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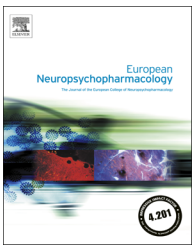

# Vortioxetine dose-dependently reverses 5-HT depletion-induced deficits in spatial working and object recognition memory: A potential role for 5-$HT_{1A}$ receptor agonism and 5-$HT_3$ receptor antagonism

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**Abstract**
We previously reported that the investigational multimodal antidepressant, vortioxetine, reversed 5-HT depletion-induced memory deficits while escitalopram and duloxetine did not. The present report studied the effects of vortioxetine and the potential impact of its 5-$HT_{1A}$ receptor agonist and 5-$HT_3$ receptor antagonist properties on 5-HT depletion-induced memory deficits. Recognition and spatial working memory were assessed in the object recognition (OR) and Y-maze spontaneous alternation (SA) tests, respectively. 5-HT depletion was induced in female Long-Evans rats using 4-cholro-DL-phenylalanine methyl ester HCl (PCPA) and receptor occupancies were determined by *ex vivo* autoradiography. Rats were acutely dosed with vortioxetine, ondansetron (5-$HT_3$ receptor antagonist) or flesinoxan (5-$HT_{1A}$ receptor agonist). The effects of chronic vortioxetine administration on 5-HT depletion-induced memory deficits were also assessed. 5-HT depletion reliably impaired memory performance in both the tests. Vortioxetine reversed PCPA-induced memory deficits dose-dependently with a minimal effective dose (MED) ≤0.1 mg/kg (~80% 5-$HT_3$ receptor occupancy; OR) and ≤3.0 mg/kg (5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_3$ receptor occupancy: ~15%, 60%, 95%) in SA. Ondansetron exhibited a MED ≤3.0 μg/kg (~25% 5-$HT_3$ receptor occupancy; OR), but was inactive in the SA test. Flesinoxan had a MED ≤1.0 mg/kg (~25% 5-$HT_{1A}$ receptor occupancy; SA); only 1.0 mg/kg ameliorated deficits in the NOR. Chronic p.o. vortioxetine administration significantly improved memory performance in OR and occupied 95%, 66%, and 9.5% of 5-$HT_3$, 5-$HT_{1B}$, and 5-$HT_{1A}$ receptors,

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respectively. Vortioxetine's effects on SA performance may involve 5-HT$_{1A}$ receptor agonism, but not 5-HT$_3$ receptor antagonism, whereas the effects on OR performance may involve 5-HT$_3$ receptor antagonism and 5-HT$_{1A}$ receptor agonism.


## 1. Introduction

Major Depressive Disorder (MDD) is a highly disabling disease with a lifetime prevalence of 17% in the United States (Kessler et al., 2005). Current predictions suggest that MDD will become the second leading cause of the global disability burden by 2020 (Michaud et al., 2001). MDD-associated disability is associated with cognitive dysfunction (Jaeger et al., 2006) across a range of domains, including speed of processing, executive function, attention, and memory function (Austin et al., 2001; Lee et al., 2011). Severe cognitive impairment may predict a poor recovery of everyday functioning, and a poor response to treatment with selective serotonin (5-HT) reuptake inhibitors (SSRIs; Jaeger et al., 2006; Dunkin et al., 2000; Withall et al., 2009). Importantly, these cognitive dysfunctions persist after recovery from mood dysfunction in many patients (Herrera-Guzman et al., 2010; Kuny and Stassen, 1995; Nebes et al., 2003), suggesting that MDD-related cognitive dysfunction is caused by a neurobiological substrate that is independent of mood and may be an important target for pharmacological remediation.

The central serotonergic system is believed to play a significant role in the pathophysiology of MDD, although the extent of its role remains enigmatic (Charney, 1998; Heninger et al., 1996; aan het Rot et al., 2009). Post-mortem analyses of brains from MDD patients indicate decreased size of the dorsal raphe nucleus, reduced brain 5-HT transporter (SERT) availability, and elevated 5-HT turnover (Barton et al., 2008). Furthermore, acute tryptophan depletion causes mood lowering in predisposed individuals and consistently impairs memory function (Benkelfat et al., 1994; Ellenbogen et al., 1996; Klaassen et al., 1999). Thus, 5-HT depletion may be a useful method to model some aspects of MDD-associated cognitive dysfunction.

Central 5-HT depletion using the irreversible tryptophan hydroxylase inhibitor, 4-chloro-DL-phenylalanine methyl ester HCl (PCPA), reliably induces cognitive deficits in rodents (Hritcu et al., 2007; Matsukawa et al., 1997; Prinssen et al., 2002). We have previously observed that a 4-day PCPA regimen impaired memory in tests of recognition and spatial working memory in rats (Pehrson et al., 2012). Treatment with a selective serotonin reuptake inhibitor (SSRI) or serotonin-norepinephrine reuptake inhibitor (SNRI) had no effect on these cognitive deficits at doses corresponding to full occupancy of the serotonin transporter (SERT). However, the investigational multimodal antidepressant vortioxetine restored 5-HT depletion-induced memory deficits in rats. Vortioxetine functions via two modes of action in human recombinant cell lines: (1) SERT inhibition ($K_i$ 1.6 nM), and (2) direct modulation of serotonergic receptors, where it acts as an antagonist at 5-HT$_3$ ($K_i$ 3.7 nM), 5-HT$_7$ ($K_i$ 19 nM), and 5-HT$_{1D}$ ($K_i$ 2.9 nM) receptors, a partial agonist at 5-HT$_{1B}$ receptors ($K_i$ 33 nM), and an agonist at 5-HT$_{1A}$ receptors ($K_i$ 15 nM) (Bang-Andersen et al., 2011; Westrich et al., 2012). Based on these data, it was hypothesized that vortioxetine's memory-restoring effect in 5-HT depleted rats derived from its direct pharmacological action on serotonergic receptors, rather than SERT inhibition.

Previous preclinical data demonstrated that 5-HT$_3$ receptor antagonists (Carey et al., 1992; Fontana et al., 1995; Boast et al., 1999; Arnsten et al., 1997; Petkov et al., 1995; Staubli and Xu, 1995) and 5-HT$_{1A}$ receptor agonists (Bertrand et al., 2001; Horiguchi and Meltzer, 2012; Depoortere et al., 2010) are able to improve memory in some preclinical cognitive dysfunction models. Thus, the goal of the present study was to investigate the role of these receptors in vortioxetine's ability to remediate 5-HT depletion-induced memory deficits. Although 5-HT$_3$ receptor antagonism improves memory function under some conditions, we hypothesized that it would be ineffective in reversing 5-HT-depletion induced memory dysfunction. However we hypothesized that 5-HT$_{1A}$ receptor activation would contribute to improvements in these deficits in rats.

## 2. Experimental procedures

### 2.1. Animals

Female adult Long-Evans rats (223–312 g; Charles River Laboratories, Wilmington, MA, USA) were used. Rats were group-housed, three per cage (Rat IVC Green Line Sealsafe plus cages; Tecniplast USA, Philadelphia, PA), in a temperature (20.6–21.6 °C) and humidity (30–70%) controlled environment on a 12 h light/dark cycle (lights on at 6 a.m.). All animals had *ad libitum* access to standard rat chow and water in their home cages. Rats were housed for approximately 1 month before initiation of experimental procedures. Except for the first week, the rats were handled once every second day. All procedures were approved by the Lundbeck Research USA Institutional Animal Care and Use Committee prior to the start of experiments and were in line with the NIH *Guide to the Care and Use of Laboratory Animals*.

