfluramine [38,39]. Similarly, based on the results described in this report (Table 1), fenfluramine binding affinity (K_i) was in the nanomolar range for σ_1 and 5-HT_{1A} receptors, with functional assays suggesting weak stimulation of σ_1 receptor activity by fenfluramine and weak-to-substantial inhibition by norfenfluramine (Supplemental Table 3; Fig. 1A, B). Previous studies support 5-HT and σ_1 receptor binding, activation, and interaction. Using a mutant *scn1Lab*$^{-/-}$ zebrafish model of Dravet syndrome, Sourbron et al. demonstrated that fenfluramine antiepileptic activity is mediated by combined antagonism at σ_1 and agonism at 5-HT_{1D} and 5-HT_{2C} receptors and possibly, 5-HT_{2A} [13,14]. In previous in vitro reports, fenfluramine behaved as an agonist at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors but lacked functional activity at 5-HT_{1A} [2,3]. Although some studies provide evidence for fenfluramine activity at 5-HT_{1A} receptors [12], our results support reports in zebrafish showing minimal activity of fenfluramine on 5-HT_{1A} receptors [11,14]. Although both fenfluramine and lorcaserin have agonist activity at 5-HT_{2C} [3,11,14], only fenfluramine potentiated BiP/σ_1 dissociation (Fig. 1C, D). These results suggest that 5-HT receptor activity alone does not necessarily result in 5-HT receptor coordination with σ_1 receptors, and therefore, fenfluramine antiseizure pharmacology may differ from other serotonergic compounds.

Although our study does not measure antiseizure activity directly, our results from a validated mouse model of σ_1 receptor activity (i.e., reversal of dizocilpine-induced learning impairment; Figs. 2 and 3) may point to σ_1 receptor positive modulation as a potential additional mechanism of action for fenfluramine. Recent studies in mice and zebrafish provide a link between our pharmacological data supporting fenfluramine as a positive modulator of the σ_1 receptor and fenfluramine's antiseizure efficacy in models of Dravet syndrome [12,14]. The first evidence for σ_1 allosteric modulation as an antiseizure mechanism was presented by Guo and colleagues [40]. Bermack et al. [41] provided evidence for σ_1 and 5-HT receptor interaction, demonstrating that σ_1 agonists could potentiate 5-HT-dependent neuronal firing at rates much more rapid than 5-HT ligands alone.

The σ_1 receptor is a target of other AEDs [12,42,43], is expressed throughout the brain [44], and has neuromodulatory effects on major neurotransmitter systems, including serotonergic and glutamatergic synapses [12,42]. Both dexfenfluramine and cannabidiol have been reported to act as σ_1 receptor antagonists in a mouse model of NMDA-mediated seizures [12,42], though in the dizocilpine-induced amnesia model reported in our study and the zebrafish model of Dravet syndrome [14], σ_1 positive modulation appears to provide the improvement. Conversely, the anticonvulsant E1R, a positive allosteric σ_1 receptor modulator, has been shown to be effective in reducing seizures in several other rodent seizure models [43]. Taken together, these data and our observation that fenfluramine has σ_1 receptor activity at

doses similar to those used in clinical trials support a pharmacological role for σ_1 in fenfluramine's antiseizure efficacy.

The efficacy of fenfluramine in reversing dizocilpine-induced memory deficits provides support for the hypothesis that fenfluramine interacts with the σ_1 receptor. The dizocilpine assays used in this study are validated in vivo models of σ_1 activity in contextual [30,31] and spatial [27,29,31] learning and memory. Some AEDs (e.g., valproate and topiramate) cause learning and memory deficits [45]. However, in phase 3 clinical studies, patients treated with fenfluramine actually showed both short- and longer-term improvement in executive function outcomes as measured by Behavior Rating Inventory of Executive Function (BRIEF®) scores after 14 weeks and 1 year of treatment, respectively [4,8]. Taken together with these data, our results suggest that fenfluramine may protect against chemically induced neurocognitive damage by positively modulating σ_1 receptors (Figs. 2 and 3). Coupled with the phase 3 clinical data, our mouse models support that fenfluramine is unlikely to cause the cognitive deficits associated with some AEDs. Further, our data may suggest a mechanism whereby long-term (≥1 year) treatment with fenfluramine, with meaningful reductions in convulsive seizures, also resulted in sustained improvements in updated BRIEF®2 metrics of executive function [8]. Recent data from mouse models corroborate these clinical findings by demonstrating a centrally acting mechanistic role for fenfluramine in improving aspects of cognitive function in rodent models [9]. Although it is unclear how improvements in dizocilpine-induced amnesia may inform these clinical observations, σ_1 receptors have been associated with neuroprotection and improvements in executive function for conditions other than epilepsy [9,46]. It remains unknown, however, whether these observed improvements in executive function and cognition are solely due to reductions in seizures and/or if there is also a component due to an inherent activity of fenfluramine, such as due to this σ_1 activity.

If fenfluramine acts as a positive σ_1 modulator to reduce seizures and/or improve cognitive outcomes, the question arises of what endogenous compound(s) it may modulate. One possibility is serotonin; another may be neuroactive steroids. Stimulation of chronic release of endogenous neuroactive steroids can facilitate positive modulation at GABAergic and glutamatergic synapses to reduce seizure frequency long-term [47,48], and a synthetic neuroactive steroid has been shown to reduce seizure frequency in Dravet mouse models [48]. In addition, endogenous neuroactive steroids may have σ_1 receptor agonist or antagonist properties of their own, suggesting there may be a network involving serotonin, σ_1 activity, and the GABAergic and glutamatergic systems that regulates neuronal excitation and inhibition [37,47]. A clear clinical correlation between σ_1 receptor activity, endogenous neuroactive steroids, and improvements in seizure reduction and cognition remains to be established.

5. Conclusions

In conclusion, our research suggests that fenfluramine's mechanism of action likely extends beyond previously characterized serotonergic modulation and may include a dual mechanism of action that involves σ_1 receptor modulation. The effects were concentration-dependent, with synergy between fenfluramine and the σ_1 agonist PRE-084 found at low doses where each alone had no effect. This novel mechanism of action may be responsible for the profound and long-lasting efficacy as well as executive function improvement demonstrated in phase 3 clinical trials of fenfluramine in the treatment of seizures in children and young adults with Dravet syndrome.

Declaration of competing interest

Dr. Martin, Dr. Gammaitoni, Dr. Farfel, and Dr. Galer are employees of, and/or own stock in, Zogenix, Inc. Dr. de and Dr. Maurice Witte received consultancy fees from Zogenix.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yebeh.2020.106989.

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