The rapid recovery of 5-HT cell firing induced by the antidepressant vortioxetine involves 5-HT$_3$ receptor antagonism

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Abstract

The therapeutic effect of current antidepressant drugs appears after several weeks of treatment and a significant number of patients do not respond to treatment. Here, we report the effects of the multi-modal antidepressant vortioxetine (Lu AA21004), a 5-HT$_3$ and 5-HT$_7$ receptor antagonist, 5-HT$_1$B receptor partial agonist, 5-HT$_1$A receptor agonist and 5-HT transporter (SERT) inhibitor, on rat 5-HT neurotransmission. Using in vivo electro-physiological recordings in the dorsal raphe nucleus of anaesthetized rats, we assessed the acute and subchronic effects of vortioxetine and/or the selective 5-HT$_3$ receptor agonist, SR57227 or the selective 5-HT$_1$A receptor agonist flesinoxan, on 5-HT neuronal firing activity. Using ex-vivo autoradiography, we correlated SERT occupancy and presumed 5-HT firing activity. The selective serotonin reuptake inhibitor, fluoxetine, was used as comparator. Importantly, the recovery of 5-HT neuronal firing was achieved after 1 d with vortioxetine and 14 d with fluoxetine. SR57227 delayed this recovery. In contrast, vortioxetine failed to alter the reducing action of 3 d treatment of flesinoxan. Acute dosing of vortioxetine inhibited neuronal firing activity more potently than fluoxetine. SR57227 prevented the suppressant effect of vortioxetine, but not of fluoxetine. In contrast, flesinoxan failed to modify the suppressant effect of vortioxetine acutely administered. Differently to fluoxetine, vortioxetine suppressed neuronal firing without saturating occupancy at the SERT. Vortioxetine produced a markedly faster recovery of 5-HT neuronal firing than fluoxetine. This is at least partly due to 5-HT$_3$ receptor antagonism of vortioxetine in association with its reduced SERT occupancy.

Received 30 March 2012; Reviewed 8 May 2012; Revised 13 August 2012; Accepted 15 August 2012; First published online 22 October 2012

Key words: Antidepressant, dorsal raphe nucleus, SERT occupancy, 5-HT$_3$ receptors, 5-HT$_1$A receptors.

Introduction

Depression is one of the most common psychiatric disorders. In the United States, the lifetime prevalence is > 12% in men and 20% in women (Belmaker & Agam, 2008). Despite many available pharmacotherapies, treatment of depression remains unsatisfactory (Berton & Nestler, 2006). Indeed, patients frequently receive several agents to obtain an antidepressant response and only 67% experience a therapeutic response according to STAR*D (Rush et al. 2009). Moreover, a therapeutic delay of weeks or months is often observed. This lag time can be critical, since it may be associated with an increased risk of suicide (Blier, 2003). Since most antidepressants, including selective serotonin reuptake inhibitors (SSRIs), have a direct effect on 5-HT neurotransmission, a key role of the 5-HT system has been suggested in the pathophysiology of depression (Hindmarch, 2002). When acutely administered, SSRIs initially elevate 5-HT levels in the rat forebrain, whereas they induce a strong decrease of dorsal raphe nucleus (DRN) 5-HT neuronal activity. This can be partly explained by a stimulation of inhibitory somatodendritic 5-HT$_1$A autoreceptors. During chronic treatment, these receptors are gradually desensitized, leading to the recovery of normal firing (Blier & de Montigny, 1999; Celada et al. 2004). The rate of this desensitization may be associated with the therapeutic delay (Blier, 2001).

Recently, new strategies have emerged to reduce therapeutic delay and improve therapeutic response. These strategies involve blockade or desensitization of inhibitory 5-HT$_1$A autoreceptors to overcome the initial suppression of the 5-HT neurotransmission produced by SSRIs, or enhancement of neurotransmitter systems in addition to the serotonergic. Examples of this are
vilazodone, a 5-HT transporter (SERT) inhibitor and 5-HT₁A receptor partial agonist that enhances 5-HT neurotransmission beyond SSRI levels (Hughes et al. 2005) and DOV 216 303, an inhibitor of 5-HT, noradrenaline (NA) and dopamine (DA) transporters (Skolnick et al. 2006). A screening programme identified vortioxetine (Lu AA21004) as a 5-HT₃ receptor antagonist (Kᵢ = 3.7 nm, IC₅₀ = 12 nm, h₅-HT₃A receptor), 5-HT₁A receptor agonist (Kᵢ = 15 nm, EC₅₀ = 200 nm, efficacy = 96%, h₅-HT₁A receptor) and SERT inhibitor (Kᵢ = 1.7 nm, IC₅₀ = 5.4 nm), it was also a partial agonist at 5-HT₁B receptors (Kᵢ = 33 nm; EC₅₀ = 120 nm, 55 % intrinsic activity, h₅-HT₁B receptor) and an antagonist at 5-HT₇ receptors (Kᵢ = 19 nm, IC₅₀ = 450 nm, h₅-HT₇ receptor) in recombinant cell systems (Bang-Andersen et al. 2011). This target profile resulted in both a disinhibition of 5-HT autoreceptors and activation of neurotransmitter systems beyond the serotonergic. Thus, microdialysis studies in free-moving rats reveal that vortioxetine increases extracellular 5-HT, NA and DA levels in brain regions relevant for depression (Mørk et al. 2011). Vortioxetine demonstrates antidepressant efficacy in clinical studies (Alvarez et al. 2011; Baldwin et al. 2011). This original profile of vortioxetine may be linked to its 5-HT₃ receptor antagonism. Indeed 5-HT₃ receptors may be an interesting target for depression (Bétry et al. 2011). 5-HT₃ receptors are the only ligand-gated ion channel of the 5-HT receptor family. They are present both in the peripheral and central nervous system and are localized in several areas involved in mood regulation (e.g. hippocampus or prefrontal cortex). Preclinically, they have been shown to be involved in regulation of various neurotransmitter systems of relevance for depression, including 5-HT, DA, NA and γ-aminobutyric acid (GABA; Alex & Pehek, 2007; Ashby et al. 1992; Bagdy et al. 1998; Chameau & van Hooft, 2006; Puig et al. 2004). Moreover, 5-HT₃ receptor antagonists present antidepressant-like properties in different behavioural tests (Martin et al. 1992; Ramamoorthy et al. 2008; Redrobe & Bourin, 1997).