### 2.2. Drugs

Vortioxetine HBr (H Lundbeck A/S, Valby, Denmark) was dissolved in 20% hydroxypropyl-β-cyclodextrin (Roquette America, Keouk, IA, USA). Flesinoxan HCl (H. Lundbeck A/S, Valby, Denmark), ondansetron HCl and 4-chloro-DL-phenylalanine methy ester HCl (PCPA; Sigma-Aldrich, St. Louis, MO, USA) were dissolved in saline. All doses are expressed in mg or μg of base/kg body weight and administered as a *subcutaneous* (*s.c.*) injection at 1 mL/kg, except PCPA where 4 mL/kg was used. The doses used for each drug are described below in the experimental design section. For chronic p.o. vortioxetine administration experiments, vortioxetine HBr was incorporated into Purina 5001 rodent chow at a concentration of 0.6 g/kg of food weight (Research Diets, Inc., New Brunswick, NJ, USA). All ligands used to define nonspecific binding in *ex vivo* autoradiography experiments, i.e., 5-HT, ondansetron (both Sigma-Aldrich, St. Louis, MO, USA) and SB216641 (Tocris Bioscience,

Minneapolis, MN, USA) were dissolved in dimethylsulfoxide (Sigma-Aldrich).

*Radioligands*: [$^3$H]LY278584 (83 Ci/mmol; 0.2 mCi/mL) was synthesized at Amersham (Piscataway, NJ, USA). [$^3$H] 8-OH-DPAT (154.2 Ci/mmol; 1 mCi/mL) and [$^3$H]GR125743 (79.6 Ci/mmol; 0.2 mCi/mL) were purchased from Perkin Elmer (Waltham, MA, USA).

## 2.3. Behavioral assessments

Rats were pretreated with PCPA, 86 mg/kg/day (*s.c.*) for 4 consecutive days, with the final day referred to as day zero. The magnitude of central 5-HT depletion induced by this PCPA treatment regimen was estimated to be 97% when measured in hippocampal tissue homogenate (Pehrson et al., 2012). This treatment regimen was chosen in order to (1) induce deficits in cognitive function and (2) eliminate the role of the 5-HT transporter in vortioxetine's mechanism of action. Rats were randomly assigned to vehicle or drug treatment groups and a control group was included in each experiment ($n$=6–13 per group). Each experiment included assessment in the object recognition (OR) and spontaneous alternation (SA) tests, and rats were only assessed once in each behavioral test. The rats were injected with drug or vehicle 1 h prior to behavioral tests, i.e., prior to the information session in the OR task and testing in the SA task. Thirty minutes prior to each behavioral assessment, the animals were transported to the experimental room for acclimation.

## 2.4. Object recognition

On the afternoon of day 0 (i.e. the final day of PCPA injections), each rat was given a habituation trial, where it was individually placed in the open field for 5 min of free exploration. On day 1 (i.e. 24 h after the final PCPA injection), rats received acute injections according to their experimental condition 1 h before the training session. In the training session, each rat was re-habituated to the open field for 1 min. The rat was then placed in a holding cage while two identical objects were positioned in the open field. Subsequently, the rat was positioned facing the wall and allowed to freely explore for 15 min. Upon completion, the rat was returned to its home cage and remained in the testing room during the retention period.

The testing session occurred 45 min after completing information session. Two objects were again placed in the arena, one identical to the objects used during training and one novel. Object pairs were validated prior to use to ensure no bias existed towards one of the objects in the pair. The position of the novel object was pseudorandomly assigned (left or right) and counter-balanced for each treatment group. The rat was positioned facing the wall and was allowed to freely explore for 3 min.

A camera recorded all behavioral sessions, which were analyzed for track lengths using Viewer III software (Bioserve GmbH, Fort Lee, NJ, USA). Exploration time for each object was recorded with a stopwatch from the videos. Exploration was defined as directing the nose towards an object at a close distance or touching the object. Sitting on the object was not considered as an exploratory behavior. An animal was excluded from the study if it failed to accumulate a total exploration time $>$6 s, failed to explore both objects, knocked an object over, or jumped out of the arena.

## 2.5. Y-maze spontaneous alternations

SA testing occurred 2 days after the last PCPA dose. One hour following acute drug treatment, each rat was placed in the center of the Y-maze facing the division point of two arms and allowed to freely explore the maze for 6 min. Following each rat, the maze was thoroughly cleaned to prevent olfactory cues. The sequence of arm entries was recorded manually from the videos. An entry was defined as a rat crossing a virtual line between the central ends of two parallel walls with its entire body excluding the tail. An exit was defined as any part of the animal crossing the same virtual line. An alternation was defined as any three-entry-sequence of one visit to each arm. The performance of an animal was excluded if the total arm entries were $<$7 or the rat jumped out of the arena.

## 2.6. Ex vivo autoradiography: 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_3$ receptor occupancies

### 2.6.1. Tissue preparation

*Ex vivo* autoradiography experiments were always conducted in behaviorally naïve animals. One hour after acute drug treatment, PCPA treated rats were anesthetized with $CO_2$ and euthanized by decapitation. Brains were dissected from the skull, frozen on powdered dry ice, and stored at −20 °C until use. For each region of interest, 20 μm thick coronal tissue slices were cut at −20 °C using a microtome-cryostat (Microm, Walldorf, Germany). A minimum of three replicate slices per brain were included for each experiment. Slice collection began at approximately 1.2–1.5 mm anterior to Bregma for 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor binding studies and at −4.8 to −5.36 mm posterior to Bregma for 5-HT$_3$ receptor binding studies (see Section 2.8. for exact brain regions). The sections were mounted on slides and stored in a slide-box with desiccant pellets at −20 °C.

### 2.6.2. General assay procedures

Procedures for each of the *ex vivo* autoradiography assays used in this study followed a similar pattern. Briefly, slide-boxes were defrosted at room temperature (RT) under a constant air stream before opening. Following a brief preincubation (in the 5-HT$_{1B}$ and 5-HT$_3$ receptor assays only), slides were incubated in assay buffer containing the tritiated radioligand corresponding to the receptor of interest. Nonspecific binding was determined by adding a high concentration of a non-radioactive competitor for the target of interest. Following incubation with the radioligand, the slides were rinsed twice in assay buffer (4 °C) and briefly dipped in distilled water (4 °C). Subsequently, the slides were air-dried for 30 min, transferred to a vacuum desiccator and dried for an additional 60 min. Finally, slides were processed in a Beta Imager (Biospace Lab, Paris, France) for 16–24 h depending on the radioligand used. Detailed descriptions of the 5-HT$_{1B}$ and 5-HT$_3$ receptor assay methods were reported previously (Pehrson et al., 2013). Specific details for each of the receptors occupancy assays are listed in Table 1 Radioligand concentrations were chosen based on saturation binding experiments conducted in this laboratory. Regions of interest for each assay are detailed below under Section 2.8.

