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# Allosteric modulation of the effect of escitalopram, paroxetine and fluoxetine: in-vitro and in-vivo studies

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## Abstract

Clinical and preclinical studies have shown that the effect of citalopram on serotonin (5-HT) reuptake inhibition and its antidepressant activity resides in the S-enantiomer. In addition, using a variety of in-vivo and in-vitro paradigms, it was shown that R-citalopram counteracts the effect of escitalopram. This effect was suggested to occur via an allosteric modulation at the level of the 5-HT transporter. Using in-vitro binding assays at membranes from COS-1 cells expressing the human 5-HT transporter (hSERT) and in-vivo electrophysiological and microdialysis techniques in rats, the present study was directed at determining whether R-citalopram modifies the action of selective serotonin reuptake inhibitors (SSRIs) known to act on allosteric sites namely escitalopram, and to a lesser extent paroxetine, compared to fluoxetine, which has no affinity for these sites. In-vitro binding studies showed that R-citalopram attenuated the association rates of escitalopram and paroxetine to the 5-HT transporter, but had no effect on the association rates of fluoxetine, venlafaxine or sertraline. In the rat dorsal raphe nucleus, R-citalopram (250 µg/kg i.v.) blocked the suppressant effect on neuronal firing activity of both escitalopram (100 µg/kg i.v.) and paroxetine (500 µg/kg i.v.), but not fluoxetine (10 mg/kg i.v.). Interestingly, administration of R-citalopram (8 mg/kg i.p.) attenuated the increase of extracellular levels of 5-HT ([5-HT]<sub>ext</sub>) in the ventral hippocampus induced by both escitalopram (0.28 µM) and paroxetine (0.75 µM), but not fluoxetine (10 µM). In conclusion, the present in-vitro and in-vivo studies show that R-citalopram counteracts the activity of escitalopram and paroxetine, but not fluoxetine, by acting at the allosteric binding site of the 5-HT transporter, either located in the dorsal raphe nucleus or post-synaptically in the ventral hippocampus. This conclusion is strengthened by the observation that the inhibitory effect of fluoxetine, which has no stabilizing effect on the radioligand/hSERT complex, was not blocked by co-administration of R-citalopram.

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**Key words:** Allosteric sites, dorsal raphe, escitalopram, hippocampus, serotonin, serotonin transporter, 5-HT<sub>1A</sub> autoreceptor.

## Introduction

Selective serotonin reuptake inhibitors (SSRIs) have been used clinically to treat a number of human brain disorders, including major depressive disorder,

obsessive-compulsive disorder, panic disorder, and bulimia (see Fuller, 1995, for a review). SSRIs require several weeks of administration to induce the full therapeutic effect, indicating that initial rapid increases in serotonin (5-HT) are not sufficient and that critical adaptive modifications are needed. For several classes of antidepressant drugs, these could be mediated via different mechanisms, such as post-synaptic sensitization to 5-HT, desensitization of the somatodendritic and/or terminal 5-HT autoreceptors, or a desensitization of  $\alpha_2$ -adrenergic heteroreceptors

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located on 5-HT terminals (Blier and de Montigny, 1994; Haddjeri et al., 1997; Mongeau et al., 1997). Several animal studies showed that long-term administration of SSRIs results in the desensitization of somatodendritic 5-HT<sub>1A</sub> autoreceptor function in the dorsal raphe nucleus resulting in recovery of their firing rate in the presence of antidepressant drugs. These adaptations have been hypothesized to play an important role in the delayed therapeutic onset of SSRIs (see Blier and de Montigny, 1999, for a review).

Clinical and preclinical studies have shown that the effect of citalopram on 5-HT reuptake inhibition and its antidepressant activity resides in the S-enantiomer (Hyttel et al., 1992; Lepola et al., 2004). In addition, using a variety of in-vivo and in-vitro paradigms, it was shown that R-citalopram counteracts the effect of escitalopram (El Mansari et al., 2005; Mørk et al., 2003; Sánchez, 2003; Sánchez et al., 2003a,b; Sánchez and Kreilgaard, 2004). The exact mechanism by which this action takes place is still unknown, but a modulation of the serotonin transporter (SERT) activity has been proposed (Mørk et al., 2003; Sánchez et al., 2004).

A recent neurochemical study revealed that R-citalopram induces minimal 5-HT increase, but attenuates the increase in extracellular 5-HT ([5-HT]<sub>ext</sub>) levels induced by escitalopram in the frontal cortex (Mørk et al., 2003). In addition, the potentiation of 5-hydroxytryptophan (5-HTP)-induced behaviours by escitalopram in mice was shown to be antagonized by the prior injection of R-citalopram (Stórustovu et al., 2004). Prevention of escitalopram activity by R-citalopram has been reported in the foot-shock-induced ultrasonic vocalization model to evaluate anxiolytic activity (Sánchez, 2003). Similar results were obtained using the conditioned fear stress model, another model predictive of anxiolytic activity (Sánchez et al., 2003b).