In clinical studies, 5-HT₃ receptor antagonists have also presented antidepressant and anxiolytic properties (Harmer et al. 2006; Johnson et al. 2003; Lecrubier et al. 1993; McCann et al. 1997; Piche et al. 2005).

To investigate the actions of vortioxetine on serotonergic neurotransmission in further detail and the putative role of 5-HT₃ receptor antagonism, we have used in vivo electrophysiological and ex vivo autoradiography methods to compare the effects of vortioxetine and the SSRI, fluoxetine. More specifically, we have investigated:

(i) The acute and chronic effects of vortioxetine or fluoxetine on 5-HT neurotransmission in the DRN alone or after administration of the 5-HT₁A receptor agonist SR57227.

(ii) The acute and subacute effects of vortioxetine on 5-HT neurotransmission in the DRN alone or after administration of the 5-HT₁A receptor agonist flesinoxan.

(iii) The relationship between SERT occupancy and DRN serotonergic neuronal firing.

(iv) The acute effect of flesinoxan in controls and vortioxetine-treated rats for 3 d followed by 1 d wash-out.

(v) The action of a 5-HT depletion on the acute effect of vortioxetine on 5-HT neuronal firing.

Method

Animals

The experiments were carried out in male Sprague-Dawley rats (Harlan, France) weighing 250–300 g at the time of the experiments. All animals arrived at the animal facilities at least 1 wk prior to experiments to allow acclimatization. Animals were kept under standard laboratory conditions (12–12 h light–dark cycle, lights on 07:00 hours, with free access to food and water). Experiments were performed in compliance with the European Community Directive (86/609 ECC) for the care and use of laboratory animals and with the approval of the Regional Animal Care Committee (University Lyon 1).

Drugs

Vortioxetine and fluoxetine were provided by H Lundbeck A/S (Denmark). They were dissolved in 10% 2-hydroxypropyl-β-cyclodextrin (w/v). The selective 5-HT₁A receptor agonist flesinoxan, the selective 5-HT₁A receptor antagonist WAY-100 635 and the tryptophan hydroxylase-depleting drug, 4-chloro-DL-phenylalanine (PCPA) were purchased from Sigma Aldrich (France) and were dissolved in saline (0.9 %). The selective 5-HT₁A receptor agonist, SR57227 (1-(6-chloro-2-pyridinyl)-4-piperidinamine) was purchased from Tocris Bioscience (USA) and was dissolved in distilled water (Bachy et al. 1993).

Electrophysiological experiments

All animals were anaesthetized with chloral hydrate (400 mg/kg i.p.) and mounted in a stereotaxic apparatus. A lateral tail vein was cannulated with a 24-gauge catheter for the i.v. administration of drugs. Extracellular recordings were performed with single-barrelled glass micropipettes preloaded with fibreglass filaments. The tip was broken back to 2–4 μm and filled with a 0.5 M Na-Acetate solution saturated with Blue Chicago dye (El Mansari et al. 2007).

Altogether, 817 presumed dorsal raphe 5-HT neurons have been recorded and encountered over a distance of 1 mm starting immediately below the ventral border of the Sylvius aqueduct, according to the atlas of Paxinos & Watson (1998). These neurons were identified using the criteria of Aghajanian (1978): a slow (0.5–2.5 Hz) and regular firing rate and long-duration (0.8–1.2 ms) positive action potentials (Haddjeri & Blier, 1995). It is
effects were mediated via 5-HT

Acute administrations

Once a putative 5-HT neuron was identified, a baseline firing rate was established over 2–3 min. Drugs were then administered with a delay of about 80 s between injections. First, saline (0.9% NaCl) was administered. Successive administrations of vortioxetine (250 μg/kg i.v.) or fluoxetine (1 mg/kg i.v.) were performed in order to fully suppress the firing activity. Vortioxetine was administered at a maximum dose of 1500 μg/kg and fle sinoxan was then used to suppress firing, if necessary. In other experiments, prior administration of the 5-HT receptor agonist, SR57227 (1 or 5 μg/kg i.v.), was performed before successive administrations of vortioxetine or fluoxetine. Similarly, prior administration of flesinoxan (50 μg/kg i.v.) was performed before successive administrations of vortioxetine. We also evaluated prior administration of vortioxetine (250 μg/kg i.v.) before successive administrations of flesinoxan (100 μg/kg i.v.). At the end of the experiments, WAY-100,635 (50 μg/kg i.v.) was administered to confirm that the suppressant effect was mediated via 5-HT1A receptor activation. Finally, rats were treated for 3 d with PCPA (100 mg/kg i.p.) or saline for control rats and the acute electrophysiological experiments with vortioxetine were performed the following day.

