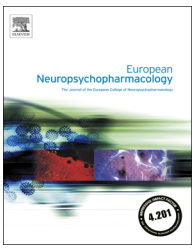
European Neuropsychopharmacology (2014) 24, 148–159



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Vortioxetine, but not escitalopram or duloxetine, reverses memory impairment induced by central 5-HT depletion in rats: Evidence for direct 5-HT receptor modulation

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Received 10 April 2013; received in revised form 18 September 2013; accepted 25 October 2013

KEYWORDS
Vortioxetine;
Lu AA21004;
Major depressive disorder;
Cognitive impairment;
Memory;
5-HT depletion

Abstract
Depressed patients suffer from cognitive dysfunction, including memory deficits. Acute serotonin (5-HT) depletion impairs memory and mood in vulnerable patients. The investigational multimodal acting antidepressant vortioxetine is a 5-HT_3, 5-HT_7 and 5-HT_{1D} receptor antagonist, 5-HT_{1B} receptor partial agonist, 5-HT_{1A} receptor agonist and 5-HT transporter (SERT) inhibitor that enhances memory in normal rats in novel object recognition (NOR) and conditioned fear (Mørk et al., 2013). We hypothesized that vortioxetine's 5-HT receptor mechanisms are involved in its memory effects, and therefore investigated these effects in 5-HT depleted rats. Four injections of the irreversible tryptophan hydroxylase inhibitor 4-chloro-DL-phenylalanine methyl ester hydrochloride (PCPA, 86 mg/kg, s.c.) induced 5-HT depletion, as measured in hippocampal homogenate and microdialysate. The effects of acute challenge with vortioxetine or the 5-HT releaser fenfluramine on extracellular 5-HT were measured in PCPA-treated and control rats. PCPA's effects on NOR and spontaneous alternation (SA) performance were assessed along with the effects of acute treatment with 5-hydroxy-L-tryptophan (5-HTP), vortioxetine, the selective 5-HT reuptake inhibitor escitalopram, or the 5-HT norepinephrine reuptake inhibitor duloxetine. SERT occupancies were estimated by *ex vivo* autoradiography. PCPA depleted central 5-HT by >90% in tissue and microdialysate, and impaired NOR and SA performance. Restoring central 5-HT with 5-HTP reversed these deficits. At similar SERT occupancies (>90%) vortioxetine, but not escitalopram or duloxetine, restored memory performance. Acute fenfluramine significantly

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http://dx.doi.org/10.1016/j.euroneuro.2013.10.011

increased extracellular 5-HT in control and PCPA-treated rats, while vortioxetine did so only in control rats. Thus, vortioxetine restores 5-HT depletion impaired memory performance in rats through one or more of its receptor activities.

1. Introduction

Patients suffering from major depressive disorder (MDD) often present with clinically significant cognitive dysfunction (Kuny and Stassen, 1995), including impaired executive function, episodic memory, and spatial working memory (Airaksinen et al., 2004; Austin et al., 2001; Lee et al., 2012). Recent data suggest that cognitive dysfunction hinders functional recovery (Jaeger et al., 2006), and that greater severity of cognitive dysfunction predicts a poorer response to selective serotonin (5-HT) reuptake inhibitors (SSRIs; Dunkin et al., 2000; Kampf-Sherf et al., 2004; Kaneda, 2009).

MDD has for decades been associated with decreased central serotonergic tone. Support of the 5-HT hypothesis of depression is partly ascribed to the success of SSRIs and other monoamine-centered therapies, but is also supported by the observation that acute depletion of the 5-HT precursor, tryptophan, induces low mood in vulnerable patients (Booij et al., 2005; Delgado et al., 1990, 1994; Benkelfat et al., 1994), and causes cognitive dysfunction (Reidel et al., 1999; Booij et al., 2005; Sobczak et al., 2002). Thus, in combination with the observation that cognitive dysfunction predicts responsiveness to SSRI treatment (Dunkin et al., 2000; Kampf-Sherf et al., 2004; Kaneda, 2009), these data suggest that the relationship between MDD and central 5-HT tone may extend beyond mood and also encompass cognitive function.

Consistent with this hypothesis, serotonergic treatments attenuate depression-related dysfunction in cognitive domains such as episodic memory, working memory, attention, and executive function (Cassano et al., 2002; Constant et al., 2005; Herrera-Guzman et al., 2009, 2010a; Levkovitz et al., 2002), although it should be noted that some studies fail to find significant treatment effects (Ferguson et al., 2003; Nebes et al., 2003). Norepinephrine reuptake inhibitors (NRIs) or serotonin norepinephrine reuptake inhibitors (SNRIs), while not as extensively studied, also appear to have a benefit in cognitive domains such as episodic memory and to a lesser extent, working memory, mental processing speed, and motor performance in patients with MDD (Ferguson et al., 2003; Herrera-Guzman et al., 2009, 2010b; but see Levkovitz et al., 2002). These data also suggest that SNRI treatment may be superior to SSRIs at improving a subset of cognitive functions (Herrera-Guzman et al., 2009, 2010b). However, it is important to note that MDD-related cognitive deficits often persist in a substantial proportion of patients even after mood dysfunction has ameliorated (Herrera-Guzman et al., 2010a,b; Kuny and Stassen, 1995; Nebes et al., 2003). Thus, remediation of cognitive dysfunction remains an unmet need for this patient population.

The investigational antidepressant vortioxetine (Lu AA21004) is one of the first antidepressants with a multimodal mechanism of action, so named because of its two separate modes of action: (1) 5-HT reuptake inhibition and (2) direct pharmacological action at serotonergic receptors. Vortioxetine acts as a 5-HT_3, 5-HT_{1D}, and 5-HT_7 receptor antagonist, 5-HT_{1B} receptor partial agonist, 5-HT_{1A} receptor agonist, and a 5-HT transporter (SERT) inhibitor in recombinant cell lines (Bang-Andersen et al., 2011; Mørk et al., 2012, Westrich et al., 2012). A recent double-blind placebo controlled clinical study in elderly depressed patients found in its exploratory endpoints that vortioxetine significantly improved performance in the Digit Symbol Substitution Test and the Rey Auditory Verbal Learning Task compared to placebo (Katona et al., 2012). In preclinical studies, vortioxetine enhances memory performance in the novel object recognition (NOR) and conditioned fear tests (Mørk et al., 2013). Several of vortioxetine's receptor activities, for example 5-HT_3 receptor antagonism (Petkov et al., 1995; Staubli and Xu, 1995) as well as 5-HT_{1A} receptor agonism (Bertrand et al., 2001; Horiguchi and Meltzer, 2012) have been implicated in enhancing memory function in rats. We therefore hypothesized that one or more of vortioxetine's receptor activities are involved in mediating its memory enhancing activities. To reduce the impact of vortioxetine's SERT inhibitory activity we investigated its effects on memory performance in rats with low central 5-HT tone. Thus, the present study investigated the ability of vortioxetine, the SSRI escitalopram and the SNRI duloxetine, to rescue object recognition and spatial working memory deficits induced by the irreversible tryptophan hydroxylase inhibitor 4-chloro-DL-phenylalanine methyl ester HCl (PCPA), which depletes 5-HT by stopping the rate-limiting step in its synthesis. The degree of depletion was measured from hippocampal tissue homogenate and microdialysate. Furthermore, extracellular 5-HT in the ventral hippocampus was measured in PCPA-treated and control rats after dosing with vortioxetine or the 5-HT releaser, fenfluramine. The level of SERT occupancy after vortioxetine, escitalopram and duloxetine were measured by *ex vivo* autoradiography.