## 2.7. Experimental design

### 2.7.1. Assessing vortioxetine's effects on 5-HT depletion-induced object recognition (OR) and SA deficits

On day 1 following cessation of PCPA treatment, PCPA-treated rats randomly received acute injections of vehicle or vortioxetine at 0.0001, 0.1, 3, or 10 mg/kg 1 h prior to the start of the information session in OR. On day 2 following PCPA cessation, animals received the same treatment 1 h prior to the SA test. These doses were chosen based on known acute dose–receptor occupancy relationships (Pehrson et al., 2013), and were designed to gradually add 5-HT$_3$ receptor antagonism, 5-HT$_{1B}$ receptor partial agonism, and 5-HT$_{1A}$ receptor agonism to vortioxetine's pharmacological actions. Additionally, this range of vortioxetine doses encompasses those where it has been found active in preclinical models of anxiety or depression (2–8 mg/kg; Mørk et al., 2012).

**Table 1** Methods for *ex-vivo* autoradiography assays for the 5-$HT_{1A}$, 5-$HT_{1B}$, and 5-$HT_3$receptors. N/A—not applicable.

| Target receptors | 5-$HT_{1A}$ receptors | 5-$HT_{1B}$ receptors | 5-$HT_3$ receptors |
|---|---|---|---|
| **Preincubation buffer** | N/A | 170 mM Tris-HCl 4 mM $CaCl_2$ 0.01% L-ascorbic acid | 50 mM Tris-HCl 150 mM NaCl |
| **Preincubation time** | N/A | 1 × 3 min | 1 × 5 min |
| **Assay buffer** | 170 mM Tris-HCl 4 mM $CaCl_2$ 0.01% L-ascorbic acid 10 μM pargyline | 170 mM Tris-HCl 4 mM $CaCl_2$ 0.1% L-ascorbic acid 10 μM pargyline | 50 mM Tris-HCl 150 mM NaCl 4 mM $CaCl_2$ |
| **Radioligand** | [$^3$H]8-OH-DPAT (3 nM) | [$^3$H]GR125743 (1 nM) | [$^3$H]LY278584 (2 nM) |
| **Nonspecific binding agent** | 5-HT (10 μM) | SB216641 (10 μM) | Ondansetron (10 μM) |
| **Incubation time** | 1 h | 1 h | 1 h |
| **Rinse in cold assay buffer** | 2 × 5 min | 2 × 5 min | 2 × 10 min |
| **Cold water dip** | Yes | Yes | Yes |
| **Acquisition time** | 16 h | 16 h | 24 h |

#### 2.7.2. Assessing the effects of 5-$HT_3$ receptor antagonism and 5-$HT_{1A}$ receptor agonism on 5-HT depletion-induced OR and SA deficits

The goal of this experiment was to assess whether a selective 5-$HT_3$ receptor antagonist or 5-$HT_{1A}$ receptor agonist affects OR or SA performance in 5-HT depleted animals. On day 1 after PCPA cessation, rats in the experiment addressing effects of 5-$HT_3$ receptor antagonism received acute injections of vehicle, 0.1 mg/kg vortioxetine, or the selective 5-$HT_3$ receptor antagonist ondansetron at 0.001, 3, or 300 μg/kg. The vortioxetine dose chosen here was used because it selectively binds to 5-$HT_3$ receptors (see Table 2, below), and is thus the best comparison for ondansetron. Ondansetron was used because it is the most selective 5-$HT_3$ receptor antagonist available (Macor et al., 2001).

Rats in the experiments addressing effects of 5-$HT_{1A}$ receptor agonism received acute injections of vehicle, 10 mg/kg vortioxetine, or the selective 5-$HT_{1A}$ receptor agonist flesinoxan at doses of 0.001, 1, or 2.5 mg/kg. The 10 mg/kg vortioxetine dose was used in this case because it occupied 5-$HT_{1A}$ receptors, whereas lower doses did not. Thus 10 mg/kg vortioxetine was the most appropriate comparison for flesinoxan. Flesinoxan was used because it is a highly selective 5-$HT_{1A}$ receptor agonist (Boess and Martin, 1994). Additionally, similarly to vortioxetine, flesinoxan is a full agonist at this receptor (Schoeffter and Hoyer, 1988).

All injections were administered 1 h prior to the start of the information session in OR. On day 2 following the last dose of PCPA, animals received the same treatment 1 h prior to the SA test.

#### 2.7.3. Assessing the effects of chronic vortioxetine administration on 5-HT depletion-induced OR and SA deficits

The goal of this experiment was to determine whether vortioxetine had sustained effects on OR and SA performance in 5-HT depleted rats after chronic p.o. administration. Rats were given *ad libitum* access to vortioxetine-infused food (0.6 g of vortioxetine per kg of food weight) or a control diet (Purina 5001 rodent chow) with exactly the same nutritional makeup for 23 days. The rats consumed 7–10 g of food per 100 g of body weight per day, giving an approximate dose of 42–60 mg/kg/day p.o. On days 18 through 21 day of vortioxetine food administration, rats received either vehicle or PCPA injections as previously described, and were subsequently assessed in the OR and SA tasks on days 22 and 23 of vortioxetine food administration.

Vortioxetine-infused food was used in this experiment to achieve chronic administration for two reasons: (1) to avoid stressing animals by giving multiple daily vortioxetine injections, and (2) to avoid using chronically in-dwelling osmotic minipumps, which have previously resulted in poor vortioxetine exposure for dosing periods greater than 3 days.

#### 2.7.4. *Ex vivo* autoradiography: assessing receptor occupancies at 5-$HT_{1A}$, 5-$HT_{1B}$, and 5-$HT_3$ receptors

These experiments had two goals: (1) to determine the relationship between vortioxetine dose and occupancies at 5-$HT_3$, 5-$HT_{1B}$ and 5-$HT_{1A}$ receptors in 5-HT depleted animals; (2) to relate 5-$HT_3$ or 5-$HT_{1A}$ receptor occupancies for ondansetron or flesinoxan to those of vortioxetine in the acute behavioral experiments.

For all *ex vivo* autoradiography experiments, rats received PCPA as described above. On the day following PCPA cessation, PCPA-treated animals were randomly assigned to receive acute injections of vehicle, vortioxetine (0.1, 3, or 10 mg/kg), ondansetron (0.001, 1, 3, 300, or 1000 μg/kg), or flesinoxan (0.1, 1, 2.5, 10, or 25 mg/kg) as described above. The sample size for each drug condition was set at $n=3$ rats, with each rat having 3 replicate determinations per experiment.

In addition, a separate set of experiments were conducted in rats given chronic *ad libitum* access as described above to vortioxetine-infused food (0.6 g/kg of food weight, Research Diets) or control diet for 22 days. Given that rats tend to consume the majority of their food at night, tissue collection experiments occurred in a separate set of animals in the morning and afternoon, to assess whether occupancy levels remained stable over a day.

### 2.8. Data analysis and statistical methods

Results are expressed as mean ± standard error of the mean (SEM). All statistical analyses consisted of one-way ANOVA with Newman-Keuls *post hoc* tests where appropriate. Statistical significance was defined at $p<0.05$. GraphPad Prism version 4.02 (GraphPad software, San Diego, CA, USA) or MATLAB (the Mathworks, Inc, Natick, MA, USA) was used for statistical analysis and graph design. Outliers were eliminated if Pierce's criteria (Ross, 2003) were met.