Chronic treatments

Osmotic mini-pumps (Alzet, USA, purchased through Charles River, France) were placed s.c. on the back of the animal. Rats were treated with vortioxetine, 5 mg/kg.d, or fluoxetine, 10 mg/kg.d. There were six different treatment durations for vortioxetine (5 h, 10 h, 1, 3, 7 and 14 d) and three different treatment durations for fluoxetine (7, 14 and 21 d). Other groups of rats were treated with SR57227 (1 mg/kg.d) alone or in combination with vortioxetine for 3 d or with flesinoxan (2.5 mg/kg.d) alone or in combination with vortioxetine for 3 d. The electrophysiological recordings were performed at the end of the treatment with the mini-pump in place. To determine possible changes in the spontaneous firing activity of 5-HT neurons, 4–5 successive descents were performed along the dorsal raphe in each rat. Another group of rats was treated for 3 d with vortioxetine (5 mg/kg.d) followed by a 24-h wash-out period. The same protocol as for dose–response curve establishment was followed using the full 5-HT1A receptor agonist flesinoxan (100 μg/kg i.v.) (Haddjeri et al. 1999). Successive administrations were performed until the firing rate of the recorded neuron was suppressed. In the control group, the same electrophysiological protocol was performed.

Medial prefrontal cortex (mPFC) electrolytic lesion

A bipolar stimulating electrode (SNEX-100; Rhodes Medical Instruments, USA) was positioned stereotaxically in the mPFC (mm: 3.2 anterior and ±0.5 lateral to bregma; 5.5 ventral from the skull) in anaesthetized rats (Paxinos & Watson, 1998). Bilateral electrolytic lesions were made with constant direct current in the mPFC (0.5 mA for 10 s; Garcia et al. 2006) and the stimulating electrodes were removed at the end of the stimulation. The electrolytic lesions were made at the same time as the implantation of mini-pumps for the 3-d treatment.

Determination of SERT occupancy using ex vivo autoradiography

Rats were euthanized 20 min after the last i.v. administration of drug for acute treatment or at the end of subchronic treatment. The brain was dissected from the skull and frozen in isopentane at –40 °C for 5 min, then stored at –20 °C until needed. Brains were sectioned coronally for autoradiography using a cryostat and frozen sections were mounted on slides. Slices were set at 20 μm thickness and sectioning began at approximately +1.2 mm anterior from bregma. Slides were stored for at least 24 h at –20 °C before being used in autoradiography experiments.

Methods used for determination of SERT occupancy are discussed in detail elsewhere (Pehrson et al. 2012). Briefly, boxes containing slides were defrosted at room temperature under a constant stream of air for 30–45 min prior to use. SERT was labelled by incubating slides for 90 min at room temperature in buffer (50 mM Tris-HCl, 150 mM NaCl and 5 mK Cl, pH 7.4) containing the SERT-specific radioligand [3H]DASB at a concentration of 0.5 nM. We used 1 μM escitalopram as an in vitro competitor for [3H]DASB in order to define non-specific binding. Slides were washed three times in buffer at 4 °C for 5 min and then briefly dipped in distilled water and air-dried. The slides were transferred to a desiccator and dried for at least another 60 min. Finally, the slides were exposed at room temperature using a Beta imager (Biospace Lab, France) for 16 h prior to analysis.

Data analysis and statistics

To normalize data, the mean firing activity prior to and after drug administration was calculated and the result was expressed as a percentage of the basal value. Data are expressed as mean ± S.E.M. We used Sigma Plot Ink 8.0 to calculate the effective dose (ED)x. All other electrophysiological statistics were performed by using Statview software (version 5; SAS Institute Inc., USA). We used two-way repeated-measures analysis of variance (ANOVA) or Student’s t test for acute administrations analyses and one-way ANOVA for subchronic treatments analyses, followed by Dunnett’s or partial least significance test.
squares difference (PLSD) Fisher’s post hoc test when multiple comparisons were necessary.

For autoradiography experiments, surface radioactivity (expressed as cpm/mm²) was measured using Beta Vision + software version 2.0 (Biospace Lab, France) from a region of interest including the lateral septum, medial septum and olfactory tubercle, which was defined a priori on the basis of receptor mapping experiments conducted by this laboratory. Specific binding was determined by subtracting non-specific binding from total binding. Specific binding for each individual subject was normalized to the average specific-bound surface radioactivity in vehicle-treated subjects and expressed as a percentage of vehicle binding. These percentages were then subtracted from 100 to obtain the percentage of SERT occupied by fluoxetine or vortioxetine.