2. Experimental procedures

2.1. Animals

Female Long-Evans rats (Charles River Laboratories, Wilmington, MA, USA) weighing 225–350 g were used for all experiments. Rats were group-housed, three per cage in plastic cages (Rat IVC Green Line Sealsafe plus cages; Tecniplast USA, Philadelphia, PA) except that rats used in microdialysis experiments were individually housed after surgeries. All animals had ad libitum access to food and water in their home cages, in a temperature (69–71°F) and humidity controlled environment (30–70%) on a 12 h light/dark cycle (lights on at 6 a.m.). Rats were housed for at least a one-week acclimation period before any experimental procedures began. All procedures were approved by the Lundbeck Research USA Institutional Animal Care and Use Committee prior to the start of these experiments, and were in line with the NIH's *Guide for the Care and Use of Laboratory Animals*.

2.2. Drugs

Vortioxetine HBr, escitalopram oxalate, and duloxetine oxalate were synthesized by H. Lundbeck A/S. Paroxetine maleate was purchased from Tocris Bioscience (Minneapolis, MN, USA). 4-Chloro-DL-phenylalanine methyl ester HCl (PCPA), carbidopa, 5-hydroxy-L-tryptophan (5-HTP), and serotonin HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]escitalopram (70 Ci/mmol; 1 mCi/mL) was synthesized at Amersham (Piscataway, NJ, USA).

Vortioxetine and duloxetine were dissolved in 20% hydroxypropyl-β-cyclodextrin (Roquette America, Keouk, IA, USA). PCPA, 5-HTP and escitalopram were dissolved in saline. Carbidopa was dissolved in water. Except for PCPA pretreatment and carbidopa, all injections were administered subcutaneously (s.c.) at a volume of 1.0 mL/kg. PCPA was injected s.c. at a volume of 4 mL/kg, while carbidopa was injected i.p. All doses are expressed in mg or μg of base per kg body weight. The specific doses for each drug used in a given experiment are discussed below in the drug treatments section.

In *ex vivo* autoradiography experiments, paroxetine was dissolved in dimethylsulfoxide (Sigma-Aldrich).

2.3. 5-HT concentration in hippocampal tissue

To determine the PCPA dose for subsequent studies, rats (n=5 per group) randomly received PCPA injections of 0.86, 2.6, 8.6, 26, or 86 mg/kg/day or vehicle s.c. for four consecutive days and were euthanized under CO_2 anesthesia 24 h after the final PCPA injection. Hippocampi were collected, flash frozen on powdered dry ice, and stored at −21 °C. Subsequently, the brain tissue samples (10–40 mg wet weight) were mixed at a ratio of 1:3 (w/v) with 0.2% acetic acid/water and homogenized at 20 °C for 4 min in a Covaris sonicator using 100 μL sample tubes. The homogenate was transferred into Amicon Ultra filters (3 kDa; Millipore) and centrifuged at 4 °C for 1 h at 13,500 × g. The resultant solution was transferred into CMA tubes for LC/MS/MS analysis of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA).

2.3.1. LC/MS/MS method

A Waters Acquity HPLC system equipped with an YMC ODS AQ 2 mm × 100 mm, 3 μm particle column provided separation of 5-HT and 5-HIAA prior to detection by a Waters Quattro Premier XE triple quadrupole mass spectrometer operating in the MS/MS mode. Column and pre-column tubing were maintained at 40 °C while eluting the analytes with a mobile phase consisting of an aqueous component (A: 0.5% formic acid in milliQ water) and an organic component (B: 1% formic acid in acetonitrile). Gradient elution included a 2 min hold at 100% A followed by a shallow gradient of 0–30% B with a total run time of 9 min. Full loop injections (5 μL) were performed with a 3× overfill to achieve adequate limits of detection for brain tissue samples (5 ng/mL 5-HT and 10 ng/mL 5-HIAA).

For all subsequent experiments, PCPA treatment consisted of 86 mg/kg/day for four consecutive days.

2.4. Microdialysis: extracellular 5-HT in the ventral hippocampus

The effects of vortioxetine (10 mg/kg), fenfluramine (10 mg/kg, s.c.) or vehicle on extracellular 5-HT levels were measured in PCPA- or vehicle-treated rats using *in vivo* microdialysis (n=5–6 per group). Rats were anesthetized using isoflurane (2%, 800 mL/min O_2). Lidocaine was used for local anesthesia and Rimadyl (1 mg/rat p.o.) was used as a pre- and peri-operative analgesic. Rats were placed in a stereotaxic frame (Kopf instruments, USA) and CMA12 guide cannulas (Harvard Apparatus, Hollingston, MA, USA) were implanted into the ventral hippocampus with the following coordinates: anteroposterior (AP) = −5.3 mm to bregma, mediolateral (ML) = −4.8 mm to midline and dorsoventral (DV) = −4.0 mm relative to dura (Paxinos and Watson, 1998). The ventral hippocampus was chosen as a target region because it allows for the use of a longer dialysis membrane. This allowed us to maximize the chance that 5-HT recovery would be sufficient to surpass our limit of detection, even in 5-HT depleted animals. In addition, this region has a known relationship to memory function (for example, see Hori et al., 2007).

On the evening prior to the microdialysis experiment (after a 7-day surgical recovery period), rats were placed into a round plastic enclosure with bedding. CMA12 PAES microdialysis probes (4 mm active membrane, Harvard Apparatus, Holliston MA, USA) were inserted into the guide cannula and were connected to a 2 channel swivel (Instech Laboratories, Plymouth Meeting, PA USA) using FEP tubing. An artificial cerebrospinal fluid consisting of 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$ and 0.85 mM $MgCl_2$ was perfused at a rate of 1.2 μL/min overnight. Rats were given approximately 18 h of recovery time after probe insertion before microdialysis experiments started. On the day of the experiment, which occurred on the day after the final PCPA or vehicle injection, microdialysis samples were collected at 30 min intervals at a rate of 1.2 μL/min into a refrigerated fraction collector (Bioanalytical Systems, West Lafayette, IN, USA). PCPA- or vehicle-treated animals received a challenge of 10 mg/kg vortioxetine or fenfluramine s.c. in vehicle (1 mL/kg volume) during the microdialysis experiment. Microdialysis samples were stored at −80 °C pending analysis.

