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Vortioxetine (Lu AA21004), a novel multimodal antidepressant, enhances memory in rats☆

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ABSTRACT

The serotonergic system plays an important role in cognitive functions via various 5-HT receptors. Vortioxetine (Lu AA21004) in development as a novel multimodal antidepressant is a 5-HT_3, 5-HT_7 and 5-HT_{1D} receptor antagonist, a 5-HT_{1B} receptor partial agonist, a 5-HT_{1A} receptor agonist and a 5-HT transporter (5-HTT) inhibitor in vitro. Preclinical studies suggest that 5-HT_3 and 5-HT_7 receptor antagonism as well as 5-HT_{1A} receptor agonism may have a positive impact on cognitive functions including memory. Thus vortioxetine may potentially enhance memory. We investigated preclinical effects of vortioxetine (1–10 mg/kg administered subcutaneously [s.c.]) on memory in behavioral tests, and on cortical neurotransmitter levels considered important in rat memory function. Contextual fear conditioning and novel object recognition tests were applied to assess memory in rats. Microdialysis studies were conducted to measure extracellular neurotransmitter levels in the rat medial prefrontal cortex. Vortioxetine administered 1 h before or immediately after acquisition of contextual fear conditioning led to an increase in freezing time during the retention test. This mnemonic effect was not related to changes in pain sensitivity as measured in the hotplate test. Rats treated with vortioxetine 1 h before training spent more time exploring the novel object in the novel object recognition test. In microdialysis studies of the rat medial prefrontal cortex, vortioxetine increased extracellular levels of acetylcholine and histamine. In conclusion, vortioxetine enhanced contextual and episodic memory in rat behavioral models. Further demonstration of its potential effect on memory functions in clinical settings is warranted.

1. Introduction

The most frequently used pharmacological treatments for major depressive disorder (MDD) include the selective serotonin reuptake inhibitors (SSRIs) and the serotonin and norepinephrine (NE) reuptake inhibitors (SNRIs) whereas the older tricyclic antidepressants and monoamine oxidase inhibitors are less used because of their adverse effects. While the SSRIs and SNRIs may generate only a limited antidepressant response (Rush et al., 2006), improved responses have been seen with different or combined therapies involving additional mechanisms, such as the allosteric serotonin reuptake inhibitor (ASRI) escitalopram (Cipriani et al., 2009; Montgomery et al., 2011; Zhong et al., 2012), as well as combination medications, such as venlafaxine plus bupropion (Fatemi et al., 1999), SSRIs plus pindolol (Artigas et al., 2006), or SSRIs augmented with atypical antipsychotics such as aripiprazole (Nelson and Papakostas, 2009).

Significant unmet needs still exist in the treatment of depression, including deficits in memory and executive functions, symptoms that may persist beyond clinical recovery (Hasselbalch et al., 2011). Ameliorating deficits in cognitive functions may also be an important part of treatment of depression and other CNS disorders (Clark et al., 2009; Marazziti et al., 2010). Studies combining neuropsychological testing, psychiatric examination, and neuroimaging showed that patients with depressive disorders suffer from widespread cognitive dysfunction (Ravnkilde et al., 2002; Bhardwaj et al., 2010). One of the current cognitive theories of depression suggests that depression may present as an increased elaboration of negative information, difficulties in disengaging from negative

Abbreviations: 5-HT, Serotonin; 5-HTT, 5-HT transporter; ACh, Acetylcholine; HA, Histamine; ANOVA, Analysis of Variance; ASRI, Allosteric serotonin reuptake inhibitor; CS, Conditioned stimulus; MDD, Major depressive disorder; PCP, Phencyclidine; s.c., Subcutaneous injection; SSRI, Selective 5-HT uptake inhibitor; US, Unconditioned stimulus.

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thoughts, and deficits in cognitive control when processing negative information (Gotlib and Joormann, 2010). Efforts directed at reducing cognitive dysfunctions in depression may have the potential to reduce disability (Naismith et al., 2007), and interventions targeting cognitive dysfunctions may be an important strategy for the treatment of depression.

Some potential pharmacological interventions to address cognitive dysfunctions in mental disorders have been reported (for a recent review, see (Wallace et al., 2011)). A large body of evidence indicates that the 5-HT system plays an important role in cognitive functions, such as learning and memory, as demonstrated through the activation or blockade of 5-HT receptor subtypes, such as the 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_6, and 5-HT_7 receptors (Buhot, 1997; Meneses and Hong, 1997; Roman and Marchetti, 1998; Buhot et al., 2000; Meneses, 2007; King et al., 2008). Among them, the 5-HT_3 and 5-HT_7 receptors are particularly interesting. Compelling preclinical data from studies in rodents suggest beneficial effects on memory of 5-HT_3 receptor antagonists such as ondansetron (Fontana et al., 1995; Akhondzadeh et al., 2009), itasetron (Pitsikas and Borsini, 1996), WAY-100579 (Hodges et al., 1996), and the 5-HT_7 receptor antagonist SB-269970 (Meneses, 2004; McLean et al., 2009; Horiguchi et al., 2011; Horisawa et al., 2011). However, none of these compounds has yet reached clinical practice for the treatment of cognitive deficits.

Vortioxetine a novel multimodal antidepressant (Adell, 2010; Alvarez et al., 2012) currently under development for the treatment of MDD, is a 5-HT_{3A} receptor antagonist ($K_i = 3.7$ nM), 5-HT_7 receptor antagonist ($K_i = 19$ nM), 5-HT_{1D} receptor antagonist ($K_i = 54$ nM), 5-HT_{1B} receptor partial agonist ($K_i = 33$ nM), 5-HT_{1A} receptor agonist ($K_i = 15$ nM) and 5-HTT inhibitor ($K_i = 1.6$ nM) as measured in recombinant cell lines expressing these human 5-HT receptors and 5-HT transporter (Bang-Andersen et al., 2011; Westrich et al., 2012) (see Table 1). Preclinical and clinical studies have demonstrated the antidepressant properties of vortioxetine (Adell, 2010; Mørk et al., 2012; Alvarez et al., 2012; Katona et al., 2012), and a recent clinical study in elderly depressed patients showed superiority to placebo in cognitive tests of speed processing, verbal learning and memory (Katona et al., 2012). Based on these clinical observations and the pharmacological profile of vortioxetine, we investigated its potential and related mechanisms to enhance memory in behavioral and microdialysis studies in rats.