Results

Effect of sustained treatment with vortioxetine or fluoxetine on the firing activity of DRN 5-HT neurons

We evaluated the spontaneous firing activity of DRN 5-HT neurons after chronic treatment with vortioxetine or fluoxetine. Successive descents were performed along the dorsal raphe in each rat and all neurons on the track were recorded. The mean spontaneous firing activity of dorsal raphe 5-HT neurons in control rats was 1.08 ± 0.12 Hz (Fig. 1; n = 47 cells in six rats). Vortioxetine (5 mg/kg,d) administered s.c. via osmotic mini-pumps for 5 h, 10 h, 1 d, 3 d, 7 d and 14 d resulted in a significant overall effect on spontaneous firing activity of dorsal raphe 5-HT neurons (F₅,₃₈₉ = 4.079, p = 0.0063; one-way ANOVA followed by Dunnett’s test). In rats treated for 7 d (n = 48), there was a 55% decrease in the spontaneous firing activity of dorsal raphe 5-HT neurons (0.49 ± 0.10, p < 0.01 compared to control). No significant differences to control rats were observed after 14 and 21 d of treatments, i.e. 0.84 ± 0.11 (n = 54) and 0.88 ± 0.14 (n = 51), respectively (Fig. 1b).

Thus, recovery of firing of DRN 5-HT neurons was achieved after only 1 d treatment with vortioxetine, whereas 14 d treatment was necessary with fluoxetine.

Evaluation of the desensitization of DRN 5-HT₁₄ autoreceptors after 3 d treatment with vortioxetine

In order to evaluate the sensitivity of 5-HT₁₄ autoreceptors after a short chronic treatment of vortioxetine, we chronically treated rats for 3 d followed by 1 d wash-out without any treatment. We then acutely administered the 5-HT₁₄ receptor agonist flesinoxan to compare the dose–response curve in treated and control rats.

In control rats, acute i.v. administration of flesinoxan (0–300 μg/kg) induced a dose-dependent inhibition of 5-HT neuronal firing rate with ED₅₀ = 113 μg/kg (n = 5 rats; Fig. 2a). In rats treated with vortioxetine (5 mg/kg,d) for 3 d followed by 1 d wash-out, the ED₅₀ increased to 355 μg/kg (n = 6; Fig. 2b). Two-way repeated-measures ANOVA indicated a significant effect of 3-d pretreatment with vortioxetine (F₁,₉ = 14.42, p = 0.0042) on the potency of flesinoxan (F₆,₄₄ = 70.66, p < 0.0001) and a significant interaction (F₆,₄₄ = 8.00, p < 0.0001; Fig. 2c). Since the degree of inhibition induced by flesinoxan is used as a reliable index of the sensitivity of 5-HT₁₄ autoreceptors in...
Fisher’s test revealed a significant effect of treatment ($F_{3,26}=8.43$, $p<0.0001$). Three days of treatment with SR57227 increased the mean spontaneous firing rate by 35% ($1.46 \pm 0.11$, $n=50$ cells, $p<0.05$; Fig. 3a). Interestingly, this increase was prevented by electrolytic lesion of the mPFC ($0.73 \pm 0.10$, $n=42$ cells, $p<0.0001$ compared to SR57227 treatment group). Indeed, one-way ANOVA followed by post hoc PLSD Fisher’s test revealed a significant effect of treatment ($F_{3,24}=7.08$, $p=0.0002$). Differently, electrolytic lesion had no effect in control rats (Fig. 3b). Co-administration of vortioxetine and SR57227 decreased the mean spontaneous firing rate by 37% ($0.68 \pm 0.10$, $n=53$ cells, $p<0.05$).

**Acute effects of vortioxetine and fluoxetine alone or after administration of the 5-HT$_1$A receptor agonist, SR57227, on the firing activity of DRN 5-HT neurons**

We first compared the effect of acute repeated systemic administration of vortioxetine and fluoxetine on the firing activity of DRN 5-HT neurons. Then we evaluated influence of a prior administration of the 5-HT$_1$A receptor agonist SR57227 on effect of both drugs.

Administration of vortioxetine (250–1250 μg/kg i.v.) suppressed the firing activity of DRN 5-HT neurons with ED$_{50}=440$ μg/kg ($n=5$; Fig. 4a). Prior administration of the 5-HT$_1$A receptor agonist, SR57227 (1 μg/kg, $n=6$ or 5 μg/kg, $n=6$, i.v.), dose-dependently prevented this suppressant response, as exemplified in Fig. 4b, with ED$_{50}=923$ μg/kg ($n=5$). Two-way repeated-measures ANOVA revealed a significant effect of SR57227 pre-treatment ($F_{3,14}=4.30$, $p=0.035$) on the potency of vortioxetine ($F_{3,14}=7.85$, $p<0.0001$) and a significant interaction effect ($F_{8,56}=2.33$, $p=0.031$). Dunnett’s test indicated a significant effect at a SR57227 dose of 5 μg/kg ($p<0.05$), but not at 1 μg/kg (Fig. 4c).

As previously demonstrated (Czachura & Rasmussen, 2000), fluoxetine (1–7 mg/kg i.v.) suppressed the firing activity of DRN 5-HT neurons with ED$_{50}=2.73$ mg/kg ($n=5$; Fig. 5a, b). Prior administration of SR57227 (5 μg/kg i.v.) did not modify this suppressant response, i.e. two-way repeated-measures ANOVA indicated a significant effect of fluoxetine ($F_{3,14}=90.98$, $p<0.0001$), but no significant effect of SR57227 pre-treatment ($F_{3,14}=2.80$, n.s.) and no significant interaction ($F_{8,56}=1.89$, n.s.; Fig. 5c).