5-HT analysis in microdialysis samples was conducted using HPLC with electrochemical detection (HPLC: Alliance 2795 HPLC from Waters, Electrochemical Detector: Decade II from Antec with a VT-03 cell (0.7 mm GC ISAAC), Column: Alf 105 1 mm × 50 mm with 3 μm C18 packing). An isocratic method was used with a 10% methanol/water mobile phase containing 0.1 mM EDTA, 8 mM NaCl, 500 mg/L of OSA (L-octane sulfonic acid sodium salt) and 50 mM phosphoric acid adjusted to pH 6 with aqueous NaOH. Microdialysis samples were loaded into a 96-well plate and heat-sealed prior to loading onto a refrigerated sample compartment (4 °C) for overnight analysis. Using full loop injections (5 μL) with a 3× overfill, this method resulted in a 5 pg/mL limit of detection, which was sufficient to reliably measure 5-HT in all samples.

2.5. Assessment of memory performance in 5-HT depleted rats

Rats were tested in two behavioral models, the novel object recognition (NOR) test 24 h after the last PCPA injection and the spontaneous alternation (SA) test on the following day. Sample sizes were 6–11 rats per group. Specific sample size information for each experimental group is included in Figures 3 and 4.

2.5.1. Novel object recognition (NOR)

Evaluation was conducted in a plastic arena (49 cm × 49 cm × 37 cm) with a consistent light intensity of approximately 5 lux. A camera recorded all behavioral sessions, which were subsequently analyzed for track lengths using Viewer III software (Bioobserve GmbH, Fort Lee, NJ, USA). Object pairs were validated before use to ensure that there was no bias towards one of the objects. Between each session, the field and objects were thoroughly cleaned to attenuate olfactory cues.

On the final day of PCPA treatments (day 0), rats were individually habituated to the open field for 5 min. The training session was conducted 24 h after habituation (day 1). Rats were rehabituated for 1 min and then placed in a holding cage while two identical objects were placed in the arena. The familiar object's identity was pseudorandomly assigned for each rat and counter-balanced between treatment groups to minimize any residual bias that may have existed. Rats were positioned facing the arena wall with equal distance to each object and allowed to explore for 15 min. Upon

completion, the rat was returned to its home cage. The testing session occurred 45 min after the training session finished. Two objects were once again placed in the arena: one identical to the training objects, and one novel object. The position of the novel object was pseudorandomly assigned and counter-balanced for each treatment group. The rat was placed in the arena facing the wall with equal distance to each object and was allowed to explore for 3 min.

The exploration time for each object was manually recorded with a stopwatch from the video recordings. Exploration was defined as directing the nose to the object at a close distance or touching the object. Sitting on the object was not considered exploration. An animal's performance was excluded if it (i) failed to accumulate a total exploration time of more than 6 s, (ii) failed to explore both objects, (iii) knocked an object over during the test session, or (iv) jumped out of the arena.

2.5.2. Spontaneous alternation (SA)

On the day following NOR testing (i.e. day 2 after cessation of PCPA treatment), rats were evaluated in the SA task. This test was conducted in a plastic beige-colored Y-maze. Each arm was 50.8 cm long and 10.2 cm wide, and the walls of the arena were 19.8 cm high. The light intensity was equal ($\sim$5 lx) in all parts of the field. A camera recorded the sessions, which were scored at a later time.

Animals were acclimated to the experimental room for 30 min prior to the start of the behavioral session. Rats were placed in the center of the Y-maze and allowed to freely explore for 6 min. The maze was thoroughly cleaned after each rat to attenuate olfactory trails. The sequence of arm entries was recorded manually from the recordings. An alternation was defined as any 3-entry sequence of one visit to each arm. The performance of an animal was excluded if the total number of arm entries was <7 or the rat jumped out of the arena.

2.6. Drug treatments

2.6.1. The role of central 5-HT in PCPA-induced memory deficits

PCPA-treated rats received vehicle injections or a combination of 1 mg/kg of the peripheral decarboxylase inhibitor carbidopa and 50 mg/kg of the 5-HT precursor 5-HTP (at 1 h and 50 min prior to assessment, respectively) before being compared to non-5-HT depleted control rats in the NOR and SA tasks. Control rats received vehicle injections at the 1 h and 50 min time points.

In a separate group of behaviorally naïve animals, hippocampal tissue homogenate was collected and analyzed for 5-HT content on days 1 or 2 following cessation of PCPA injections or on day 1 after cessation of vehicle. In addition, hippocampal tissue was collected in another group that received PCPA followed by carbidopa and 5-HTP injections as described above.

2.6.2. The effects of vortioxetine, escitalopram, or duloxetine on PCPA-induced memory deficits

The dose for each drug was chosen to produce maximum SERT occupancy based on the dose-occupancy relationships established in PCPA treated animals (see the *ex vivo* autoradiography section, below). In subsequent behavioral experiments, control or PCPA pretreated groups received injections of vehicle, 0.5 mg/kg escitalopram, 15 mg/kg duloxetine, or 10 mg/kg vortioxetine 1 h before being assessed in the NOR and SA tasks.

2.7. *Ex vivo* autoradiography

Fractional receptor occupancies for vortioxetine, duloxetine, and escitalopram were estimated using *ex vivo* autoradiography in rats pretreated with PCPA for four consecutive days as described above. On the day following the last PCPA injection, rats were randomly assigned to treatment with vehicle, vortioxetine, duloxetine, or escitalopram. The sample size was set at three rats per treatment group, with each brain represented by three replicate slices.

One hour after drug treatment, the rats were anesthetized with CO_2 and sacrificed by decapitation. Brains were dissected from the skull, flash frozen on powdered dry ice, and stored at -20 °C until use. Twenty micrometer thick coronal slices were collected using a cryostat (Microm, Walldorf, Germany) beginning at approximately 1.2–1.5 mm anterior to Bregma. Sections were mounted on slides and stored in a slide box with desiccant pellets at -20 °C.

Ex vivo autoradiography experiments for the SERT were performed as described previously (Pehrson et al., in press) with minor modifications. Briefly, slide boxes were defrosted at room temperature (RT) under a constant stream of air for at least 30 min before opening. Slides were incubated (RT) in assay buffer consisting of 50 mM Tris, 150 mM NaCl, 5 mM KCl containing 4.5 nM [^{3}H]escitalopram for 1 h. Nonspecific binding was determined on a separate slide by the addition of 1 μM of non-radioactive paroxetine to the assay buffer. Following the incubation period, the slides were rinsed twice in 4 °C assay buffer and briefly dipped in 4 °C distilled water. Subsequently, the slides were air-dried, transferred to a vacuum desiccator and dried for an additional 60 min. Finally, the slides were processed in a Beta Imager (Biospace Lab, Paris, France) for 16 h.

2.8. Data analysis and statistical methods

Results are expressed as mean$\pm$SEM. Statistical significance was accepted 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. All data sets were tested for normality using the lilliefors test. In cases where data was normally distributed, outliers were eliminated using Pierce's criterion (Ross, 2003). If data was not normally distributed, no data points were removed as outliers.