Two memory tests were used, i.e., the rat contextual fear conditioning and novel object recognition tests. In the classical fear-conditioning paradigm, animals learn to associate a neutral environment (context, as a conditioned stimulus, CS) with an aversive experience, i.e., an electrical foot-shock as an unconditioned stimulus (US). Upon re-exposure to the CS, animals will demonstrate freezing behavior as a sign of fear-related memory (Fanselow, 1980). In order to rule out possible analgesic effects of vortioxetine in affecting the behavioral results in the memory tests, the pain threshold was assessed by a hotplate method, as previously described (Montezinho et al., 2010). The novel object recognition task is a memory test that takes advantage of a rodent's innate drive to spontaneously explore novelty in its environment, and involves several brain structures, including the hippocampus and the medial prefrontal cortex (Ennaceur and Delacour, 1988; Antunes and Biala, 2012).

The present study also examined extracellular levels of acetylcholine (ACh) and histamine (HA), two neurotransmitters involved in regulation of memory function, in freely moving rats following vortioxetine administration. We have previously shown that vortioxetine increases the level of extracellular 5-HT, as well as the levels of dopamine and norepinephrine in the rat prefrontal cortex and hippocampus (Bang-Andersen et al., 2011; Mørk et al., 2012). The receptor mechanisms mediating the effects of vortioxetine may also modulate the properties of cholinergic and histaminergic neurons via their role as presynaptic heteroreceptors or postsynaptic receptors, or indirectly through interneurons.

2. Materials and methods

2.1. Animals

Studies were carried out in male Sprague–Dawley rats from Charles River (250–300 g, 7–8 weeks). All animals were housed two per cage under a 12 h light/dark cycle in a temperature- (21 ± 2 °C) and humidity- (60 ± 10%) controlled environment. Food and water were available ad libitum. Rats were used 1 week after arrival. The fear conditioning and ACh microdialysis experiments were carried out at H Lundbeck A/S, Denmark and ethical permissions were granted by the animal welfare committee appointed by the Danish Ministry of Justice. All animal procedures for these studies were carried out in compliance with the EC directive 86/609/EEC and Danish law regulating experiments on animals. The HA microdialysis study was carried out at Brainsonline (The Netherlands) as described previously (Flik et al., 2011). The novel object recognition and hotplate studies were conducted at Lundbeck Research, USA and were approved by the Institutional Animal Care and Use Committee (IACUC) of Lundbeck Research USA, Inc.

2.2. Drugs

In the fear conditioning, hotplate, and microdialysis studies, vortioxetine (1-[2-(2,4-dimethylphenyl-sulfanyl)-phenyl]-piperazine) was dissolved in 10% aqueous hydroxypropyl-ß-cyclodextrin and injected subcutaneously (s.c.), in a volume of 2.5 ml/kg. In novel object recognition studies, vortioxetine was dissolved in 20% aqueous hydroxypropyl-ß-cyclodextrin and administered s.c. in a volume of 2.0 ml/kg, 60 min prior to training. All doses used are expressed as

Table 1
The in vitro pharmacological profile and in vivo target occupancies of vortioxetine. The in vitro activities of vortioxetine at different 5-HT targets and their corresponding in vivo occupancies in the rat and human are summarized based on previous reports as referenced.

Target	Type of activity	Binding Ki or potency IC_{50} (nM)		In vivo occupancy	
		Human	Rat	Human	Rat
5-HT_{3A}	Antagonist	3.7[a]	1.1[b]	ND	100% at 1 mg/kg; ED_{50} 0.004 mg/kg[c]
5-HT_7	Antagonist	19[a]	200[d]	ND	ND
5-HT_{1D}	Antagonist	54[e]	3.7[e]	ND	ND
5-HT_{1B}	Partial agonist	33[a]	16[d]	ND	~80% at 10 mg/kg; ED_{50} 3.2 mg/kg[d]
5-HT_{1A}	Agonist	15[a]	230[a]	ND	28%, 35%, 44% at 5, 10, 20 mg/kg, respectively[c]
5-HTT	Inhibitor	5.4 (IC_{50} on uptake)[a]	5.3 (IC_{50} on uptake)[a]	~50% at 5 mg[f]	>80% at 10 mg/kg; ED_{50} 0.4 mg/kg[d]

ND, not determined.
[a] Bang-Andersen et al. (2011).
[b] Sanchez et al. (2012).
[c] Pehrson et al. (2013).
[d] Mørk et al. (2012).
[e] Westrich et al. (2012).
[f] Areberg et al. (2012).

mg/kg of the base. Hydroxypropyl-ß-cyclodextrin was used as vehicle in all experiments.

2.3. Contextual fear conditioning

2.3.1. Apparatus

Training and testing were conducted in a soundproof chamber (30×20×40 cm) kept in an isolated room and connected to a ventilation system. Illumination was provided by an incandescent white light (60 W). The floor of the chamber consisted of a metal grid attached to an electric shock generator. Prior to training and testing, the chamber was cleaned with 70% ethanol. A video camera was used for behavioral observations and recording of the training session for off-line analysis. The white light and current were controlled by FCON win software (Ellegaard Systems, Denmark). Animal behavior was recorded by EthoVision3.0 (Noldus, Netherlands).

2.3.2. Behavioral measurement

Freezing was used to assess conditioned fear as described previously (Fendt and Fanselow, 1999). During acquisition, animals were allowed to freely explore the novel environment for a 1 min habituation period, which terminated with a US consisting of one inescapable foot-shock through the electrifiable grid floor. The foot-shock had a 2-s duration and an intensity of 0.75 mA when drugs were dosed before acquisition or during memory consolidation or 1.5 mA when drugs were dosed before the retention test in order to increase the freezing behavior of the animals. Animals remained in the conditioning chamber for another 60 s after the US. Freezing behavior was scored during the first 58 s (pre-US acquisition) by an experimenter blinded to treatment in order to establish baseline-freezing responses to the context. At the end of the acquisition, animals were removed and returned to their home cages. To test fear conditioning associated with context, each rat was placed back in the chamber as a contextual conditioned stimulus (CS) for 2 min with no foot-shocks applied, 24 h after acquisition. Freezing behavior was manually scored during the whole test period and presented as percent of the total test period, again with the experimenter blinded to treatment group.

To assess the effects of vortioxetine on acquisition of fear conditioning, vehicle or vortioxetine (1, 5 or 10 mg/kg) was administered 60 min before acquisition (CS–US). The animals were tested (CS) 24 h later. To test the effect of vortioxetine on the consolidation of contextual fear memory, vehicle or vortioxetine (5 or 10 mg/kg) were given immediately after acquisition (CS–US). The animals were tested (CS) 24 h later. To investigate the effects of vortioxetine on retention (memory recall) or expression of fear conditioning, vehicle or vortioxetine (5 or 10 mg/kg) was given to the animals 24 h after acquisition (CS–US), which was 60 min before the testing of contextual conditioning (CS). These different time points for vortioxetine administration with respect to fear acquisition and contextual freezing test are shown in Fig. 1.