### 2.9. Behavioral assessments

For the OR test, the preference score (%) was defined as the time spent exploring the novel object (in seconds) divided by the total exploration time. For the SA test, the percentage of spontaneous

alternations was defined as the number of spontaneous alternations divided by the total number of entries minus 2.

Rotational bias was also calculated for each animal included in the SA experiments as described in McFarland (1989). Briefly, this measure was calculated by dividing the total number of turns by 2. This value was subtracted from the total number of left turns made. The absolute value was taken, divided by the total number of arm entries made, multiplied by 100, and added to 50. This yielded the percent bias towards the preferred direction. This measure was included to determine whether changes in the % alternation dependent measure were better explained by effects on motor function than spatial working memory.

### 2.10. *Ex vivo* autoradiography

The Beta Imager outputs were quantified using β-Vision software (Biospace Lab, Paris, France). Surface radioactivity (expressed as counts per min/mm$^2$, or cpm/mm$^2$) was measured from a region of interest (ROI) defined *a priori* based on previous receptor mapping experiments. The ROIs for 5-HT$_{1B}$ (caudate and putamen) and 5-HT$_3$ (amygdala, and piriform cortex) receptors have been described in detail elsewhere (Pehrson et al., 2013). Figure 1 shows the ROI used for the 5-HT$_{1A}$ receptor binding assay, which included the lateral and medial septum.

Total binding was determined by averaging surface radioactivity from the ROIs of three replicate slices per brain. Nonspecific binding

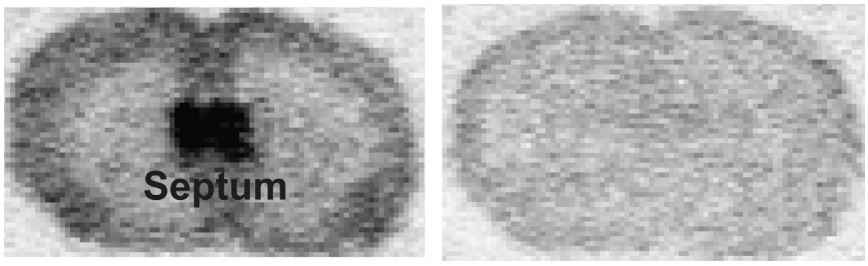


**Figure 1** Region of interest (ROI) in the *ex vivo* autoradiography 5-HT$_{1A}$ receptor assay. These brain slices represent total (left panel) and non-specific binding (right panel). The ROI included the lateral and medial septum, and exhibited a high degree of specific binding.

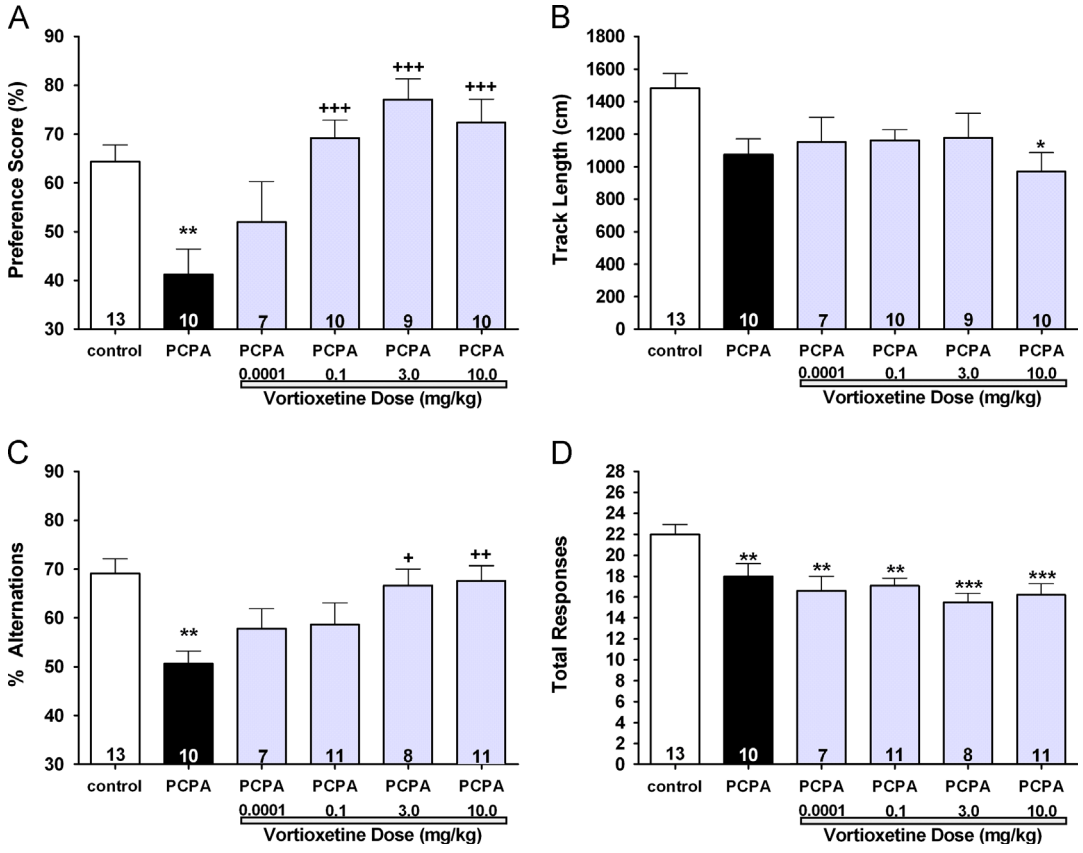


**Figure 2** Acute vortioxetine dose-dependently restores memory deficits induced by 5-HT depletion. In the NOR test, PCPA treatment impaired performance, but acute vortioxetine restored performance at doses higher than 0.1 mg/kg (Panel A). PCPA treatment did not significantly reduce locomotor activity, except after acute 10 mg/kg vortioxetine (Panel B). In the SA test, PCPA treatment impaired performance, and vortioxetine improved performance above 3 mg/kg (Panel C). PCPA treatment reduced the total number of responses in the SA test, but vortioxetine treatment did not affect this measure (Panel D). Bars represent mean ± SEM. Numbers contained within bars represent the sample size. Plus signs (+) and asterisks (*) represent statistically significant differences from PCPA and control, respectively ($+p<0.05$; $++p<0.01$; $+++p<0.001$).

was determined by averaging surface radioactivity from brain slices in from the nonspecific binding condition. Specific binding was determined by subtracting nonspecific binding from total binding. Subsequently, specific binding levels were expressed as a percentage of the average specific binding from vehicle-treated rats. These percentages were subtracted from 100 to obtain percent receptor occupancy.

## 3. Results

### 3.1. Behavioral assessments

#### 3.1.1. Acute vortioxetine dose-effect curve

As reported previously, repeated PCPA administration caused significant deficits in OR performance compared to vehicle-treated animals ($F(5,53)=7.935$, $p<0.0001$; Figure 2A). Acute vortioxetine treatment dose-dependently improved novel object preference scores at doses above 0.1 mg/kg. Although PCPA-treated rats had numerically lower track lengths compared to controls, these decreases were not significant, except where animals also received 10 mg/kg vortioxetine ($F(5,53)=2.95$, $p<0.05$; Figure 2B).