2.8.1. Central 5-HT depletion

Tissue data were normalized to the mean of vehicle-treated animals. Because these data were not normally distributed, data were analyzed using a Kruskal–Wallis nonparametric ANOVA followed by a Holm–Bonferroni corrected Mann–Whitney *U post hoc* where appropriate. The 5-HT turnover was defined as the quotient of the concentration of 5-HIAA (%) and 5-HT (%): 5-HT turnover$=$[5-HIAA]/[5-HT].

Statistical methods for the analysis of microdialysis data were conducted as discussed previously (Pehrson et al., 2013) with minor modifications. Briefly, the mean of three pretreatment microdialysis samples was used to define the baseline for each animal; all samples from that animal were normalized to baseline and expressed as a percentage of this value. Subsequently, the area under the curve was determined for each animal using the trapezoid method. Because these data were not normally distributed, significance testing was conducted using the Kruskal–Wallis nonparametric ANOVA with Holm–Bonferroni corrected Mann–Whitney *U post hoc* tests where appropriate. Basal extracellular 5-HT concentrations for animals treated with vehicle or PCPA once per day for four days were normalized and expressed as a % of vehicle concentrations. These data were also not normally distributed, therefore statistical analysis was conducted using a Mann–Whitney *U* test.

2.8.2. Behavioral assessments

For the NOR test, preference score was defined as the time spent exploring the novel object divided by the total exploration time (i.e. time exploring both novel and familiar objects). Track length, measured in centimeters, was recorded by Bioobserve software.

For the SA test, the percentage of spontaneous alternations (% alternations) was calculated using the following formula: $A/(T-2) \times 100$, where A is the number of spontaneous alternations, and T represents the total number of arm entries. 2 was subtracted from the total number of arm entries to determine the number of possible alternations.

Rotational bias was also calculated for each animal included in the spontaneous alternation experiments as described in McFarland (1989). To calculate this measure, the total number of turns made was divided by 2 and subtracted from the total number of left turns. The absolute value was taken and divided by the total number of arm entries. This value was multiplied by 100 and added to 50 to yield the percent bias towards turning in the preferred direction. Although some rotational bias is normal in the SA test, this measure makes it possible to determine whether alternation score changes are better explained by motor effects than spatial working memory effects.

All behavioral dependent measures were analyzed using a one-way ANOVA followed by Newman-Keuls *post hoc* test.

2.8.3. *Ex vivo* autoradiography

Beta Imager data 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 an *a priori*-defined region of interest (ROI), based on previous receptor mapping experiments. The ROI for SERT has been described in detail elsewhere (Pehrson et al., 2013), and included the lateral and medial septum, and the olfactory tubercle.

Total binding was determined by averaging cpm/mm^2 from the ROI of three replicate slices from each rat brain. Nonspecific binding was determined by averaging cpm/mm^2 from brain slices in the nonspecific binding condition. Specific binding was determined for each brain by subtracting nonspecific binding from total binding. Specific binding levels for each brain were subsequently normalized to the vehicle condition and expressed as a percentage of the average specific binding in vehicle-treated brains. Receptor occupancies were determined by subtracting these percentages from 100.

3. Results

3.1. 5-HT levels in hippocampus homogenate

PCPA administration for four consecutive days dose-dependently decreased the average 5-HT concentration in hippocampal tissue homogenate to approximately 3% of control values at 86 mg/kg/day (Table 1), and this dose was used in all subsequent experiments. 5-HT depletion reached statistical significance at 26 mg/kg/day ($H(5, n=25)=23.4$, $p<0.001$). The ED_{50} of PCPA treatment was 17.3 mg/kg/day. The profound depletion of 5-HT observed at this dose ($H(3, n=20)=16.58$, $p<0.001$; Figure 1A) remained stable for at least 2 days after the cessation of PCPA administration, which encompassed the entire time during which behavioral and neurochemical testing occurred. In addition, 5-HIAA ($H(3, n=20)=17.58$, $p<0.001$; Figure 1B) and 5-HT turnover ($H(3, n=20)=17.31$, $p<0.001$; data not shown) were also significantly decreased by PCPA treatment over this timeframe.

Acute treatment with 1 mg/kg carbidopa and 50 mg/kg 5-HTP 24 h after the last PCPA dose significantly increased hippocampal 5-HT (Figure 1A) and 5-HIAA levels (Figure 1B), and 5-HT turnover (data not shown) compared to the control and PCPA groups.

Table 1 The effect of ascending PCPA doses on hippocampal tissue homogenate 5-HT concentrations. PCPA treatment for 4 consecutive days dose-dependently decreased 5-HT concentrations with an ED_{50} of 17.3 mg/kg (95% confidence interval: 12.5-24 mg/kg/day).

PCPA dose (mg/kg/day × 4, s.c.)	% control 5-HT concentration (tissue homogenate)
Vehicle	100 ± 9.6
0.86	114 ± 12
2.6	121 ± 10
8.6	82 ± 5.5
26	28 ± 6.2**
86	3.2 ± 0.3**

Data are presented as mean ± SEM.
**Significant differences from the vehicle condition ($p<0.01$).

3.2. Extracellular 5-HT concentrations in the ventral hippocampus

Basal extracellular 5-HT levels in animals treated with vehicle once per day for four days was 156 ± 29 pg/mL, while PCPA treated animals had basal extracellular 5-HT levels of 11 ± 2.3 pg/mL (data not shown; $U(n=34)=17$, $p<0.0001$). Thus, 86 mg/kg/day PCPA reduced extracellular 5-HT concentrations to approximately 7% of control, similar to the level of depletion observed in tissue homogenate.

Treatment with acute vortioxetine (10 mg/kg) significantly increased extracellular 5-HT in the hippocampus of vehicle-treated animals (Figure 2A and B; $H(3, n=22)=12.61$, $p<0.01$). Although vortioxetine treatment lead to numerical increases in extracellular 5-HT in PCPA-treated animals, these increases did not reach statistical significance ($p=0.03$, Holm-Bonferroni adjusted alpha=0.025). Interestingly, acute fenfluramine treatment (10 mg/kg) elicited a significant increase in extracellular 5-HT not only in vehicle-treated animals but also in PCPA-treated animals ($H(3, n=23)=16.79$, $p<0.001$; Figure 2C and D). Note that in the vortioxetine and fenfluramine challenge experiments, extracellular 5-HT values for each rat were normalized to its own average basal extracellular 5-HT concentration. Thus, fractions 1-3 are by definition close to a value of 100%, whether rats were vehicle- or PCPA-treated.