2.4. Novel object recognition

2.4.1. Apparatus

The novel object recognition task was adapted from previous studies (Ennaceur and Delacour, 1988). The experimental apparatus consisted of a rectangular opaque plastic chamber (61 cm×42 cm×37 cm) with bedding on the floor. The objects were either a goblet (11 cm in height, 6.5 cm in diameter) or a conical candlestick holder (11 cm in height, 6 cm in diameter) both made of green, nontransparent glass. Multiple copies of each object were used for training and testing and object pairs were randomly assigned between and within conditions. During each day of experimentation, as described below, the rats were transported in their home cages from the vivarium to a dimly lit (5–6 lx) testing room and acclimated to the room for approximately 45 min before being tested. In addition, after testing each rat, the objects and chamber walls were thoroughly cleaned with a Clidox solution to remove any residual olfactory cues.

2.4.2. Behavioral measurement

On day 1, each rat was individually placed in the empty training–testing chamber for 5 min of free exploration. Before being transported back to the vivarium, the rat was returned to its home cage and remained in the testing room until all rats were habituated. On day 2, training for each rat began in the empty chamber, positioned with nose centered to a long wall. After 1 min of re-habituation, the rat was removed from the chamber and placed in a holding cage for approximately 10 s while two identical training objects were positioned 8 cm from the center of the short walls. The rat was returned to the chamber for 15 min of exploration. Movements were observed via a camera located above the chamber and recorded. Before being transported back to the vivarium, the rat was returned to his home cage and remained in the testing room until all rats were trained.

Testing of each rat began in the empty chamber 24 h later. The test procedure was similar to the training procedure, except that one object was identical to the training object, while the other was novel. The position of the novel object was randomly assigned (left or right) and balanced for each rat and treatment group. The rat was allowed to actively explore for 15 min or until 60 s of total object exploration was accumulated. Object exploration was only scored if the rat's nose was within 1 cm of the object and its vibrissae were moving. Failure to explore both objects resulted in exclusion of the data of this animal from the study.

Rats were randomly assigned to one of 5 treatment groups (vehicle or vortioxetine, 2.5, 5 or 10 mg/kg or the positive control donepezil 1.0 mg/kg) and dosed 60 min prior to novel object recognition training. The acetylcholinesterase inhibitor donepezil was used since it has been often used as a positive control compound in different memory tests done in animals in our previous studies. During the test, exploration times of the familiar and novel objects were recorded and the mean time spent exploring each object was calculated. In addition, percent novel exploration, expressed as the percentage of time spent exploring

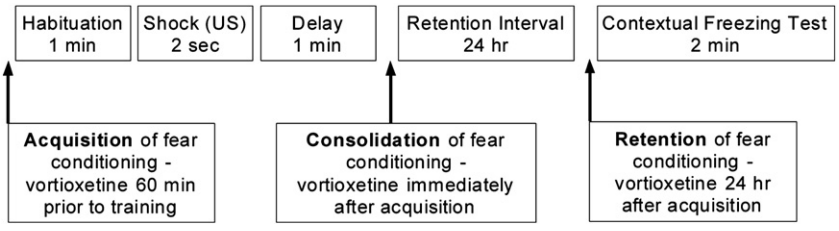

Fig. 1. Diagram showing the different time points for vortioxetine administration with respect to fear acquisition and contextual freezing test. Vortioxetine was administered 60 min before acquisition, immediately after acquisition, or 24 h after acquisition but 60 min before the contextual freezing test, in order to assess possible effects on acquisition of fear conditioning and the consolidation or retention of contextual memory, respectively.

the novel object in relation to the total time spent exploring both objects [100∗(novel time)/(novel time + familiar time)] was analyzed.

2.5. Hotplate test

The hotplate apparatus (IITC Life Science, USA) was equipped with a built-in heater and a square Plexiglas enclosure to contain the animals. Vortioxetine (2.5, 5 or 10 mg/kg) or vehicle was administered s.c., 30 min prior to testing. The animals were placed on the hotplate at 50 °C. The latency to a discomfort reaction was scored manually with a stopwatch by an experimenter blind to the drug condition of the subjects. The response endpoint was defined as either withdrawal or licking of the forepaw or hind paw. Test time was limited to 120 s to minimize the discomfort of the animal.

2.6. Microdialysis

The effect of vortioxetine (2.5, 5 or 10 mg/kg) on extracellular levels of ACh and HA in the medial prefrontal cortex was determined by microdialysis in freely moving rats. The surgery and microdialysis procedures were performed as previously reported (Flik et al., 2011; Mørk et al., 2012). When studying effects on extracellular levels of ACh, the microdialysis perfusion solution contained 0.5 μM neostigmine (Sigma-Aldrich, USA) to increase the sensitivity of the method and to increase the basal ACh levels to detectable levels. The measured content of neurotransmitters in the dialysates was not been corrected for in vitro recovery of the microdialysis probes.

2.6.1. Analysis of acetylcholine in dialysate

Concentrations of ACh in the dialysates were analyzed by means of HPLC with electrochemical detection using a mobile phase consisting of 100 mM disodium hydrogen phosphate, 2.0 mM octane sulfonic acid, 0.5 mM tetramethyl-ammonium chloride and 0.005% MB (Environmental Sciences Associates, ESA), pH 8.0. A pre-column enzyme reactor (ESA), containing immobilized choline oxidase, removed choline from the injected sample (10 μl) prior to separation of ACh on the analytical column (ESA ACH-250); flow rate 0.35 ml/min, temperature 35 °C. After the analytical column the sample passed through a post-column solid phase reactor (ESA) containing immobilized acetyl cholinesterase and choline oxidase. The latter reactor converted ACh to choline and subsequently choline to betaine and H_2O_2. The latter was detected electrochemically using a platinum electrode (Analytical cell, ESA, model 5040).