In the SA test, repeated PCPA administration significantly decreased % alternations compared to control rats ($F(5,54)=4.49$, $p<0.01$; Figure 2C). Acute vortioxetine administration significantly improved alternation behavior compared to the PCPA group at the 3 and 10 mg/kg doses. Interestingly, although lower vortioxetine doses did not significantly improve alternation behavior compared to PCPA, rats in these groups also did not perform significantly worse than controls. As reported previously, PCPA treatment was associated with reductions in the number of arm entries ($F(5,54)=5.89$, $p<0.001$; Figure 2D), which vortioxetine did not significantly affect. Finally, no treatments were associated with changes in response bias ($F(5,54)=0.9$, n.s.; data not shown).

#### 3.1.2. Acute ondansetron dose effect curve

PCPA treatment again significantly reduced novel object preference scores ($F(5,40)=12.18$, $p<0.0001$; Figure 3A). Acute ondanstetron significantly reversed this impairment at doses above 0.001 μg/kg. Additionally, the ability of acute 0.1 mg/kg vortioxetine to significantly improve novel object preference scores was replicated in this experiment. Once again, repeated PCPA treatment significantly reduced

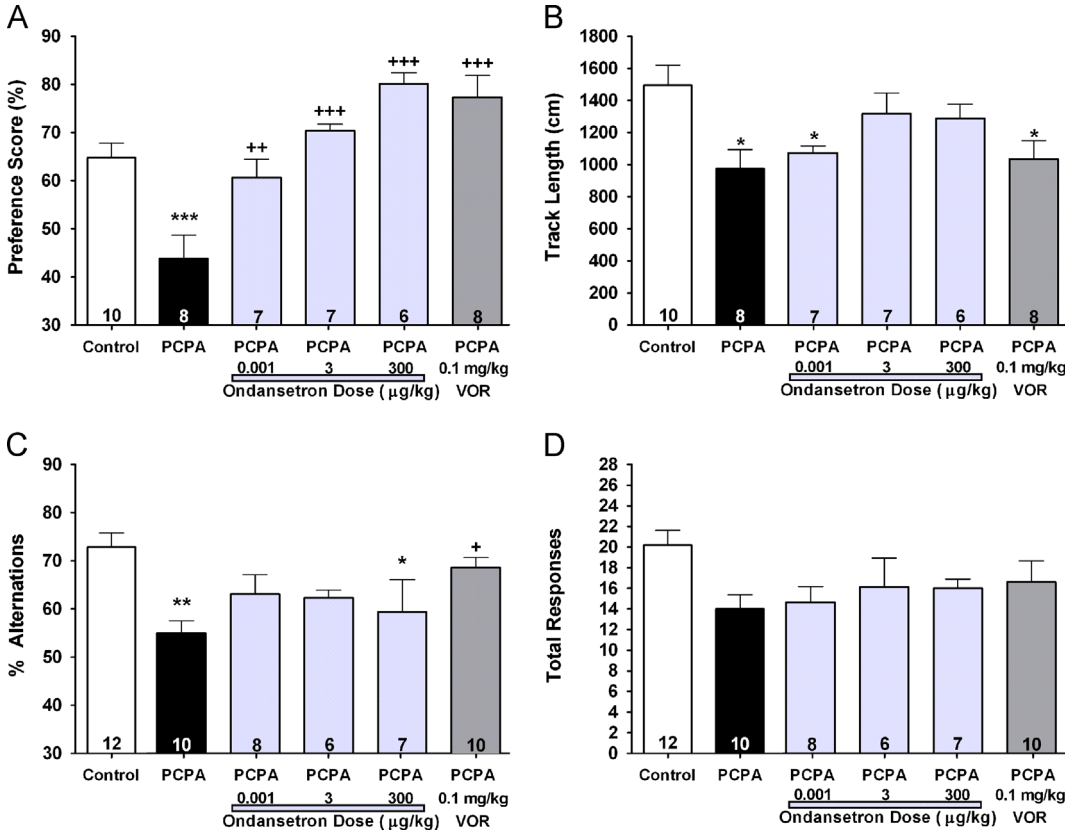


**Figure 3** Acute ondansetron restored memory deficits induced by 5-HT depletion in the NOR test, but not in the SA test. PCPA treatment significantly impaired NOR preference scores (Panel A) and reduced locomotor activity during the NOR test (Panel B). Acute treatment with ondansetron or 0.1 mg/kg vortioxetine significantly improved preference scores compared to PCPA alone. Neither ondansetron nor vortioxetine altered PCPA's effects on locomotor activity. In the SA test, PCPA treatment significantly impaired alternation scores (Panel C), but did not affect total responses. Ondansetron did not significantly improve alternation scores at any dose, however 0.1 mg/kg vortioxetine did improve alternation scores compared to PCPA alone. Neither vortioxetine nor ondansetron affected total responses in the SA task. Bars represent mean ± SEM. Numbers contained within bars represent the sample size. Plus signs (+) and asterisks (*) represent statistically significant differences from PCPA and control, respectively (+$p<0.05$; ++$p<0.01$; +++$p<0.001$).

locomotor activity ($F(5,40)=3.59$, $p<0.01$; Figure 3B). Significant reductions in track length were also observed in the 0.001 μg/kg ondansetron dose and the 0.1 mg/kg vortioxetine dose. However at the 3 and 300 μg/kg doses of ondansetron, no significant differences from controls were observed.

In the SA task, PCPA treatment significantly impaired alternation scores ($F(5,47)=4.25$, $p<0.01$; Figure 3C). Acute ondansetron treatment did not improve the % alternation dependent measure compared to the PCPA only group at any dose; however the 0.001 and 3 μg/kg doses were not significantly different from the control group either. Although it should be noted that the 300 μg/kg ondansetron group had significantly lower alternation scores compared to controls. Interestingly, in this cohort the 0.1 mg/kg vortioxetine dose significantly improved alternation scores compared to the PCPA only group, although it did not do so in the vortioxetine dose effect curve (see above). Also differently from the acute vortioxetine dose effect curve, PCPA treatment did not significantly decrease the number of arm entries, though there was a trend towards significance ($F(5,47)=2.04$, $p=0.09$; Figure 3D). Once again, none of the pharmacological treatments were associated with changes in the response bias measure ($F(5,47)=0.64$, n.s.; data not shown).

### 3.1.3. Acute flesinoxan dose effect curve

Repeated PCPA administration again significantly impaired novel object preference scores ($F(5,46)=8.94$, $p<0.0001$; Figure 4A). Acute administration of either 1 mg/kg flesinoxan or 10 mg/kg vortioxetine in PCPA-treated animals restored novel object preference scores back to control levels. However, 0.001 or 2.5 mg/kg flesinoxan did not significantly improve OR scores. As observed previously, 5-HT depletion significantly decreased locomotor behavior during the OR test session ($F(5,46)=3.36$, $p<0.05$; Figure 4B). Although track lengths in the acute flesinoxan (at 1 or 2.5 mg/kg) or 10 mg/kg vortioxetine groups were not significantly greater than those of the PCPA group, neither were they significantly lower than in control animals.