3.3. 5-HT depletion impairs performance in the NOR and SA tests

PCPA impaired NOR performance, significantly reducing novel object preference ($F(2.22)=9.6$; $p<0.01$; Figure 3A) and track lengths during the test session ($F(2,22)=26.4$, $p<0.001$; Figure 3B). Acute administration of 1 mg/kg carbidopa and 50 mg/kg 5-HTP in PCPA treated animals restored novel object preference scores to control levels while further reducing track lengths. Non-quantified observations indicate that this group of animals displayed a high frequency of anxiety-related behaviors, such as stretch-attend postures (Capone et al., 2005) and thigmotaxia, that

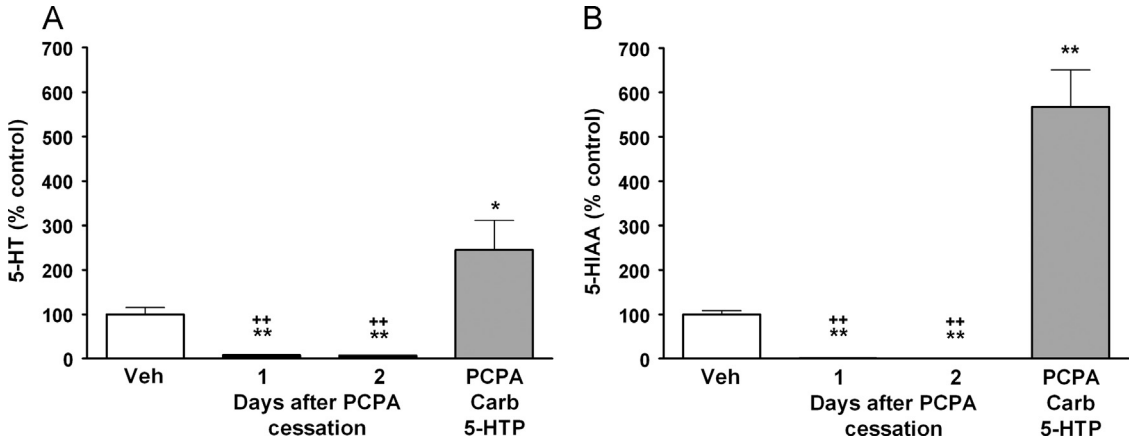

Figure 1 PCPA administration depletes hippocampal 5-HT (Panel A) and 5-HIAA (Panel B) in tissue homogenate for at least 2 days after PCPA cessation. This timeframe encompasses the entire period within which testing took place. The combination of 1 mg/kg carbidopa and 50 mg/kg 5-HTP significantly increased 5-HT and 5-HIAA levels in PCPA-treated rats. Data are presented as mean ± SEM. Asterisks represent significant differences from vehicle ($^{*}p<0.05$; $^{**}p<0.01$). Plus signs represent significant differences from the PCPA+carbidopa+5-HTP condition ($^{+}p<0.05$; $^{++}p<0.01$).

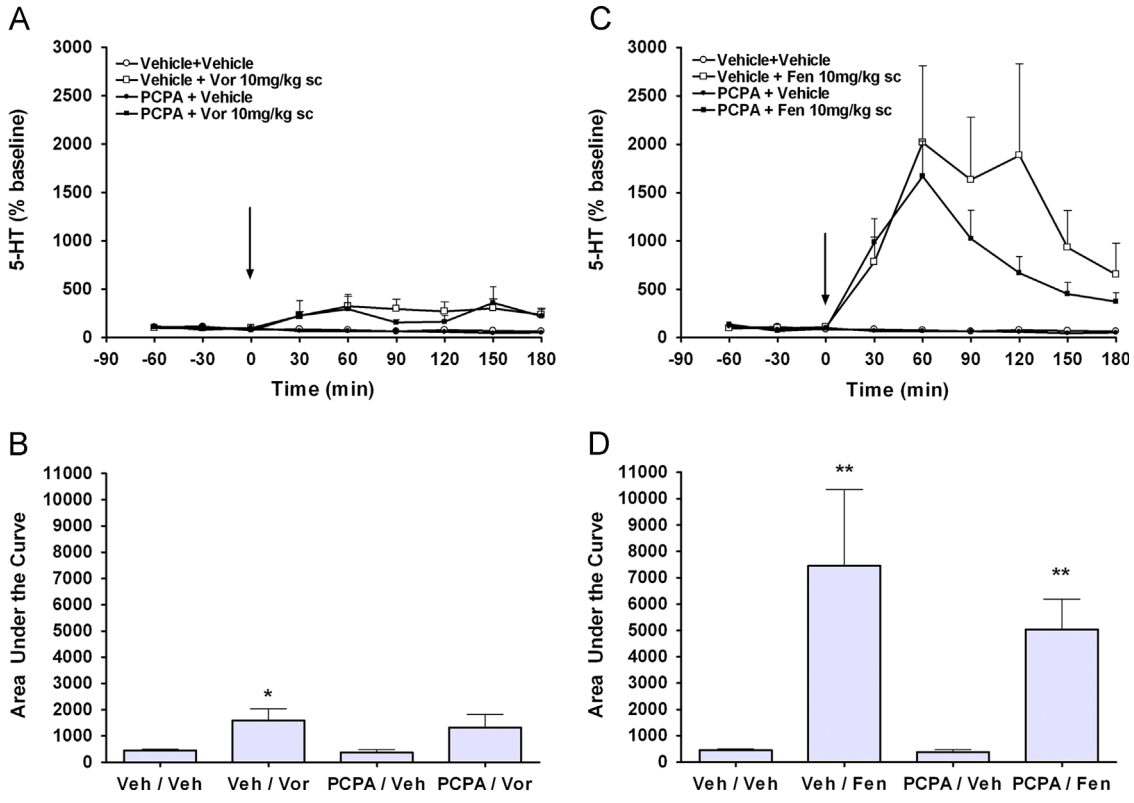

Figure 2 The effects of acute vortioxetine or fenfluramine on hippocampal extracellular 5-HT in vehicle or PCPA treated animals. In panels A and C, symbols represent average extracellular 5-HT concentrations. For each animal, 5-HT concentrations were normalized to the animal's average baseline concentration, thus, extracellular 5-HT at time points 1–3 are close to 100% by definition for both vehicle and PCPA treated groups. Arrows indicate acute injection with vortioxetine, fenfluramine or vehicle. In panels B and D, bars represent area under the curve for extracellular 5-HT. Vortioxetine (panels A and B) significantly increased extracellular 5-HT in vehicle treated animals but not in PCPA-treated animals ($p=0.03$, with a Holm–Bonferroni adjusted alpha level of 0.025). Fenfluramine significantly increased extracellular 5-HT in vehicle and PCPA-treated rats (panels C and D). All data are expressed as mean ± SEM. Asterisk represents a significant difference from the Veh/Veh group ($^{*}p<0.05$; $^{**}p<0.01$).

were not observed in the control or PCPA groups. Thus, it may be that the reduced track length observed in the carbidopa/5-HTP group reflects increased anxiety.