2.6.2. Analysis of histamine in dialysate

Concentrations of HA were determined by HPLC combined with tandem mass spectrometry (MS/MS) detection using an internal standard. Samples were mixed with the internal standard solution, and were derivatized with SymDAQ (symmetrical dialdehyde quaternary ions). After a reaction time of 2 min 50 μl of the mixture was injected into the LC system by an automated sample injector (SIL-10 AD vp, Shimadzu, Japan). Chromatographic separation was performed on a reverse phase Hypersil 50×2.1 mm (1.9 μm particle size) column held at a temperature of 35 °C. Components were separated using a linear gradient of acetonitrile/0.1% formic acid in 0.1% formic acid (flow rate 0.2 ml/min). MS analyses were performed using an API 4000 MS/MS system consisting of an API 4000 MS/MS detector and a Turbo Ion Spray interface (both from Applied Biosystems, The Netherlands). Data were calibrated and quantified using the Analyst™ data system (Applied Biosystems, version 1.4.2). The mean value of 3 to 4 consecutive dialysis samples immediately preceding compound administration served as the basal level for each experiment and data were converted to percentage of basal (mean basal pre-injection values normalized to 100%).

2.7. Data and statistical analyses

Data were analyzed with GraphPad Prism 4 (GraphPad, San Diego, CA). In contextual fear conditioning and hot-plate tests, statistical analyses were done using one-way Analysis of Variance (ANOVA), followed by Dunnett's post hoc test comparing all dose groups versus the control group. In the novel object recognition test (for raw seconds of novel versus familiar object exploration) and microdialysis studies, statistical analyses were conducted using two-way repeated measures ANOVA, followed by Bonferroni's or Student–Newman–Keuls post-hoc test. For the percent novel exploration analysis in the novel object recognition test, one-way ANOVA was used followed by Dunnett's Multiple Comparisons Test.

3. Results

3.1. Contextual fear conditioning

All groups displayed increased freezing rates compared to baseline (before acquisition) when exposed to the conditioning chamber 24 h after acquisition (Fig. 2a), indicating successful retention of the aversive memory towards the CS. Animals treated with vortioxetine 60 min before acquisition significantly increased freezing [$F(3, 34) = 4.3$, $P<0.05$, one-way ANOVA], suggesting enhanced contextual memory formation during acquisition and/or consolidation. Post hoc tests indicated a significant increase in freezing at 10 mg/kg compared to the vehicle control group ($P<0.01$). This effect appeared to be dose dependent in that 1 mg/kg did not have any effect, whereas 5 mg/kg produced a non-significant increase. Administration of vortioxetine 60 min before acquisition did not affect freezing at baseline, i.e., prior to the exposure to the foot shock during acquisition (Fig. 2a). This indicates that the drug had no nonspecific effect on freezing behavior.

To isolate the effect of vortioxetine on the consolidation phase, we administered the drug immediately after acquisition. Here, interestingly, vortioxetine treatment also caused increased freezing rates during retention [$F(2, 43) = 3.5$, $P<0.05$, one-way ANOVA], an effect that reached statistical significance by post hoc tests at the 5 mg/kg dose compared to vehicle ($P<0.05$) (Fig. 2b). At 10 mg/kg dose, there was a trend towards an increase that did not reach statistical significance.

Finally, when vortioxetine was administered 60 min before the retention test to assess its possible effect on the expression of fear conditioning, there was no significant effect on freezing [$F(2, 34) = 1.4$, $P = 0.25$, one-way ANOVA] (Fig. 2c).

3.2. Novel object recognition

To test vortioxetine in an episodic-like memory test that does not rely on aversive memory formation, we applied the novel object recognition task. We used a 24-h interval between acquisition and retention trials, which has been shown to be sufficient for the natural forgetting of familiar objects (Ennaceur and Delacour, 1988). Indeed, vehicle-treated animals explored the objects to a similar degree (Fig. 3a), and the performance of both groups was that expected by chance (Fig. 3b). Rats treated with 2.5 or 5 mg/kg vortioxetine prior to acquisition showed average exploration times of 29 and 33 s for the novel object, respectively (Fig. 3a). These correspond to 51% and 52% of the total exploration time (Fig. 3b), which were similar to the times for the vehicle group. Two-way repeated measures ANOVA indicated that there were a significant discrimination between novel versus familiar objects [$F(1, 35) = 14.9$, $P<0.001$], and a significant treatment × object interaction [$F(4, 35) = 3.5$, $P<0.05$], though the dose effect was not significant [$F(4, 35) = 1.1$, NS] (Fig. 3a). Post hoc analyses showed that pretreatment with vortioxetine at 10 mg/kg significantly enhanced novel versus familiar object recognition time to 49 s as compared to the 28 s for vehicle ($P<0.01$, Fig. 3a). In addition, no significant difference was observed between 5 and 10 mg/kg doses of vortioxetine on time spent with the