Once again, repeated PCPA treatment significantly reduced alternation scores in the SA task ($F(5,52)=5.2$, $p<0.001$; Figure 4C). These reductions were reversed by flesinoxan 1 or 2.5 mg/kg by vortioxetine, 10 mg/kg. The observation that PCPA treatment significantly reduced the

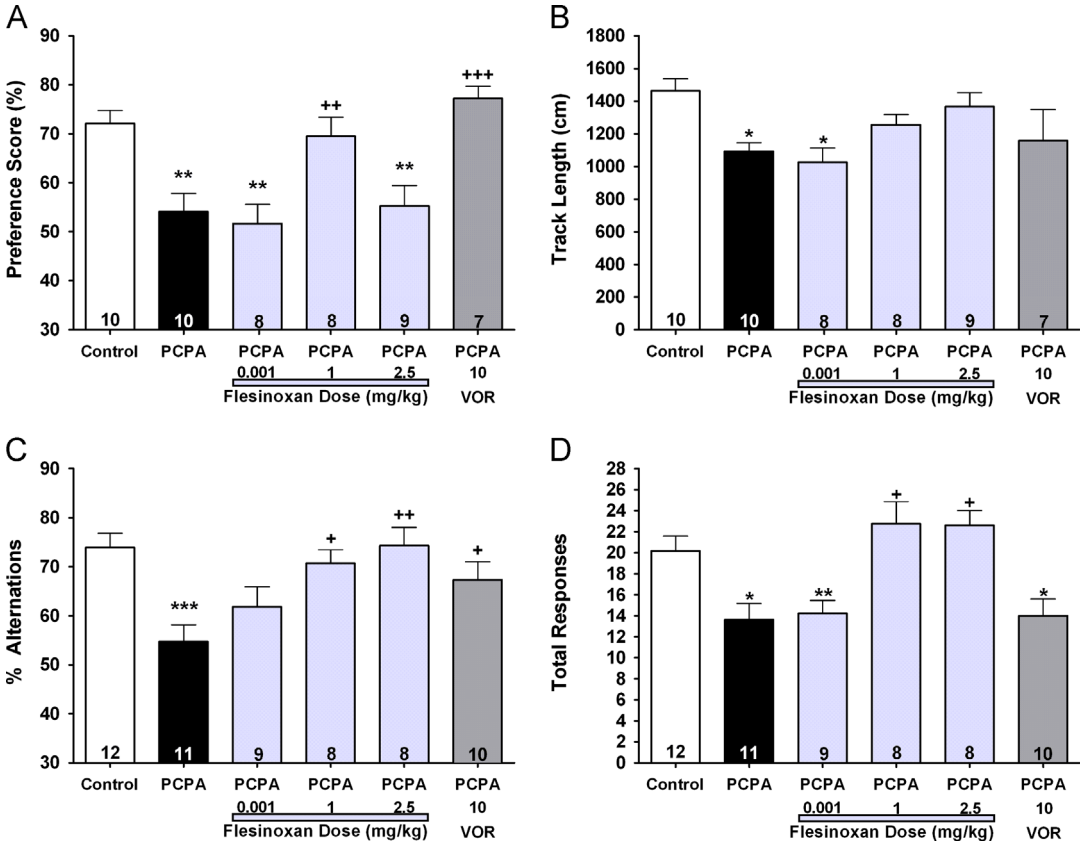


**Figure 4** Acute flesinoxan restores 5-HT depletion-induced memory deficits in the NOR and SA tests. 5-HT depletion using PCPA significantly impaired novel object preference scores (Panel A) and significantly reduced locomotor activity during the NOR task (Panel B). Acute treatment with 1 mg/kg flesinoxan or 10 mg/kg vortioxetine significantly improved preference scores compared to PCPA alone without altering locomotor activity. PCPA treatment also impaired alternation scores (Panel C) and reduced the total number of responses made (Panel D) in the SA test. 1 or 2.5 mg/kg flesinoxan significantly improved alternation scores compared to PCPA alone and reversed the reduction in total responses induced by PCPA treatment. Acute treatment with 10 mg/kg vortioxetine significantly improved alternation scores without affecting total responses. Bars represent mean ± SEM. Numbers contained within bars represent sample size. Plus signs (+) and asterisks (*) represent statistically significant differences from PCPA and control, respectively (+$p<0.05$; ++$p<0.01$).

frequency of arm entries was replicated ($F(5,52)=7.56$, $p<0.0001$; Figure 4D). Flesinoxan treatment led to significant increases in arm entries (1 and 2.5 mg/kg), while 10 mg/kg vortioxetine did not. Finally, no experimental manipulations altered response biases ($F(5,52)=1.99$, n.s.; data not shown).

#### 3.1.4. Chronic vortioxetine administration

PCPA treatment significantly impaired novel object preference scores ($F(2,20)=5.96$, $p<0.01$; Figure 5A), which were restored back to control levels by chronic vortioxetine. PCPA administration did not significantly decrease locomotor behavior in this case ($F(2,20)=1.18$, n.s.; Figure 5B).

In the SA test, although animals treated with PCPA only or PCPA and chronic vortioxetine displayed numerically lower alternation scores than controls, these differences only trended towards significance ($F(2,20)=2.99$, $p=0.073$). Similarly, no significant differences between treatment groups were observed in the total responses dependent measure ($F(2,20)=0.007$, n.s), or in the response bias measure ($F(2,20)=1.02$, n.s.; data not shown).

#### 3.1.5. Receptor occupancy

Target occupancies after acute dosing are presented in Table 2. Acute vortioxetine (0.1–10 mg/kg) treatment produced significant receptor occupancy (78–97%) at 5-HT$_3$ receptors at all doses tested ($F(3,8)=161.96$, $p<0.001$). 5-HT$_{1B}$ receptor occupancy was significantly greater than vehicle at 3 and 10 mg/kg vortioxetine ($F(3,7)=163.4$, $p<0.001$), while 5-HT$_{1A}$ receptor occupancy was significantly greater than vehicle only at 10 mg/kg vortioxetine ($F(3,8)=6.67$, $p<0.05$).

Ondansetron (0.001–1000 μg/kg) produced significant, dose-dependent increases in 5-HT$_3$ receptor occupancy ($F(5,12)=24.5$, $p<0.001$) at doses above 0.1 μg/kg, while flesinoxan (0.1–25 mg/kg) significantly occupied 5-HT$_{1A}$ receptors at all doses tested.

Chronic vortioxetine administration in food leads to similar profiles of receptor occupancy whether measured in the morning or afternoon. Fractional occupancies at the 5-HT$_{1A}$ receptor were 9.5±3.5% and 11±0.8% in the morning and afternoon, respectively. 5-HT$_{1B}$ receptor occupancies were 66±2.1% in the morning and 50±1.3% in the afternoon. Finally, occupancies at the 5-HT$_3$ receptor were 95±0.6% and 96±0.6% in the morning and afternoon, respectively.