In addition, pretreatment with PCPA impaired SA performance. The PCPA-treated group had significantly lower alternation scores ($F(2,20)=11.52$, $p<0.001$; Figure 3C)

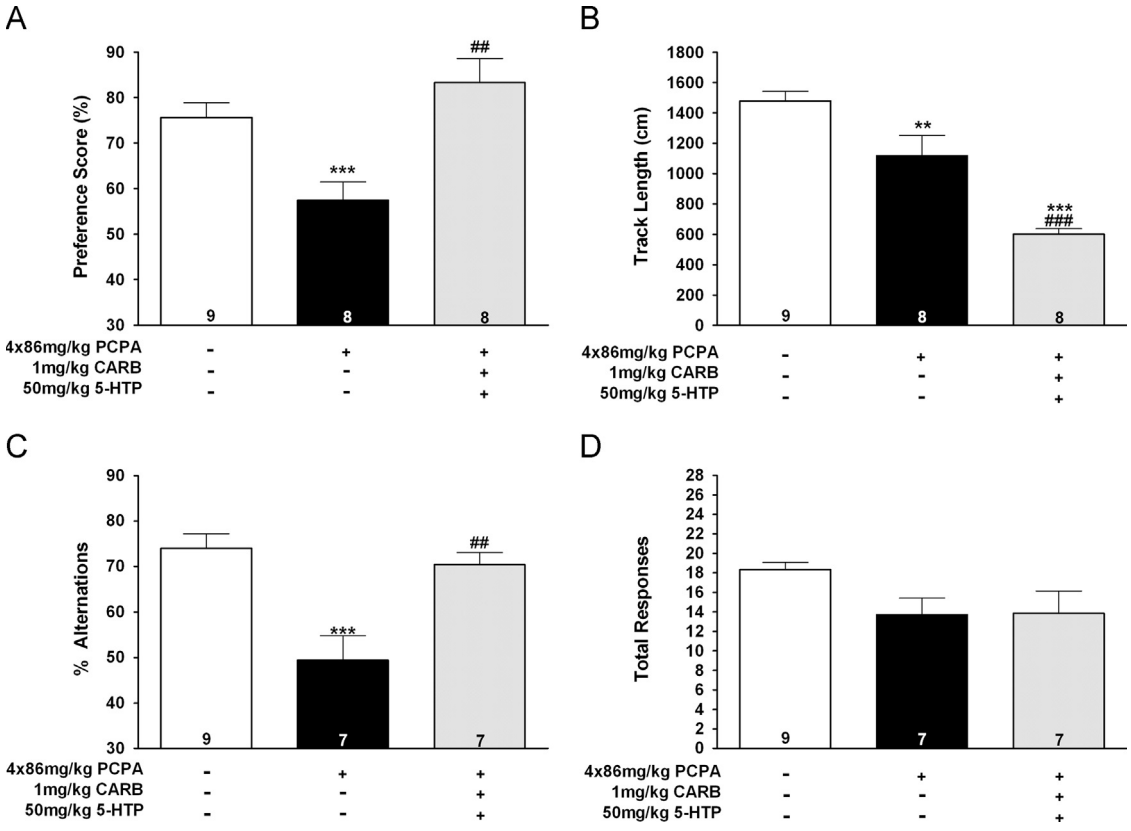

Figure 3 The effect of acute s.c. administration of 5-HTP on PCPA-induced memory deficits in the NOR and SA test. PCPA treatment significantly reduced novel object preference scores (panel A) and track lengths (panel B) in the NOR test. PCPA treatment also impaired alternation scores (panel C) but did not affect total responses in the SA test. 1 mg/kg carbidopa and 50 mg/kg 5-HTP restored rats to normal novel object preference and alternation scores, but further reduced track lengths in the NOR test compared to PCPA treatment alone. Bars represent mean ± SEM. Numbers within the bars represent sample size. Asterisks represent significant differences from vehicle (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Plus signs represent significant differences from the PCPA condition ($^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$).

than control animals, while the carbidopa/5-HTP combination significantly improved alternation scores compared to animals given PCPA only. Although the PCPA and carbidopa/5-HTP groups had numerically lower numbers of arm entries than the control group, these differences did not reach statistical significance ($F(2,20)=2.98$, $p=0.074$; Figure 3D). Finally, neither pretreatment with PCPA nor the combination of carbidopa and 5-HTP caused any significant changes in the response bias dependent measure compared to controls ($F(2,20)=0.005$, n.s.; data not shown).

3.4. Vortioxetine, but not escitalopram or duloxetine, rescues 5-HT depletion-induced deficits

Pretreatment with PCPA again impaired NOR performance by significantly reducing preference for the novel object ($F(4,40)=6.485$, $p< 0.001$; Figure 4A). Administration of 10 mg/kg vortioxetine to PCPA-treated animals significantly increased preference for the novel object compared to animals given PCPA only, thereby restoring object recognition to control levels. However, animals given equivalent doses of escitalopram (0.5 mg/kg) or duloxetine (15 mg/kg) after PCPA pretreatment remained significantly impaired relative to controls. As observed previously, PCPA pretreatment also significantly reduced locomotor activity during the NOR test session compared to control animals ($F(4,40)=12.65$, $p<0.0001$; Figure 4B). None of the pharmacological treatments used affected the reduced track length caused by PCPA treatment.

Similarly, acute treatment with 10 mg/kg vortioxetine in PCPA-pretreated rats significantly improved alternation scores in the SA task compared to animals treated with PCPA only ($F(4,40)=11.82$, $p<0.0001$; Figure 4C), while acute 0.5 mg/kg escitalopram or 15 mg/kg duloxetine failed to improve alternation scores. PCPA treatment was associated with a significant decrease in the total number of arm entries, which was not attenuated by acute vortioxetine, escitalopram, or duloxetine ($F(4,40)=6.28$, $p<0.001$; Figure 4D). Again, none of the experimental manipulations used here led to significant changes in rotational bias compared to control animals ($F(4,40)=0.32$, n.s.; data not shown).

3.5. Estimation of SERT occupancies using *ex vivo* autoradiography

Acute administration of vortioxetine (0.1–10 mg/kg), escitalopram (0.03–0.5 mg/kg), and duloxetine (1.5–15 mg/kg) in PCPA-treated rats produced dose-dependent increases in SERT occupancy (Table 2). In each case, the highest dose used engendered approximately 90% or greater occupancy