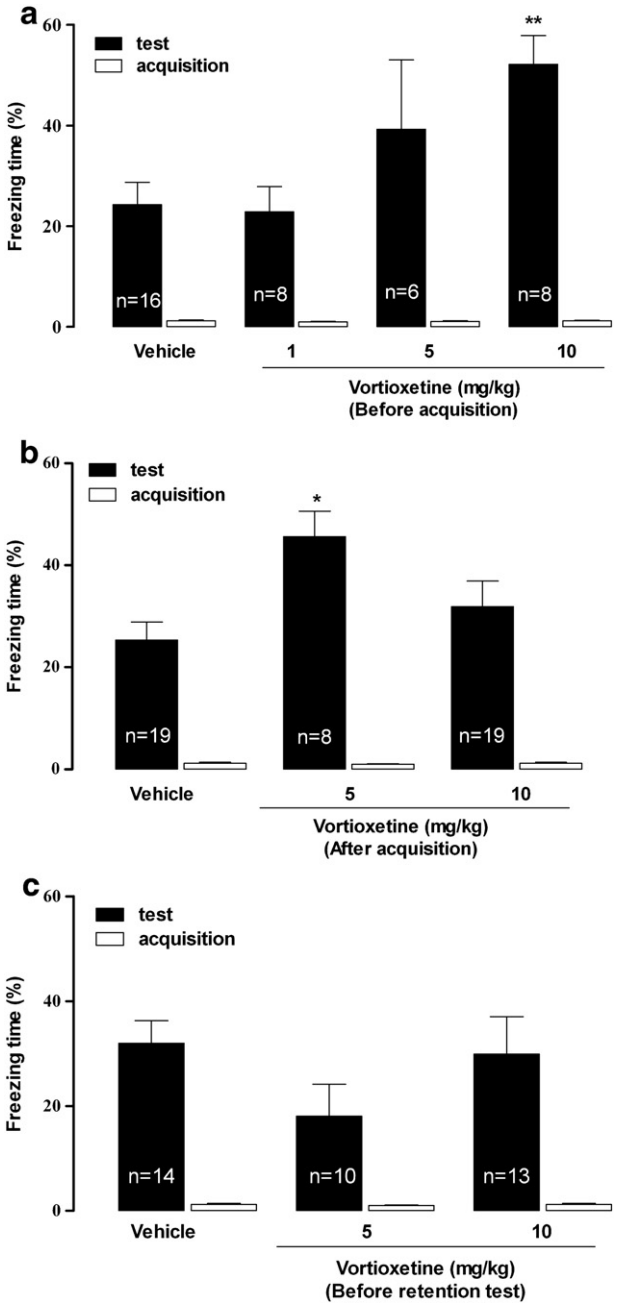

Fig. 2. The effects of vortioxetine on the time spent freezing during the retention test in a contextual fearing conditioning model. Freezing behavior was used to assess contextually conditioned fear in male Sprague–Dawley rats as described in Materials and methods. During acquisition, animals were placed in a novel environmental chamber for 1 min, which co-terminated with an unconditioned stimulus (US) consisting of one inescapable foot-shock. 24 h after acquisition, the rat was placed back in the chamber for 2 min with no foot-shock applied, during which freezing behavior was scored. The average percentage of time spent freezing for each treatment group during the 58-s habituation period prior to the foot shock US (pre-shock acquisition) (*white bars*) and when measured 24 h after acquisition (retention test) (*solid bars*) are shown. Vortioxetine was administered 60 min before acquisition (a), immediately after the acquisition (b), or 60 min prior to the retention test (c). Results are expressed as mean ± SEM (n = number of animals per group). Data were analyzed by one way ANOVA, followed by Dunnett's post hoc test. $^{*}P<0.05$, $^{**}P<0.01$ versus vehicle control.

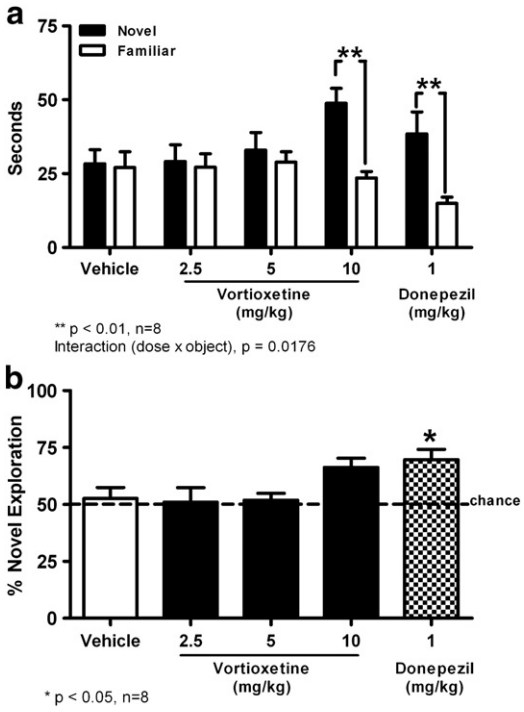

Fig. 3. Vortioxetine increased the time rats spent investigating the novel object in the rat 24-h novel object recognition test. The habituation, training and testing procedures for novel object recognition test are described in Materials and methods. After a 24-h delay post training with 2 identical familiar objects, the rats were allowed to actively explore a familiar object together with a novel object for 15 min or until 60 s of total object exploration was accumulated. Eight rats were randomly assigned to one of 5 treatment groups (vehicle or vortioxetine, 2.5, 5 or 10 mg/kg or positive control donepezil 1.0 mg/kg) and dosed 60 min prior to novel object recognition training. During the test, exploration times for both the familiar and novel objects were recorded and mean time spent exploring each object was derived (a). In addition, the percent novel exploration, expressed as the percentage of time spent exploring the novel object in relation to the total time spent exploring both objects, was calculated (b). Results are expressed as mean ± SEM (n = 8 animals per group). Data were analyzed using a two-way repeated measures ANOVA, followed by Bonferroni's test (for raw seconds of novel versus familiar exploration, interaction between dose and object), and a one-way ANOVA, followed by Dunnett's multiple comparisons test versus vehicle control (percent novel exploration). $^{*}P<0.05$, $^{**}P<0.01$, n = 8 per group.

familiar object. When these effects expressed as percent exploration were compared a significant treatment effect [$F(4, 35) = 3.7$, $P<0.05$, one way ANOVA] was observed. The numerical increase for vortioxetine compared to vehicle approached but did not reach statistical significance (66% ± 4% of total exploration time) (Fig. 3b). As a positive control, donepezil (1 mg/kg) also significantly ($P<0.01$) increased recognition memory, with a mean novel object exploration time of 38 s (Fig. 3a) or 70% of total exploration time (Fig. 3b) as compared to vehicle (28 s, or 50% of total exploration time).

During novel object recognition training, rats were allowed to explore two identical objects. During 5, 10 or 15 min periods, the accumulative exploration times were 33–36 s, 55–68 s, and 73–93 s, respectively (data not shown). There was no significant difference between treatment groups [$F(4, 10) = 0.1$, NS].

3.3. Hotplate test

The hotplate test was conducted to rule out the possibility that the effects of vortioxetine on contextual freezing might be confounded by a potential effect on pain sensitivity. The effect of vortioxetine on pain perception as measured by the hotplate test is shown in Fig. 4. Vortioxetine significantly reduced nociception, assessed as increased paw withdrawal latency as compared to vehicle injected subjects by one-way ANOVA [$F(3, 28) = 3.0$, $P<0.05$]. Post hoc tests showed a significant increase in paw withdrawal latency for vortioxetine at 10 mg/kg versus vehicle-treated subjects ($P<0.01$), while no significant effect was seen at 2.5 or 5 mg/kg.

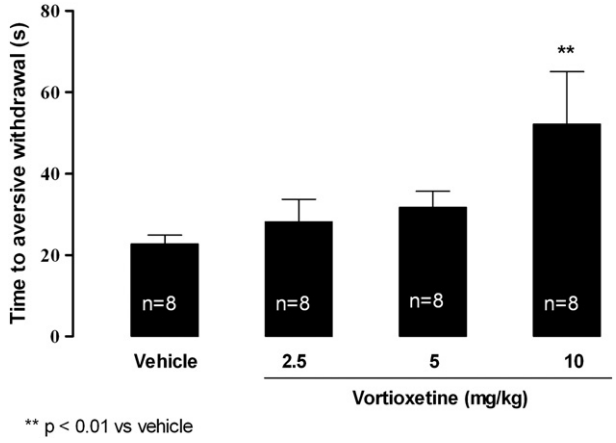

Fig. 4. Effect of vortioxetine on pain perception measured by hotplate test. The latency to the first sign of withdrawal behavior (time of aversive withdrawal) was determined. Data were analyzed using one-way ANOVA followed by Dunnett's post hoc test comparing all treatments, and are shown as mean time (s) ± SEM with the number (n) of animals per group. $^{**}P<0.01$.