## 4. Discussion

The present study demonstrated that vortioxetine reversed the 5-HT depletion-induced OR and SA deficits in a dose- and test-dependent manner. A low acute dose of 0.1 mg/kg consistently improved memory performance in 5-HT depleted rats in the OR test and less consistently in the SA task, while higher doses of 3 and 10 mg/kg consistently improved performance in both tasks. Chronic vortioxetine administration also improved 5-HT depletion-induced deficits in OR performance, but its effects on SA are unclear due to PCPA's lack of an effect on SA performance in that experiment. Moreover, vortioxetine's memory effects were independent of the locomotor activity-suppressing effects of 5-HT depletion, which were not changed by vortioxetine administration. Furthermore, the 5-HT$_3$ receptor antagonist ondansetron selectively improved OR performance without affecting the SA test. The 5-HT$_{1A}$ receptor agonist flesinoxan significantly improved OR performance at the 1 mg/kg dose, restored SA performance to normal levels at 1 and 2.5 mg/kg doses, and reversed some 5-HT depletion-induced motor deficits.

We have reported elsewhere (Pehrson et al., 2012) that the SSRI escitalopram and the SNRI duloxetine fail to restore 5-HT depletion-induced deficits in the OR and SA tests at doses corresponding to full SERT occupancy. These data suggest that SERT inhibition is insufficient to restore memory function to normal levels in 5-HT depleted animals. Thus, vortioxetine's effects on 5-HT depletion-induced memory deficits are most likely mediated via direct pharmacological action on serotonergic receptors.

The vortioxetine dose range tested here was chosen to gradually engage the different serotonergic targets in its pharmacological profile, which it did in agreement with the order of *in vitro* affinities at these receptors in the rat (Bang-Andersen et al., 2011). Our *ex vivo* autoradiography data show that 0.1 mg/kg vortioxetine fully occupied 5-HT$_3$

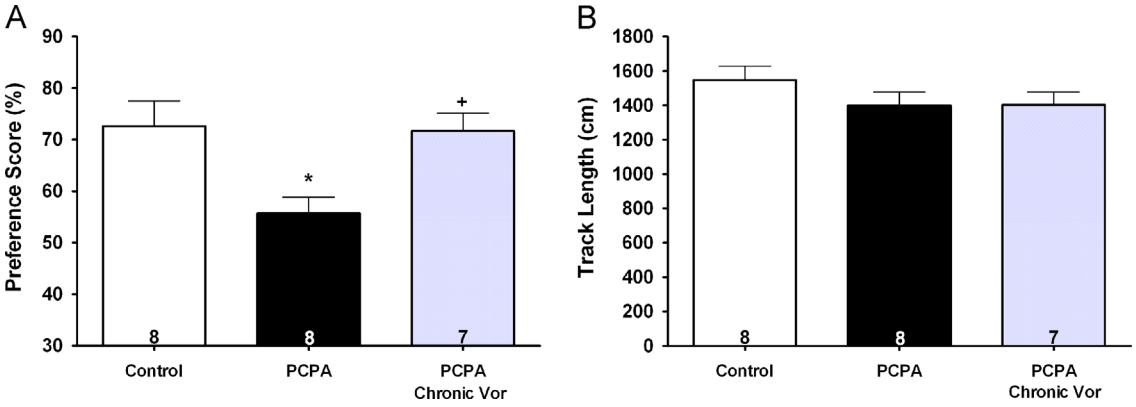


**Figure 5** Chronic vortioxetine administration blocks 5-HT depletion-induced deficits in NOR performance. 5-HT depletion using PCPA induced significant deficits in preference scores in the NOR test (Panel A), which were blocked by chronic vortioxetine administration. Neither PCPA nor chronic vortioxetine affected locomotor activity in the NOR task (Panel B). Bars represent mean±SEM. Numbers contained within bars represent sample size. Plus signs (+) and asterisks (*) represent statistically significant differences from PCPA and control, respectively ($+p<0.05$).

**Table 2** Target occupancies following acute vortioxetine, ondansetron, and flesinoxan administration in PCPA-treated female rats. All drugs exhibited a dose-dependent occupancy curve. Values are mean ± SEM. N/A—not applicable.

| Compound and dose | | 5-$HT_{1A}$ receptor occupancy (%) | 5-$HT_{1B}$ receptor occupancy (%) | 5-$HT_3$ receptor occupancy (%) |
|---|---|---|---|---|
| **Vortioxetine** (mg/kg) | 0.1 | 6 ± 13 | −2.2 ± 4.4 | 78 ± 3.8 |
| | 3 | 15 ± 5.8 | 62 ± 4.4 | 94 ± 3.8 |
| | 10 | 43 ± 0.6 | 89 ± 1.9 | 97 ± 1.7 |
| **Ondansetron** (μg/kg) | 0.001 | N/A | N/A | 9.8 ± 3.5 |
| | 1 | N/A | N/A | 24 ± 4.7 |
| | 3 | N/A | N/A | 25 ± 5.5 |
| | 300 | N/A | N/A | 39 ± 4.3 |
| | 1000 | N/A | N/A | 58 ± 4.3 |
| **Flesinoxan** (mg/kg) | 0.1 | 28 ± 4.9 | N/A | N/A |
| | 1.0 | 27 ± 3.9 | N/A | N/A |
| | 2.5 | 46 ± 5.3 | N/A | N/A |
| | 10 | 82 ± 4.4 | N/A | N/A |
| | 25 | 93 ± 2.2 | N/A | N/A |

receptors in PCPA-treated rats without significantly occupying 5-$HT_{1A}$ or 5-$HT_{1B}$ receptors, and selectively reversed the 5-HT depletion-induced deficit in OR performance without affecting SA performance. Additionally, 3 and 10 mg/kg vortioxetine, which occupied 5-$HT_{1B}$ and 5-$HT_{1A}$ receptors in addition to 5-$HT_3$ receptors, was able to reverse 5-HT depletion-induced deficits in both OR and SA performance.

### 4.1. 5-$HT_3$ receptor antagonism reverses 5-HT depletion-induced memory deficits

Considered in context with the receptor occupancy data, the selective improvement in OR performance elicited by vortioxetine 0.1 mg/kg suggests, rather surprisingly, that 5-$HT_3$ receptor antagonism is sufficient to restore normal object recognition memory in 5-HT-depleted rats. We considered it unlikely that 5-$HT_3$ antagonism would have any effect in a 5-HT depletion model such as this. However, the idea that 5-$HT_3$ antagonism has a beneficial effect under the conditions used in this study is supported by the potent, dose-dependent effect of the selective 5-$HT_3$ receptor antagonist, ondansetron, which also reversed 5-HT depletion-induced deficits in OR performance.

Based on the high level of sequence homology between 5-$HT_3$ receptors and α7 nicotinic receptors, an alternative explanation for these results may be that the effects of ondansetron or vortioxetine are mediated via effects on cholinergic, rather than serotonergic neurotransmission. However, we regard this alternative mechanism to be highly unlikely, given the extremely low binding affinity ondansetron has for α7 ($K_i$ = 3 μM) and α4β2 ($K_i$ > 10 μM) nicotinic receptors (Macor et al., 2001). Additionally, Lundbeck internal data suggests that vortioxetine has similarly negligible affinity at cholinergic receptors.