The inhibitory effects of both vortioxetine and fluoxetine were reversed by i.v. administration of the selective 5-HT$_1$A receptor antagonist WAY-100,635 (50 μg/kg), suggesting 5-HT$_1$A receptor mediation.

**Acute effects of vortioxetine on the firing activity of DRN 5-HT neurons in 5-HT depleted rats**

PCPA (100 mg/kg i.p.) treatments for 3 d were performed in order to deplete 5-HT. Thus, we evaluated the importance of 5-HT in the acute suppressant effect of vortioxetine. In control rats, successive administration
of vortioxetine (250 μg/kg i.v.) suppressed the firing activity of DRN 5-HT neurons with ED₅₀ = 0.638 ± 0.015 mg/kg (n = 6; Fig. 6a, c), while in PCPA-treated rats, significantly larger doses were necessary (Fig. 6b). Indeed, vortioxetine (500 μg/kg i.v.) suppressed the firing activity of DRN 5-HT neurons with ED₅₀ = 1.627 ± 0.429 mg/kg (n = 5, p < 0.01; Fig. 6c).

### SERT occupancy after acute dosing of vortioxetine or fluoxetine

Using ex vivo autoradiography, we evaluated the percentage of SERT occupied after acute administration of vortioxetine or fluoxetine. We found that vortioxetine, unlike fluoxetine, inhibited 5-HT neuronal firing at doses that did not saturate inhibition at the SERT, i.e. about 53% SERT occupancy was required to reduce the firing rate by 50% for vortioxetine, whereas about 90% SERT occupancy was required to achieve a 50% reduction of the firing rate with fluoxetine. Co-administration of SR57227 did not affect SERT occupancy of fluoxetine or vortioxetine (Table 1).

### Acute effects of vortioxetine and the 5-HT₁A receptor agonist flesinoxan on the firing activity of DRN 5-HT neurons

Vortioxetine has a poor affinity for rat 5-HT₁A receptors but a high affinity for human ones. In order to confirm these in vitro data, we acutely co-administered vortioxetine and the 5-HT₁A receptor agonist flesinoxan to assess an interaction between both drugs.

As demonstrated in Fig. 1c, systemic administration of vortioxetine suppressed dose-dependently the firing activity of DRN 5-HT neurons. Prior administration of the selective 5-HT₁A receptor agonist flesinoxan

### Table 1. Rat brain SERT occupancies after acute administration of vortioxetine or fluoxetine alone or preceded by treatment with SR57227, 5 μg/kg i.v.

<table>
<thead>
<tr>
<th>Vortioxetine (mg/kg i.v.)</th>
<th>Fluoxetine (mg/kg i.v.)</th>
<th>SERT occupancy (%) drug alone</th>
<th>SERT occupancy (%) drug combined with SR57227 5 μg/kg i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>0 ± 8.6</td>
<td>0 ± 3.5</td>
</tr>
<tr>
<td>0.25</td>
<td>–</td>
<td>38 ± 0.96</td>
<td>50 ± 3.9</td>
</tr>
<tr>
<td>0.50</td>
<td>–</td>
<td>56 ± 1.5</td>
<td>59 ± 4.1</td>
</tr>
<tr>
<td>0.75</td>
<td>–</td>
<td>73 ± 2.8</td>
<td>63 ± 12</td>
</tr>
<tr>
<td>1.00</td>
<td>–</td>
<td>75 ± 1.3</td>
<td>81 ± 2.0</td>
</tr>
<tr>
<td>–</td>
<td>2</td>
<td>87 ± 0.29</td>
<td>82 ± 2.3</td>
</tr>
<tr>
<td>–</td>
<td>4</td>
<td>93 ± 0.84</td>
<td>94 ± 2.5</td>
</tr>
<tr>
<td>–</td>
<td>6</td>
<td>97 ± 0.62</td>
<td>99 ± 0.27</td>
</tr>
</tbody>
</table>

SERT, 5-HT transporter

Data are expressed as mean percent SERT occupancy ± S.E.M. (n = 3 per group).