3.4. Microdialysis

The effects of vehicle and vortioxetine on extracellular levels of ACh and HA in the medial prefrontal cortex were expressed as the percentage of their baseline concentrations in the dialysates and are shown in Fig. 5.

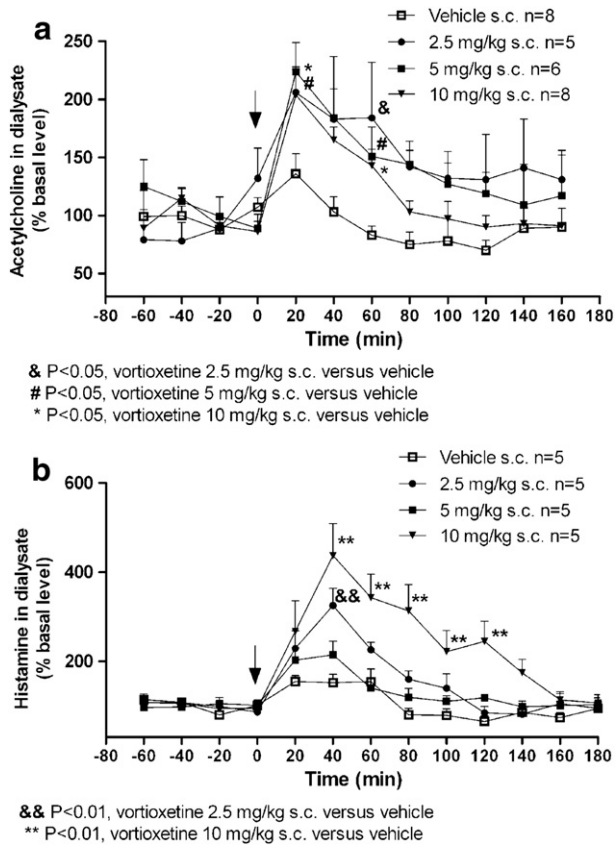

Fig. 5. Vortioxetine increased acetylcholine and histamine levels in the medial prefrontal cortex of freely-moving rats. Rats were anesthetized and intracerebral guide cannulas/microdialysis probes were implanted, followed by a two-day recovery period. For each animal, the average of the 3–4 consecutive samples collected immediately preceding compound administration was used as the basal level and was set to 100%. The percentage in relation to baseline values for acetylcholine (a) and histamine (b) were calculated for each time point. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way repeated measures ANOVA with Bonferroni post-hoc test comparing all groups versus vehicle control. * or # $P<0.05$; ** or ## $P<0.01$.

For ACh, two-way repeated measures ANOVA indicated significant effects by vortioxetine treatment [$F(3, 288) = 5.5$, $P<0.05$], and by time [$F(11, 288) = 8.0$, $P<0.001$], but no treatment × time interaction was found [$F(33, 288) = 0.7$, NS] (Fig. 5a). ACh levels of the vehicle group at 20, 40, 60 and 80 min were 146%, 103%, 83%, and 75% of baseline, respectively, with the early peak likely due to the fact that ACh in the prefrontal cortex is sensitive to injection stress. Post hoc (Student–Newman–Keuls) analyses indicated that vortioxetine at 5 and 10 mg/kg increased the levels of ACh to 224% and 204% of baseline 20 min after injection, which differed significantly from the vehicle group ($P<0.05$). An enhancing effect of vortioxetine was also seen at all three doses (2.5, 5 and 10 mg/kg) 60 min after administration, with respective ACh levels of 184%, 151%, and 143% of baseline ($P<0.05$ for all doses).

For HA, two-way repeated measures ANOVA also showed a significant effect by vortioxetine treatment [$F(3, 199) = 32.3$, $P<0.001$] (Fig. 5b). In addition, there was a significant effect by time [$F(12, 199) = 19.7$, $P<0.001$], as well as a significant treatment × time interaction [$F(36, 199) = 3.0$, $P<0.001$]. Post hoc tests indicated that the group treated with 2.5 mg/kg vortioxetine showed significantly higher HA levels in the medial prefrontal cortex relative to baseline (325%) compared to vehicle (152%) at 40 min ($P<0.01$). More pronounced effects of vortioxetine were seen at 10 mg/kg, since HA levels were significantly higher than those in the vehicle control group at more time points: 40, 60, 80, 100 and 120 min ($P<0.01$ for all indicated time points) (Fig. 5b). At 5 mg/kg, vortioxetine did not produce a significant effect on HA levels compared to vehicle-treated animals. However, it was observed that animals in this group had higher absolute baseline HA levels compared to other groups (respective HA levels: 82 ± 5, 57 ± 9, 107 ± 23, 65 ± 18 fmol/sample, for vehicle, 2.5 mg/kg, 5 mg/kg and 10 mg/kg). Furthermore, a one-way repeated measures ANOVA on pre-injection and post-injection levels of HA demonstrated that vortioxetine at 5 mg/kg significantly increased the HA levels [$F(9, 36) = 8.0$, $P<0.0001$].

4. Discussion

The results of the present study demonstrated that vortioxetine improves contextual fear memory in rats when administered before or immediately after acquisition. Vortioxetine increased the conditioned freezing time without altering baseline freezing behavior and without increasing pain sensitivity of the animals. Vortioxetine also increased episodic memory in the rat. The present work also indicates that the effects of vortioxetine on memory function may partly involve increases in extracellular ACh and HA levels in the medial prefrontal cortex.

Clinical doses of vortioxetine (5–20 mg daily) achieve 50–90% 5-HTT occupancies (Areberg et al., 2012). As summarized in Table 1, when administered in the rat at 1–10 mg/kg doses, vortioxetine would also generate this range of 5-HTT occupancies, as well as meaningful occupancies at the 5-HT_3, 5-HT_7, 5-HT_{1B}, and 5-HT_{1A} receptors as further discussed below.