It seems implausible that neutral 5-$HT_3$ receptor antagonists can improve cognitive function after the 97% depletion of 5-HT observed in tissue homogenate or 93% depletion in basal extracellular 5-HT induced by PCPA (Jensen et al., submitted for publication). However, we have found that acute fenfluramine administration, which disrupts the vesicular 5-HT storage and reverses the function of SERT (Gobbi et al., 1998), profoundly increases extracellular 5-HT concentrations not only in vehicle-treated animals, but also in those treated with PCPA (Jensen et al., submitted for publication). The source of this 5-HT and whether it is releasable via an impulse flow-dependent mechanism has yet to be determined. However, these data may suggest that a biologically significant 5-HT pool remains for synaptic signaling after the PCPA treatment regimen used here. If so, then it may be possible that the remaining 5-HT is sufficient to activate 5-$HT_3$ receptors, which could be blocked by a neutral antagonist such as ondansetron or vortioxetine.

Moreover, the notion that 5-$HT_3$ receptor antagonism can have beneficial cognitive effects is not novel. Several laboratories have reported improved cognitive function in primates and rodents using ondansetron, particularly where deficits are induced via manipulation of cholinergic neurotransmission (Carey et al., 1992; Fontana et al., 1995), glutamatergic neurotransmission (Boast et al., 1999), or as the result of aging (Fontana et al., 1995; Arnsten et al., 1997). Studies in rodents have also found beneficial effects of ondansetron on memory function (Petkov et al., 1995; Staubli and Xu, 1995), or memory-related plasticity measures such as long term potentiation (Staubli and Xu, 1995) in unimpaired animals. Despite the relatively consistent preclinical literature that 5-$HT_3$ antagonism improves memory function, it is important to note that ondansetron is viewed as having limited clinical efficacy for improving cognitive function (Costall and Naylor, 2004), although beneficial memory effects of ondansetron have been observed in the clinic (Akhondzadeh et al., 2009; Levkovitz et al., 2005).

### 4.2. 5-$HT_{1A}$ receptor agonism reverses 5-HT depletion-induced memory deficits

Ten milligram per kilogram vortioxetine significantly improved memory in the OR and SA tests, and significantly engaged 5-$HT_3$, 5-$HT_{1B}$ and 5-$HT_{1A}$ receptors. The observation that the

selective 5-HT$_{1A}$ receptor agonist flesinoxan restored PCPA-induced memory deficits in both tests could support a role for 5-HT$_{1A}$ receptor agonism in the ability of vortioxetine to reverse 5-HT depletion-induced deficits in OR or SA performance. Interestingly, 5-HT$_{1A}$ receptor activation appears to have an "inverted U" type relationship with OR performance, where 1 but not 2.5 mg/kg reversed PCPA-induced deficits. However, SA performance was improved by both 1 and 2.5 mg/kg doses of flesinoxan, suggesting that these tests are mediated by distinct and pharmacologically separable biologies.

It should be noted that preclinical cognition studies in unimpaired rodents paint a broadly consistent and negative picture of the effects of 5-HT$_{1A}$ receptor agonists such as buspirone (Quartermain et al., 1993; Wada and Fukuda, 1992) tandospirone (Quartermain et al., 1993), 8-OH-DPAT (Stiedel et al., 2000; Pitsikas et al., 2005; Seibell et al., 2003), and flesinoxan (Herremans et al., 1995; Tsuji et al., 2003) on learning and memory. However, under conditions where impairment is induced via scopolamine (Bertrand et al., 2001) or NMDA receptor antagonism (Horiguchi and Meltzer, 2012; Depoortere et al., 2010), 5-HT$_{1A}$ receptor stimulation improves cognitive function.

In the chronic vortioxetine treatment experiment, PCPA treatment significantly impaired performance in the OR task and chronic vortioxetine treatment blocked these memory impairing effects, suggesting that vortioxetine's beneficial effects on cognitive function in the context of 5-HT depletion are maintained after long periods of administration. In the SA task, PCPA treatment did not impair performance, although a nonsignificant trend towards a reduction was present. It is likely that this lack of an effect is due to low statistical power. Likewise, chronic treatment with vortioxetine did not significantly alter SA performance.

Although we focused on the potential role of 5-HT$_3$ and 5-HT$_{1A}$ receptors in this study, vortioxetine is also active at other receptor mechanisms over this dose range, which have not been fully considered here. Our data demonstrate that vortioxetine occupied meaningful 5-HT$_{1B}$ receptor occupancy levels over the doses used here. Additionally, vortioxetine has a similar affinity for 5-HT$_{1A}$ and 5-HT$_7$ receptors, suggesting that 5-HT$_7$ receptor occupancy would be similar to our observed for 5-HT$_{1A}$ receptor occupancies over these doses. Therefore, it is possible that 5-HT$_{1B}$ and 5-HT$_7$ receptor mechanisms also play a role in vortioxetine's effects on 5-HT depletion-induced memory deficits.

The current study has several limitations that should be noted. While the data presented here suggest that vortioxetine may have some beneficial effects on 5-HT depletion-induced cognitive dysfunction in rats, it is important to interpret these results cautiously. First, vortioxetine has an approximately 10-fold weaker affinity at 5-HT$_{1A}$ and 5-HT$_7$ receptors in rats compared to humans, while it has stronger effects at the 5-HT$_3$ receptor (Bang-Andersen et al., 2011; Mørk et al., 2012). Thus it is difficult to extrapolate what vortioxetine's effects on MDD-related cognitive dysfunction in humans will be, based on these data alone. Furthermore, given that the same animals were used in both the NOR and SA tasks, it is possible that behavioral history or drug history altered behavioral responses to the pharmacological treatments used in the SA task. Therefore, these data should be interpreted with increased caution. Finally, although the data presented in this manuscript support a role for 5-HT$_3$ receptor antagonism and 5-HT$_{1A}$ receptor agonism in improving OR and SA task performance, we did not attempt to pharmacologically antagonize these mechanisms in vortioxetine-treated rats. Thus, we cannot definitively assess whether these are the mechanisms by which vortioxetine is improving memory performance 5-HT depleted rats.

## 5. Conclusions

The investigational multimodal antidepressant, vortioxetine, dose-dependently reversed recognition memory and spatial working memory deficits induced by 5-HT depletion over a broad dose range, as measured in the NOR and SA tests. The effect in the NOR test may rely in part on 5-HT$_3$ receptor antagonism, whereas 5-HT$_{1A}$ receptor agonism may be relevant for both tests. Thus, the memory enhancing effects of vortioxetine in 5-HT depleted animals may be ascribed to modulation at 5-HT receptors. These preclinical results suggest that the clinical potential of vortioxetine's effect on cognitive dysfunction in MDD should be explored.

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## Contributors

Kristian Gaarn du Jardin and Jesper Bornø Jensen participated in all experiments related to behavior, and *ex vivo* receptor occupancy. Connie Sanchez participated in the design of all experiments. Alan Pehrson participated in all experiments related to *ex vivo* receptor occupancy, statistically analyzed all data, and participated in the design of all experiments. All authors contributed to and have approved the final draft of this manuscript.

## Conflict of interest

Kristian Gaarn du Jardin, Jesper Bornø Jensen, Connie Sanchez, and Alan Pehrson are employees of Lundbeck Research USA, Inc.

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