Using brain neuronal and platelet membranes, several studies revealed an allosteric modulation on the SERT protein, which modulates the association and/or dissociation rates of SERT ligands. It was concluded that at least two binding sites for citalopram are present; a primary high-affinity binding site that mediates the inhibition of 5-HT reuptake, and an allosteric low-affinity binding site that modulates the binding of ligands at the primary site (Plenge and Møllerup, 1985; Plenge et al., 1991; Wennogle and Meyerson, 1985). Moreover, using radioligand-binding studies in COS-1 cells expressing human 5-HT transporter (hSERT), it was shown that both citalopram enantiomers bind to the allosteric site, and decreased the dissociation rate of [<sup>3</sup>H]escitalopram

from the transporter. However, the allosteric potency of escitalopram is about five times higher than R-citalopram. Interestingly, the allosteric effect of escitalopram on [<sup>3</sup>H]escitalopram dissociation from hSERT was approximately double the paroxetine effect on [<sup>3</sup>H]paroxetine, while fluoxetine had no detectable effect on binding of [<sup>3</sup>H]fluoxetine at the transporter (Chen et al., 2005a).

Based on the difference shown in the effect of escitalopram, paroxetine and fluoxetine with regard to the allosteric site, the present study was undertaken to further characterize, both in vitro and in vivo, the functional interaction between the two citalopram enantiomers at the SERT. It was also aimed at determining whether R-citalopram modulates the effects of different SSRIs on rat 5-HT neurotransmission both in the dorsal raphe nucleus and hippocampus, using in-vivo electrophysiological and microdialysis paradigms, as well as in-vitro binding studies at hSERT expressed in a heterologous system.

## Methods

### Membrane preparations

COS-1 cells were cultured and transfected with hSERT as previously described (Chen et al., 2005a) and plated at 35% confluency in 150-mm dishes. Cells were grown for 64 h and were rinsed in PBS prior to harvesting. Cells were harvested with a cell scraper in buffer 1 (50 mM Tris-base, 150 mM NaCl, 20 mM EDTA; pH 7.4). After centrifugation, cells were suspended and homogenized with an Ultra-Turrax for 20 s in buffer 1. Membranes were pelleted by ultracentrifugation and homogenization was repeated. Finally, after a second ultracentrifugation, membranes were re-suspended in buffer 2 (50 mM Tris-base, 120 mM NaCl, 5 mM KCl; pH 7.4) and stored at  $-80^{\circ}\text{C}$ .

### Binding assays

A membrane suspension was dispensed into a 96-well plate (5  $\mu\text{g}$ /well). Buffer 2 containing increasing radioligand concentrations was added to each well. Membranes were incubated for 60 min on ice and transferred to a 96-well glass-fibre filter plate (Unifilter, Packard Bell, Meriden, CT, USA) pre-incubated with 25  $\mu\text{l}$  0.5% polyethyleneimine, using a Packard Bell cell harvester and subsequently washed four times with water. Filters were soaked in 40  $\mu\text{l}$  MicroScint 20 scintillation liquid (Packard Bell) and accumulated radioactivity was determined by direct counting using a Packard Bell microplate scintillation counter. Non-specific binding was

determined by assaying non-transfected cells in parallel or by adding 10  $\mu\text{M}$  RTI-55 as a displacer.

The data obtained were plotted and saturation curves were analysed by nonlinear least-squares curve-fitting using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) to estimate  $K_d$  values assuming Michaelis–Menten kinetics.

#### *Determination of hSERT association rates*

A membrane preparation was suspended in buffer 2 and preincubated for 30 min at 20 °C with increasing concentrations of R-citalopram, or (+)paroxetine, up to 100 nM. The kinetics for the association of the  $^3\text{H}$  radioligands (escitalopram, paroxetine, fluoxetine, sertraline, venlafaxine and duloxetine) was followed by incubating for increasing time intervals at 20 °C. Radioligands were incubated at concentrations corresponding to their respective  $K_d$  values (Chen et al., 2005a). Non-specific binding was determined in the presence of 10  $\mu\text{M}$  paroxetine. Reactions were terminated by filtration through GF/C glass-fibre filters on a cell harvester as described above. Bound radioactivity was determined by direct counting using a Packard Bell microplate scintillation counter. Assays were carried out in quadruplicate from at least three independent transfections. Association curves were obtained by plotting bound radioligand vs. time and were used to calculate association rates according to the second-order rate equation:

$$d[\text{LR}]/dt = k_{+1} * [\text{L}] * [\text{R}],$$

where [L], [R], and [LR] are the concentrations of ligand, receptor, and ligand–receptor complex respectively. The potencies of the ligands were determined as the drug concentration that attenuates the observed association rate by 50% compared to association in buffer, i.e. the  $\text{EC}_{50}$  value.  $\text{EC}_{50}$  values were obtained from concentration-effect curves of normalized association rates vs. log-drug concentrations and are given as mean values  $\pm$  standard deviation.