Vortioxetine increased contextual freezing when administered before acquisition, indicating enhanced fear-conditioned memory. It is interesting that vortioxetine also enhanced memory consolidation, since vortioxetine augmented contextual freezing when administered immediately after acquisition. Vortioxetine enhanced conditioned-fear memory when administered before acquisition at the 10 mg/kg dose, while a trend towards increase was observed at 5 mg/kg. With regard to memory consolidation, an enhancement was seen for vortioxetine at the 5 mg/kg dose only. It is not unusual that the dose–response effects for acquisition and consolidation are different (Colon-Cesario et al., 2006; Hubbard et al., 2007; Montezinho et al., 2010). As discussed in the previous report (Montezinho et al., 2010), exposure to novel environments induced increases in hippocampal extracellular 5-HT levels (Kobayashi et al., 2008). Moreover, an electric foot-shock significantly enhanced extracellular efflux of 5-HT in the rat hippocampus, as measured by in vivo microdialysis (Hajos-Korcsok et al., 2003). Thus,

vortioxetine may exert its effect under different homeostatic conditions, explaining that different doses are required to obtain significant changes in fear conditioning.

Several lines of evidence indicate that the effects of vortioxetine in the contextual freezing test are due to improved memory rather than non-specific effects on anxiety-like states or pain sensitivity. First, only the highest dose of vortioxetine had an effect in the hotplate test of nociception; thus drug-induced increases in shock sensitivity would not adequately explain the mnemonic effects of vortioxetine at doses lower than 10 mg/kg (Fig. 2b). Second, the effect of vortioxetine on pain sensitivity should not confound any positive mnemonic effects in the fear conditioning experiments. Previously we have used hotplate test to rule out the influence of pain sensitivity on the acquisition of the association between the conditioning fear stimulus and the context (Montezinho et al., 2010). An increase in pain sensitivity might confound fear conditioning results, although focused studies suggest that different fear responses do not seem to be directly determined by pain perception (Lehner et al., 2010). An analgesic effect of vortioxetine (10 mg/kg) would mean a reduction in shock sensitivity or lowering the aversive hedonic value of the shock in the fear conditioning experiments, and thus would only serve to reduce conditioned fear performance rather than enhance it. Furthermore, the positive effects of vortioxetine on acquisition and consolidation of contextual fear memory are unlikely to be due an anxiogenic cause. In fact, vortioxetine does have an anxiolytic effect in the social interaction and conditioned fear-induced ultrasonic vocalization tests (Mørk et al., 2012). In addition, vortioxetine did not seem to be able to increase the expression of contextual fear when it was administered 60 min before the retention test as shown in Fig. 2c. Finally, vortioxetine also showed an effect in the novel object recognition test, which does not utilize aversive or anxiogenic stimuli.

The neuro-circuitries of both contextual fear conditioning and novel object recognition in relation to learning and memory involve the prefrontal cortex and other brain regions including the amygdala and hippocampus (Bird and Burgess, 2008; Aggleton et al., 2011; Antunes and Biala, 2012). The enhanced memory elicited by vortioxetine may be mediated by modulations in neurotransmitters levels in these brain regions as a consequence of its action at the various serotonergic targets. At the neurochemical level, the extracellular outputs of 5-HT, dopamine and norepinephrine in the medial prefrontal cortex and the ventral hippocampus were increased by vortioxetine, as reported previously (Bang-Andersen et al., 2011; Mørk et al., 2012). The importance of 5-HT in the brain, especially the prefrontal cortex, with regard to cognition has been demonstrated in 5-HT depletion and augmentation studies in animals (Clarke et al., 2004, 2007; Khaliq et al., 2006). In otherwise healthy human subjects, depletion of the 5-HT precursor, tryptophan, also negatively impacts cognitive function (Riedel et al., 2002; Sambeth et al., 2007; Mendelsohn et al., 2009).

In the present study, vortioxetine also increased extracellular ACh and HA levels in the medial prefrontal cortex. ACh plays a central role in memory and learning, and the memory-enhancing effects of ACh elevations have been consistently demonstrated with acetylcholinesterase inhibitors such as donepezil and physostigmine (Degroot and Parent, 2000; Pepeu and Giovannini, 2009; Savage, 2012). It has been demonstrated that stimulation of 5-HT_{1A} receptors, located postsynaptically, increases hippocampal and cortical efflux of ACh in the rat (Izumi et al., 1994; Consolo et al., 1996). Moreover, 5-HT_3 receptors, located on GABAergic interneurons in the forebrain, maintain an inhibitory basal tone on the release of ACh and norepinephrine (Matsumoto et al., 1995; Yan, 2002). Thus, a reduced inhibitory tone via blockade of 5-HT_3 receptors may contribute to the increased extracellular levels of neurotransmitters seen in the present and previous studies (Bang-Andersen et al., 2011; Mørk et al., 2012). Central HA is important for attention and vigilance, as well as for short-term memory and long-term memory (Kay, 2000; Brioni et al., 2011; Kohler et al., 2011; Miwa et al., 2011). Accordingly, performance in the novel object recognition test was impaired in H_1 receptor knockout and H_2 receptor knockout mice compared to their respective wild-type mice (Dai et al., 2007), and both spatial and episodic memory were impaired in HA decarboxylase knockout mice (Dere et al., 2003; Acevedo et al., 2006). Various histamine H_3 receptor antagonists, which increase extracellular levels of many neurotransmitters, including HA, improved cognitive performance assessed in multiple animal behavioral models (Hancock, 2006; Wijtmans et al., 2007; Bonaventure et al., 2007; Brioni et al., 2011).

The activities of vortioxetine on 5-HT receptors and on 5-HTT have been shown to increases extracellular 5-HT levels to meaningful levels for antidepressant activity in the clinic (Mørk et al., 2012; Areberg et al., 2012). The activities of vortioxetine at the 5-HT_3, 5-HT_7, 5-HT_{1A} and 5-HT_{1B} receptors as potential mechanisms mediating the memory-enhancing effects are discussed below.