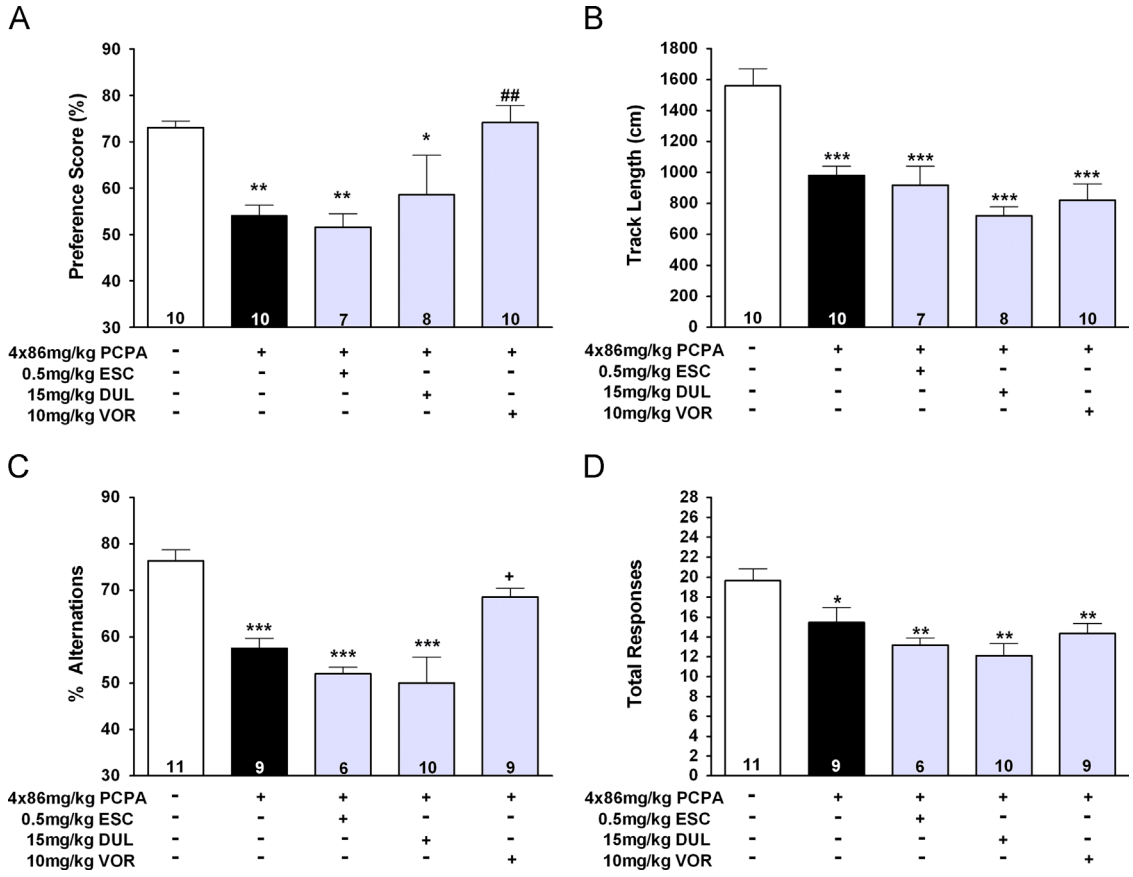

Figure 4 Effect of acute s.c. administration of escitalopram, duloxetine, and vortioxetine on PCPA-induced memory deficits in the NOR and SA test. PCPA treatment impaired novel object preference scores (Panel A), reduced track lengths in the NOR test (Panel B), reduced alternation scores (Panel C), and reduced the number of responses in the SA test. Treatment with vortioxetine, but not escitalopram or duloxetine, restored novel object preference and alternation scores to normal, without affecting track length or total responses. Bars represent mean ± SEM. Numbers within the bars represent sample size. Asterisks represent significant differences from vehicle ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$). Plus signs represent significant differences from the PCPA condition ($^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$).

Table 2 SERT occupancies for vortioxetine, escitalopram, and duloxetine. Data are presented as mean ± SEM.

Compound	Dose (mg/kg)	SERT occupancy (%)
Vortioxetine	0.1	22 ± 11
	3.0	81 ± 1.5
	10.0	95 ± 0.9
Escitalopram	0.03	29 ± 5.5
	0.1	71 ± 1.8
	0.3	83 ± 2.3
	0.5	88 ± 1.4
Duloxetine	1.5	90 ± 1.3
	3.0	98 ± 0.9
	15.0	103 ± 0.2

at SERT. This is considered to be full SERT occupancy, and thus these doses can be considered both maximal and equivalent in terms of their 5-HT reuptake inhibition.

4. Discussion

In the present study we investigated the effects of the multimodal acting antidepressant vortioxetine on memory performance in a rat model of 5-HT depletion-induced memory deficits and addressed the hypothesis that vortioxetine's effects might involve direct 5-HT receptor mediated effects.

In line with previous research we show that repeated administration of the irreversible tryptophan hydroxylase inhibitor, PCPA, results in dose-dependent reductions in brain tissue concentration of 5-HT and its metabolite, 5-HIAA, as well as 5-HT turnover. Our observation that repeated administration of PCPA (86 mg/kg daily for four days) in rats resulted in a 97% reduction in brain tissue homogenate 5-HT concentrations is comparable to the findings of other research groups, who found 80–90% depletions after a similar PCPA administration regimen (Matsukawa et al., 1997; Prinssen et al., 2002). We also demonstrated that basal extracellular 5-HT concentrations were reduced by approximately 93%, thus demonstrating a similar level of depletion compared to tissue homogenate.

Using *in vivo* microdialysis, we found that vortioxetine significantly increases hippocampal extracellular 5-HT in

non-5-HT depleted animals, as in previous reports (Pehrson et al., 2013). Vortioxetine administration in PCPA-treated animals also caused a trend towards increases in 5-HT concentrations, although these increases did not meet statistical significance. Interestingly, the 5-HT releaser fenfluramine markedly increased hippocampal extracellular 5-HT not only in control rats, but also in those pretreated with PCPA, despite the profound 5-HT depletion we observed in tissue homogenate or basal extracellular 5-HT. Given that fenfluramine acts by relocating vesicular 5-HT to the cytosol and reversing the function of the SERT (Gobbi et al., 1992), these data may suggest that there is a CNS-derived pool of 5-HT that is spared after 5-HT depletion using PCPA. Whether this pool of 5-HT is releasable in an impulse-flow dependent manner is currently unknown, however, the trend towards increased extracellular 5-HT noted in PCPA-treated rats after acute vortioxetine may suggest that it is.

As predicted, the profound central 5-HT reductions found after PCPA in rodents were associated with impaired performance of the NOR and SA tests, which are thought to model recognition memory and spatial working memory, respectively. Previous research using PCPA has demonstrated that it can also have a depleting effect on other monoamine neurotransmitters such as dopamine (DA) and norepinephrine (NE; Dailly et al., 2006). Thus, it is possible that the memory deficits we observed were not solely due to effects on the serotonergic system. Although this possibility was not directly investigated in this study, we find it unlikely that the NOR deficits performance observed here were due to depletion of DA or NE. First, Dailly et al. (2006) did not find significant decreases in DA or NE until comparatively high PCPA doses ($\geq$ 300 mg/kg). Importantly, we demonstrated that acute treatment with carbidopa and 5-HTP significantly increased tissue 5-HT concentrations in PCPA-treated animals and recovered memory performance. Thus, 5-HT neurotransmission appears to be the fundamental mechanism underlying the performance deficits induced by the repeated PCPA treatment regimen used in this study.