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**Fig. 3.** Effect on the firing activity of dorsal raphe nucleus 5-HT neurons of co-administration of vortioxetine and SR57227 for 3 d. (a) Mean (± S.E.M.) of the firing rate of dorsal raphe 5-HT neurons in controls and rats treated for 3 d with vortioxetine (5 mg/kg.d s.c.) and/or SR57227 (1 mg/kg.d). (b) Mean (± S.E.M.) of the firing rate of dorsal raphe 5-HT neurons in medial prefrontal cortex electrolytic-lesioned control rats and/or treated for 3 d with SR57227 (1 mg/kg.d). *p < 0.05 as compared to control, ***p < 0.001 as compared to SR57227 using one-way analysis of variance followed by post-hoc partial least squares difference Fisher’s test. The numbers in the bars indicate the total number of neurons recorded in each group of treated rats.
Fig. 4. Effect on the firing activity of dorsal raphe nucleus (DRN) 5-HT neurons of acute administration of vortioxetine alone or after administration of SR57227. (a) Integrated firing rate histogram of a presumed dorsal raphe 5-HT neuron showing its response to cumulative doses of vortioxetine (250–1250 µg/kg i.v.). Note that the selective 5-HT₁A receptor antagonist WAY-100,635 reversed the full inhibition induced by vortioxetine. (b) Integrated firing rate histogram of a presumed DRN 5-HT neuron showing its response to cumulative doses of vortioxetine (250–1500 µg/kg i.v.) after a prior administration of the selective 5-HT₁A receptor agonist SR57227 (5 µg/kg i.v.). The selective 5-HT₁A receptor agonist flesinoxan was used to fully suppress neuronal firing. (c) Dose–response curve of the suppressant effect of vortioxetine constructed with cumulative doses (250–1000 µg/kg i.v.) alone or with prior administration of SR57227 (1 or 5 µg/kg i.v.). Each symbol represents the mean relative to control of dorsal raphe 5-HT cell (in percentage ± S.E.M.). *p < 0.05, as compared to saline using two-way repeated-measures analysis of variance followed by post hoc Dunnett’s test.

(50 µg/kg i.v., n = 6) did not modify this suppressant response, as exemplified in Fig. 7a. Indeed, the two-way repeated-measures ANOVA revealed a significant effect of the vortioxetine doses (F₃,₄₅ = 88.79, p < 0.0001), but no significant effect of the flesinoxan pretreatment ((F₃,₄₅ = 2.83, n.s.) and no significant interaction (F₅,₄₅ = 0.68, n.s.).

As previously demonstrated, flesinoxan (100–300 µg/kg i.v., n = 5) suppressed the firing activity of DRN 5-HT neurons (Haddjeri et al. 1999). Prior administration of vortioxetine (250 µg/kg i.v., n = 5) did not modify this suppressant response, since the two-way repeated-measures ANOVA indicated a significant effect of flesinoxan dose (F₃,₄₅ = 103.38, p < 0.0001) but no significant effect of vortioxetine pretreatment (F₃,₄₅ = 2.02, n.s.) and significant interaction (F₅,₄₅ = 3.64, p = 0.027; Fig. 7b).
The main findings of the present study are summarized as follows: (i) vortioxetine induced a fast recovery of the 5-HT neuronal firing rate after only 1 d subchronic treatment due to 5-HT1A receptor desensitization; (ii) SR57227 delayed this recovery; (iii) vortioxetine dose-dependently acutely inhibited DRN 5-HT neuronal firing rate and this effect is prevented by the 5-HT1A agonist SR57227; (iv) vortioxetine displayed a SERT occupancy of about 53% for an inhibition of 50% of DRN 5-HT firing; (v) the SSRI fluoxetine suppresses DRN-5-HT neuronal firing and the SR57227 tends to increase this suppressant effect. A much higher SERT occupancy (about 90%) was needed to suppress the firing; (vi) in 5-HT depleted rats, the suppressant effect of vortioxetine was significantly reduced; (vii) both acute and subacute administrations of vortioxetine did not alter the suppressant effect of flesinoxan on DRN 5-HT firing activity.

The recovery of firing of 5-HT cells in the DRN was evaluated after sustained treatment with vortioxetine and fluoxetine. After 7 d treatment with fluoxetine, the mean firing rate of 5-HT neurons was decreased, as previously shown (Czachura & Rasmussen, 2000). Only after 14 and 21 d treatment had the firing rate returned to the control value. The reduction of this delay in normalization of DRN 5-HT neuronal firing rate is a major challenge, since it seems to be correlated with the delay of the response to antidepressant treatment (Blier et al. 1998). Consequently, it was surprising to observe a decrease of 5-HT neuronal firing in the DRN at 5 and 10 h, but a recovery after 24 h of dosing with vortioxetine. During chronic treatment with SSRIs, the gradual recovery of activity of 5-HT cells has been attributed to the desensitization of the somatodendritic 5-HT1A receptor (Blier et al. 1998). To determine the sensitivity of the 5-HT1A receptor, the responsiveness of 5-HT neurons to flesinoxan was assessed. Rapid desensitization of somatodendritic 5-HT1A receptors was observed after only 3 d treatment with vortioxetine followed by 1 d wash-out. Nevertheless, early desensitization is not unique; indeed, desensitization of somatodendritic 5-HT1A autoreceptors increases from approximately 40% after 3 d to 60–80% after 21 d treatment with the SSRIs, fluoxetine or paroxetine (Le Poul et al. 1995). Early desensitization is also observed after treatments (2 d) with the monoamine oxidase-A inhibitor befloxatone (Haddjeri et al. 1998). Interestingly, it has been shown that 2 d co-administration of paroxetine and the α2-adrenoreceptor antagonist, mirtazapine, which is a 5-HT2 receptor antagonist, attenuates the suppressant effect on 5-HT neuronal firing of paroxetine given alone (Besson et al. 2000). In depressed patients, this co-administration is a more effective antidepressant treatment than their mono-therapy (Blier et al. 2009).

In contrast to SSRIs, vortioxetine also targets several 5-HT receptors and is particularly potent at the 5-HT3

Discussion

These data suggest that vortioxetine had no significant in vivo activity on rat 5-HT1A receptors.