Overall, the serotonergic system plays a significant role in learning and memory, in particular by interacting with the cholinergic, glutamatergic, dopaminergic or GABAergic systems (Buhot et al., 2000; Meneses, 2004; Ogren et al., 2008). Interestingly, benzimidazole–arylpiperazine derivatives with mixed 5-HT_{1A} receptor partial agonism and 5-HT_3 receptor antagonism have been shown to exert effects in a passive avoidance learning test (Lopez-Rodriguez et al., 2004). This supports the memory enhancing effects of vortioxetine in the acquisition and consolidation of contextual fear conditioning and novel object recognition tests. Vortioxetine is a potent 5-HT_3 receptor antagonist ($K_i = 3.7$ nM). Blockade of 5-HT_3 receptors may contribute to cognition directly and indirectly as the result of downstream effects on the cholinergic system. Previous data have suggested the beneficial effects of 5-HT_3 receptor antagonists on cognition and memory, though such compounds have not yet reached clinical practice in this respect (Brambilla et al., 1993; Fontana et al., 1995; Pitsikas and Borsini, 1996; Roychoudhury and Kulkarni, 1997; Arnsten et al., 1997). Preclinical evidence of cognition- and memory-enhancing effects by 5-HT_3 receptor antagonism includes itasetron in a multiple choice avoidance behavioral task (Pitsikas and Borsini, 1996). In scopolamine-treated mice, ondansetron attenuated the performance deficits (Roychoudhury and Kulkarni, 1997), suggesting the role of 5-HT_3 receptors in the normalization of the cholinergic system disrupted by scopolamine. Furthermore, blockade of 5-HT_3 receptors may also enhance glutamate transmission since these receptors can suppress both the spontaneous firing and N-methyl-D-aspartic acid-evoked responses of the pyramidal neurons in the rat medial prefrontal cortex (Ashby et al., 1991; Liang et al., 1998). As previously mentioned, the 5-HT_3 receptor seems to exert an inhibitory tone on the ACh efflux in the brain. In the present study, the effect of vortioxetine on the extracellular levels of ACh did not increase further by raising the dose from 2.5 to 10 mg/kg. If the 5-HT_3 receptor is involved in this effect the maximal effect at 2.5 mg/kg may be explained by the fact that vortioxetine occupies the 5-HT_3 receptor by 100% after administration of only 1 mg/kg (Table 1).

Vortioxetine is a 5-HT_7 receptor antagonist ($K_i = 19$ nM). Memory enhancing effects of 5-HT_7 antagonists have been shown in preclinical models (Meneses, 2004; McLean et al., 2009; Horiguchi et al., 2011; Horisawa et al., 2011). In a Pavlovian/instrumental autoshaping learning task, the 5-HT_7 receptor antagonists SB-269970 and DR 4004 reversed scopolamine- or dizocilpine-induced amnesia (Meneses, 2004). The 5-HT_7 receptor antagonist SB-269970 also significantly attenuated sub-chronic phencyclidine (PCP)-induced reversal learning deficits in female rats (McLean et al., 2009), and similarly, SB-269970 dose-dependently reversed PCP-induced deficits in a novel object recognition test (Horiguchi et al., 2011). An enhancement of glutamate transmission may play a role in the effects of 5-HT_7 receptor antagonism, since 5-HT_7 receptor activation can decrease the glutamate release of glutamatergic cortico-raphe neurons directly or via GABAergic neurons in the dorsal raphe (Harsing, 2006; Duncan and Congleton, 2010). Another recent report showed that SB-269970 improved recognition memory with a 24 h delay in the novel object recognition test as used in the present study (Waters et al., 2012), further supporting the

assumption that 5-HT_7 antagonism may be one of the key mechanisms for the memory-enhancing effect of vortioxetine. Interestingly, SB-269970 at an inactive dose augmented the cognition-enhancing efficacy of escitalopram in a rat prefrontal cortex-dependent attentional set-shifting task (Nikiforuk, 2012), indicating a synergistic effect between 5-HT_7 antagonism and 5-HTT inhibition, both of which are part of the target profile of vortioxetine. It should be noted that vortioxetine has a 10-fold lower in vitro affinity for rat 5-HT_7 (K_i = 200 nM) compared with human 5-HT_7 receptors (K_i = 19 nM) (Mørk et al., 2012). Thus, the contribution of the 5-HT_7 receptor in the clinic may be underestimated when predicted from preclinical models.

The inhibitory 5-HT_{1A} receptor serves as both a somatodendritic autoreceptor and a postsynaptic heteroreceptor with important functions in learning and memory in brain regions such as the dorsal raphe, entorhinal cortex, hippocampus, and central amygdala (Chalmers and Watson, 1991; Polter and Li, 2010). Agonists of the 5-HT_{1A} receptor have a memory enhancing profile (Meneses and Hong, 1999; Meeter et al., 2006; Newman-Tancredi et al., 2009; Depoortere et al., 2010). For example, the activation of 5-HT_{1A} receptors by 8-OH-DPAT can reverse the learning deficit induced by scopolamine and dizocilpine in an autoshaping learning task (Meneses and Hong, 1999). Furthermore, similar to the affinity of vortioxetine for 5-HT_7 receptors, its in vitro affinity at the 5-HT_{1A} receptors (K_i = 230 nM) is ~15-fold lower than at human 5-HT_{1A} receptors (K_i = 15 nM) (Bang-Andersen et al., 2011), raising the possibility that activity at 5-HT_{1A} receptors may contribute to the clinical activity of vortioxetine to a higher degree than what is apparent from animal studies. However, after administration of the highest dose tested in this study (10 mg/kg) vortioxetine occupies the 5-HT_{1A} receptor by approximately 35% (Table 1). Thus, a 5-HT_{1A} receptor contribution of vortioxetine can be expected in preclinical studies at high doses.

Brain 5-HT_{1B} receptors function as inhibitory autoreceptors on serotonergic neurons and as heteroreceptors on neurons of other neurotransmitters such as ACh and glutamate (Pazos and Palacios, 1985; Olivier and Oorschot, 2005). In the dorsal subiculum, 5-HT_{1B} receptors are located on CA1 pyramidal axon terminals as inhibitory heteroreceptors (Ait et al., 1995), and activation of these receptors attenuates glutamate transmission in the hippocampus (Boeijinga and Boddeke, 1996; Mlinar et al., 2003). Administration of a selective 5-HT_{1B} receptor antagonist NAS-181 caused a dose-dependent increase in ACh levels in the frontal cortex and ventral hippocampus of freely moving rats (Hu et al., 2007). There is evidence that the 5-HT_{1B} receptor is involved in learning and memory (Meneses, 2001, 2007; Eriksson et al., 2008). For example, in an associative autoshaping learning task, a 5-HT_{1B} receptor inverse agonist (SB-224289) facilitated learning consolidation and was able to reverse the cognitive deficits induced by either the cholinergic inhibitor scopolamine or the glutamatergic antagonist dizocilpine (Meneses, 2001). In an aversive contextual learning task in mice, NAS-181 dose-dependently improved passive avoidance retention performance (Eriksson et al., 2008). Thus, the partial agonist activity of vortioxetine at the 5-HT_{1B} receptor (K_i = 33 nM) may also play a role in its memory-enhancing properties.

5. Conclusions

The present study demonstrated that vortioxetine enhances contextual and episodic memory in rat behavioral models and increases extracellular ACh and HA levels in the rat medial prefrontal cortex. The multimodal effect of vortioxetine through various 5-HT targets, may contribute to improving cognitive dysfunction in MDD as observed in a recent clinical study (Katona et al., 2012).

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