The idea that central 5-HT depletion impairs some aspects of memory function is well supported by the literature. This laboratory has previously demonstrated that the PCPA administration regimen used here consistently impairs object recognition memory (du Jardin et al., in press). Reducing central 5-HT concentrations using 5,7 dihydroxytryptamine (5,7-DHT) lesions of the dorsal raphe nucleus (Lieben et al., 2006) or acute or chronic tryptophan depletion also impairs object recognition memory (Rutten et al., 2007; Jans et al., 2010). Similarly, PCPA administration (du Jardin et al., in press) and 5,7-DHT lesions (Hritcu et al., 2007) impair spatial working memory as assessed in the *y*-maze spontaneous alternation paradigm, lending further support to the effects of 5-HT depletion found in the present study.

Although 5-HT depletion consistently impairs some aspects of memory function, it does not affect all forms. While *y*-maze spontaneous alternations are impaired by reduced central serotonergic neurotransmission, other behavioral tasks that putatively assess spatial working memory, such as the 8-arm radial maze (Hritcu et al., 2007) and the delayed nonmatch to position tasks (Jakala et al., 1993), are not affected by 5-HT depletion. Additionally, spatial reference memory assessed in the 8-arm radial maze (Hritcu et al., 2007) and Morris water maze (Matsukawa et al., 1997) is not significantly affected in 5-HT depletion paradigms. Based on these data, it is plausible that central 5-HT function plays an important role in some, but not all aspects of memory.

However, the data on the role of 5-HT in memory should be interpreted with caution. Reducing 5-HT neurotransmission on such a broad scale may set off compensatory adaptations that mask the normal role of this neurotransmitter system in memory. Thus, it is possible that selective modulation of 5-HT receptor targets under normal serotonergic tone may reveal a role for this neurotransmitter system in aspects of memory that are not seen under depleted conditions. For example, Staubli and Xu (1995) demonstrated that selective 5-HT_3 receptor antagonism improves performance in an 8-arm radial maze task. Further discussion of selected 5-HT receptor systems and their role in cognitive function can be found in du Jardin et al. (in press) and Pehrson and Sanchez (in press).

We hypothesized that antidepressants whose mechanism of action is mediated primarily by 5-HT reuptake inhibition would be incapable of restoring memory deficits induced by PCPA treatment, given the level of 5-HT depletion we observed. But because vortioxetine's mode of action includes direct modulation of 5-HT receptors, we hypothesized that vortioxetine could reverse 5-HT depletion-induced deficits in memory. This hypothesis was supported by our observation that vortioxetine administration restored memory function in the NOR and SA tasks to normal levels, while the SSRI escitalopram and the SNRI duloxetine failed to restore memory performance in PCPA-treated rats despite similarly high SERT occupancies ($>$ 90%) for all three antidepressants. Thus, it is likely that vortioxetine's ability to restore NOR and SA performance are driven by its effects at serotonergic receptors, rather than SERT inhibition. An alternative interpretation is that vortioxetine restores performance by reinstating extracellular 5-HT tone to a sufficient degree for normal memory function to occur. However, the inability of 5-HT reuptake blockade to significantly improve NOR or SA performance again suggests that any increases in extracellular 5-HT driven by acute vortioxetine must be a result of serotonin receptor modulation.

In addition to the changes observed in the novel object preference and % alternation measures, PCPA treatment reduced locomotor activity. Thus, it may be argued that the behavioral deficits observed in this experiment are a result of impaired motor function. However, acute treatment with vortioxetine reversed the impairments in novel object preference and % alternation without altering PCPA's locomotor effects in either task, suggesting that the cognitive and locomotor effects of 5-HT depletion occur *via* independent mechanisms.

One potential explanation for the reduced locomotor activity observed in PCPA-treated rats is related to body temperature regulation. There is a long-recognized relationship between body temperature and locomotor activity in mammals such that periods of higher body temperature are tightly associated with increased levels of locomotor activity (Refinetti, 1999), although the consensus is that these increases in body temperature are not caused by increased locomotor activity (Refinetti, 1999; Bolles et al., 1968). Given that acute PCPA injections are known to cause

marked reductions in body temperature (Lin et al., 1978; Satinoff et al., 1991), the PCPA-induced reductions in locomotor activity may be the result of reduced body temperature. However, more recent investigations into the relationship between PCPA and body temperature have revealed that although an initial dose of PCPA produces reductions in body temperature, temperatures normalize over time and are unaffected by further PCPA injections (Satinoff et al., 1991). Given that reduced locomotor activity was observed five to six days after the initial PCPA injection, it seems more likely that the PCPA-induced reduction in locomotion is independent of changes in body temperature.

The spontaneous alternation experiments in this study have a few limitations that should be considered. First, the animals used in the SA task are the same that were used on the previous day in the NOR experiments. Thus, it cannot be ruled out that an animal's behavioral or drug history may have altered their response to acute administration of the carbidopa/5-HTP combination, escitalopram, duloxetine or vortioxetine in the SA task. Additionally, although the SA task is generally regarded as a measure of spatial working memory, there is no established method to assess whether the spatial memory deficits induced by 5-HT depletion are delay-dependent, which is the gold standard of an effect on working memory. Based on these limitations, the results of the SA task should be interpreted with caution.

In conclusion, this study demonstrated that 5-HT depletion *via* administration of the irreversible tryptophan hydroxylase inhibitor PCPA reliably impaired memory performance, as assessed by the NOR and SA tests. Subsequently increasing central 5-HT concentrations using acute 5-HTP and carbidopa administration rescued these memory deficits, thereby indicating that central 5-HT receptor activity is a fundamental mechanism of this model. Unlike an SSRI and SNRI, acute treatment with vortioxetine reversed 5-HT depletion-induced memory deficits as assessed in the NOR and SA tests. Vortioxetine's effects on 5-HT-depletion-induced memory deficits appear to be mediated by its direct pharmacological actions on serotonergic receptors.

Role of the funding source

This study was funded by H. Lundbeck A/S and the Takeda Pharmaceutical Company, Ltd. Employees of Lundbeck played a role in the design of experiments, as well as the collection, analysis and interpretation of data. Lundbeck employees also played a role in the writing of and the decision to submit the present study.

Contributors

Jesper Bornø Jensen and Kristian Gaarn du Jardin participated in all experiments related to behavior, assessment of 5-HT concentration in hippocampal tissue, and *ex vivo* receptor occupancy. Dekun Song conducted the *in vivo* phase of all microdialysis experiments. David Budac analyzed 5-HT concentrations from microdialysate and hippocampal tissue homogenate. Gennady Smagin participated in the design of microdialysis experiments. Connie Sanchez participated in the design of all experiments. Alan Pehrson participated in all experiments related to assessment of 5-HT concentration in hippocampal tissue, and *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 paper.

Conflict of interest

Jesper Bornø Jensen, Kristian Gaarn du Jardin, Dekun Song, David Budac, Gennady Smagin, Connie Sanchez, and Alan Pehrson are employees of Lundbeck Research USA, Inc.

Acknowledgments

The authors wish to thank Mr. David J. Simpson, who assisted with proofreading this manuscript.

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