**Effect of co-administration of vortioxetine and flesinoxan on the recovery of the firing activity of DRN 5-HT neurons**

Rats were treated for 3 d with vortioxetine (5 mg/kg s.c.), flesinoxan (2.5 mg/kg s.c.) or with co-administration of both drugs. The mean spontaneous firing activity of DRN 5-HT neurons in control rats was 1.01 ± 0.09 (n = 41 cells). One-way ANOVA followed by Dunnett’s test revealed a significant effect of treatment ($F_{3,41} = 9.69$, $p < 0.001$; Fig. 8). Flesinoxan inhibited DRN 5-HT neuronal firing (0.61 ± 0.13, $n = 32$, $p < 0.05$) and in a similar potency than when co-administered with vortioxetine and flesinoxan (0.38 ± 0.06, $n = 39$, $p < 0.01$).
receptor subtype. Therefore, results from the present study suggest that the 5-HT3 receptor antagonism of vortioxetine might be responsible, at least in part, for this surprisingly rapid recovery of 5-HT neuronal firing. We demonstrated that it is delayed by co-administration of SR57227 for 3 d, suggesting a crucial role for 5-HT3 receptors. Cortical 5-HT3 receptors are known to be localized on GABAergic interneurons (Morales & Bloom, 1997; Morales et al., 1996; Puig et al., 2004) and their stimulation induces an activation of GABAergic interneurons, which consequently inhibit the firing activity of pyramidal cells (Zhang et al., 2011) and by a feedback long-loop decrease the dorsal raphe 5-HT neuronal firing rate (Celada et al., 2001; Puig & Gulledge, 2011). Differently, 5-HT2A receptors are essentially localized on pyramidal neurons, thus their stimulation induced an excitatory effect (Jakab & Goldman-Rakic, 1998; Santana et al., 2004). Acutely, 5-HT induced an overall suppressant effect on pyramidal neuronal activity while chronically 5-HT3 (but not 5-HT2A) receptors are desensitized (Kondaurova et al., 2012; Zhou & Hablitz, 1999). We postulate that 3-d treatment with SR57227 increased the DRN 5-HT neuronal firing rate via stimulation of cortical pyramidal neurons and desensitization of 5-HT3 receptors. This hypothesis is supported by the fact that a cortical electrolytic lesion prevented the enhancement of DRN 5-HT neuronal firing induced by the subchronic treatment with SR57227. One may assume that co-administration of Lu AA21004 and SR57227 may result in a decreased firing rate because of the lack of 5-HT3 receptor desensitization. Further studies are necessary to validate such a hypothesis. However, our data suggest that the blockade of 5-HT3 receptors is important to obtain a shorter recovery time of the DRN 5-HT neuronal firing rate.

We evaluated the acute effect of fluoxetine and Lu AA21004, with or without SR57227 acutely, to confirm that 5-HT3 receptors may be responsible for the different effects between both drugs. As previously demonstrated (Czachura & Rasmussen, 2000), acute administration of fluoxetine suppressed 5-HT neuronal firing in the DRN. While both vortioxetine and fluoxetine have in vitro 5-HT reuptake inhibitory potencies in the same order of magnitude, i.e. 1.6 and 8 nM, respectively (Bang-Andersen et al., 2011; Wong et al., 1995), vortioxetine suppressed 5-HT neuronal firing about 6-fold more potently than...
fluoxetine (ED_{50} = 0.44 mg/kg and 2.73 mg/kg i.v., respectively). This difference may at least partly be due to a contribution of 5-HT, receptor antagonism by vortioxetin. As previously hypothesized, the 5-HT_3 receptor agonists may decrease the DRN 5-HT neuronal firing rate via a cortical feedback long loop. The effects of SR57227 and fluoxetine seem to be in accordance with this statement since we found a tendency to a synergistic suppressant effect. On the other hand, we found that SR57227 partially blocked the suppressant effect of Lu AA21004, suggesting a different interaction than this classical SSRI.

In a previous study, it has been shown that the SSRI, paroxetine, on DRN 5-HT neuronal firing rate (unpublished observations). To our knowledge, the full receptor agonistic property of SR57227 has not yet been demonstrated and one may assume that this compound may behave as a partial agonist as is the case with the 5-HT_3 receptor agonist, S 21007, which displays in vitro partial agonistic activity (Delagrange et al. 1996). As previously reviewed (Etievant et al. 2010; Tammenga, 2002), while a receptor agonist mimics the action of a natural neurotransmitter, a partial receptor agonist induces a reduced signalling response compared to the maximum achievable response by a full receptor agonist. The intrinsic activity or efficacy of a partial receptor agonist depends on the sensitivity and responsiveness of the receptors, thus partial receptor agonists can behave as an agonist or antagonist, depending on the target receptor population and the local concentration of the natural neurotransmitter (Etievant et al. 2010; Tammenga, 2002). Paradoxically, Lu AA21004 induced a higher release of 5-HT in comparison to the classical SSRIs at a low SERT occupancy (Pehrson et al. 2012). We hypothesize that SR57227 in the presence of fluoxetine may further stimulate 5-HT_3 receptors. Differently, Lu AA21004 has 5-HT_3 receptor antagonist properties, which can induce competition for the 5-HT_3 receptor. Moreover, if SR57227 behaves as a partial agonist receptor, in the presence of Lu AA21004, it may block 5-HT_3 receptors, because of the increased level of extracellular 5-HT induced by Lu AA21004. Moreover, the K_i values of Lu AA21004 for the SERT and the 5-HT_3 receptor are similar, i.e. 1.7 and 3.7 nm respectively. Thus, with a low dose of Lu AA21004, 5-HT_3 receptors are probably partially occupied identically to the occupation of the SERT (about 38% for 250 µg/kg). Hence, in the presence of a low dose of Lu AA21004, SR57227 may occupy 5-HT_3 receptors; at a higher dose of Lu AA21004, the 5-HT_3 receptors may be fully occupied by Lu AA21004. This could explain the trend we observed with 1 µg/kg SR57227 (Fig. 4c). Indeed, the difference between the effects of co-administration of SR57227 and Lu AA21004 and Lu AA21004 alone is observable even at a low dose of Lu AA21004.

We used PCPA to induce 5-HT depletion. PCPA is an inhibitor of tryptophan hydroxylase, the rate-limiting enzyme in 5-HT biosynthesis (Koe & Weissman, 1968). It has been previously shown that the ‘antidepressant-like’ effect of different SSRIs such as paroxetine or fluoxetine was prevented by treatment with PCPA in the forced swim test (Page et al. 1999; Redrobe et al. 1998). Correspondingly, we demonstrated that PCPA significantly reduced the suppressant effect of vortioxetine on the DRN 5-HT neuronal firing rate (ED_{50} = 0.638 ± 0.015 mg/kg in controls vs. 1.627 ± 0.429 mg/kg in PCPA-treated rats).

Vortioxetine also differs from SSRIs in its capacity to decrease 5-HT neuronal firing and to enhance 5-HT release at low SERT occupancy. In the present study, vortioxetine inhibited neuronal firing activity by 50% at a dose 0.44 mg/kg (i.v.), which produced about 53% SERT occupancy, whereas fluoxetine inhibited neuronal firing activity by 50% at a dose of 2.73 mg/kg (i.v.), which produced about 90% SERT occupancy. This difference strongly supports the view that vortioxetine modulates 5-HT neuronal firing by a mechanism that goes beyond inhibition of the SERT. Clinical PET imaging studies have shown that at least 80% SERT occupancy is observed at therapeutic doses of SSRIs (e.g. fluoxetine or paroxetine) or serotonin-noradrenaline reuptake inhibitors (e.g. duloxetine; Meyer et al. 2004; Takano et al. 2006; Talbot & Laruelle, 2002). Interestingly, vortioxetine induces a rapid increase in rat cortical 5-HT release at low SERT occupancy. In fact, the rat microdialysis study of Mørk et al. (2009) revealed a significant increase of extracellular 5-HT levels after only 3 d treatment with vortioxetine (5 mg/kg.d s.c.) corresponding to 40% SERT occupancy. This is qualitatively different from the 80% SERT occupancy and 14 d treatment usually required for SSRIs studied under similar test conditions.

Although vortioxetine is a potent 5-HT_{1A} receptor in humans, it has been demonstrated that vortioxetine has a weaker activity on rat 5-HT_{1A} receptor (K_i = 230 nM; Bang-Andersen et al. 2011). Our data show that flesinoxan failed to modify the acute suppressant effect of vortioxetine on 5-HT DRN neuronal activity. Similarly, prior acute administration of vortioxetine has no effect on flesinoxan suppressant effect suggesting no competition for 5-HT_{1A} receptor between both compounds. We also demonstrated that subacute administrations of vortioxetine did not alter the suppressant effect of flesinoxan. Thus, we could conclude that vortioxetine has no intrinsic activity on the 5-HT_{1A} receptor confirming that vortioxetine was devoid of rat 5-HT_{1A} receptor activity. Nevertheless, the ‘silent’ 5-HT_{1A} receptor antagonist WAY-100 635 reversed the inhibition induced by both fluoxetine and vortioxetine. It may be assumed that the inhibition of 5-HT neuronal activity induced by vortioxetine is mediated through an indirect 5-HT_{1A} receptor activation, i.e. the release of extracellular 5-HT.
In conclusion, vortioxetine, a 5-HT₁ and 5-HT₇ receptor antagonist, 5-HT₁₈ receptor partial agonist and human 5-HT₁₆ receptor agonist and inhibitor of the SERT, clearly differs from the SSRI fluoxetine with regard to effects on DRN 5-HT neuronal firing. The acute suppressant effect of firing is obtained at low SERT occupancy and there is a rapid recovery of firing after sustained treatment in this preclinical study. These effects are at least partly due to the 5-HT₇ receptor antagonism of vortioxetine. The distinct pharmacological profile of vortioxetine compared to currently used antidepressants may lead to a different clinical profile.

Acknowledgements

The technical assistance from Magali Novo-Perez was greatly appreciated. This research was supported by Claude Bernard University-Lyon 1 and H. Lundbeck A/S.

Statement of Interest

N. Haddjeri, INSERM employee, has received grants from Lundbeck and Solvay Pharmaceuticals. B. Ebert, A. Pehrson, and C. Sánchez are Lundbeck employees. Dr Frazer, the EIC of the IJNP, is on a Lundbeck advisory board for vortioxetine. Consequently, he removed himself from the review process such that all aspects of the review including the final decision were handled by a field editor.

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