#### *Extracellular unitary recordings of dorsal raphe 5-HT neuron*

Experiments were performed in accordance with the European Communities Council Directives 86/609, OJ L 358,1, 12 December 1987, for the care and use of laboratory animals. All experiments were performed with the approval of the Regional Animal Care Committee (Faculty of Medicine, Claude Bernard University–Lyon 1).

The in-vivo experiments were carried out with male Sprague–Dawley rats (Harlan, Gannat, France)

weighing 250–300 g on the day of the experiment. Extracellular recordings were performed with single-barreled glass micropipettes in rats anaesthetized with chloral hydrate (400 mg/kg i.p.). The tip was broken back to 2–4  $\mu\text{m}$  and filled with a 2 M NaCl solution saturated with Pontamine Sky Blue. Presumed dorsal raphe 5-HT neurons were encountered over a distance of 1 m starting immediately below the ventral border of the Sylvius aqueduct. 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. To normalize data we calculated the mean firing activity prior to and after a drug administration and transposed the result as a percentage of basal values. To determine a putative interaction between the R-enantiomer of citalopram and each SSRI on the firing activity of 5-HT neurons, 250  $\mu\text{g}/\text{kg}$  (i.v.) of R-citalopram was injected prior to escitalopram ( $\text{ED}_{100} = 100 \mu\text{g}/\text{kg}$  i.v.), paroxetine ( $\text{ED}_{100} = 500 \mu\text{g}/\text{kg}$  i.v.) and fluoxetine ( $\text{ED}_{100} = 10 \text{ mg}/\text{kg}$  i.v.). In the electrophysiological studies, the firing rate of 5-HT neurons quoted is the average number of spikes in six, 10-s bins at baseline, or three, 10-s bins after administration of a drug. The estimation of blockade by R-citalopram was done first by determining the time necessary to produce a complete cessation of neuron firing by SSRIs, then by estimating, after that same time, the effect of the SSRI on the discharge of the neuron in the presence of R-citalopram.

#### *Microdialysis in the ventral hippocampus*

Male Sprague–Dawley rats (280–320 g, Harlan) were anaesthetized with urethane (1.15 g/kg i.p.). The microdialysis probe was implanted into the right ventral hippocampus at the following coordinates relative to bregma: anterior 5.2 mm, lateral 5.0 mm, ventral 8.5 mm below the brain surface. As previously described (Benturquia et al., 2005), concentric microdialysis probes were constructed in our laboratory from regenerated cellulose dialysis tubing and fused-silica capillary tubing, the body of the probe being made of a 3-cm 26G stainless-steel tube. The collection of 30-min samples was initiated after a 3-h waiting period. Samples were collected in PCR tubes (Abgene, Epsom, UK) and immediately stored at  $-20$  °C for <1 wk before separation by capillary electrophoresis on a SpectroPhoresis100 module (ThermoSeparation Products, Les Ulis, France) using a modular ZetaLIF 266 detector (Picometrics, Ramonville, France). The excitation was performed with solid-state laser (266 nm, 2 mW). Separations were carried out with

a 120 cm  $\times$  50  $\mu$ m ID fused silica capillary (Composite Metal Services, Worcester, UK) with an effective length of 60 cm. On-column laser induced fluorescence detection was carried out through a 5-mm-wide window opened by removing the polyimide cover of the capillary. Separation was performed under an applied voltage of 30 kV. The running buffer was 80 mmol/l citrate buffer (pH 2.5) containing 20 mmol/l hydroxypropyl- $\beta$ -cyclodextrin. The basal value of  $[5\text{-HT}]_{\text{ext}}$  was calculated from the mean of 30-min sample collection. All subsequent sample values calculated as the amount of 5-HT outflow collected during the 0- to 150-min period from the ventral hippocampus are expressed as a percentage of basal values. The limit of sensitivity for  $[5\text{-HT}]_{\text{ext}}$  was  $\sim 2.5 \times 10^{-10}$  M per sample (signal-to-noise ratio = 2). The artificial cerebrospinal fluid (aCSF) used for probe perfusion had the following composition (in mmol/l): NaCl, 145.0; KCl, 2.7;  $\text{MgCl}_2$ , 1.0;  $\text{CaCl}_2$ , 1.2;  $\text{NaH}_2\text{PO}_4$ , 0.45;  $\text{Na}_2\text{HPO}_4$ , 1.55 (pH 7.4).

#### Data analysis and statistics

Statistical analysis was performed with Statview software. Values are shown as the mean  $\pm$  s.e.m. Statistical significance is shown as \*  $p < 0.05$ , \*\*  $p < 0.01$  and #  $p < 0.01$ . The non-parametric Mann-Whitney  $U$  test was used for the electrophysiological experiments and one- and two-way analysis of variance (ANOVA) followed by the PLSD Fisher post-hoc test for the microdialysis data.

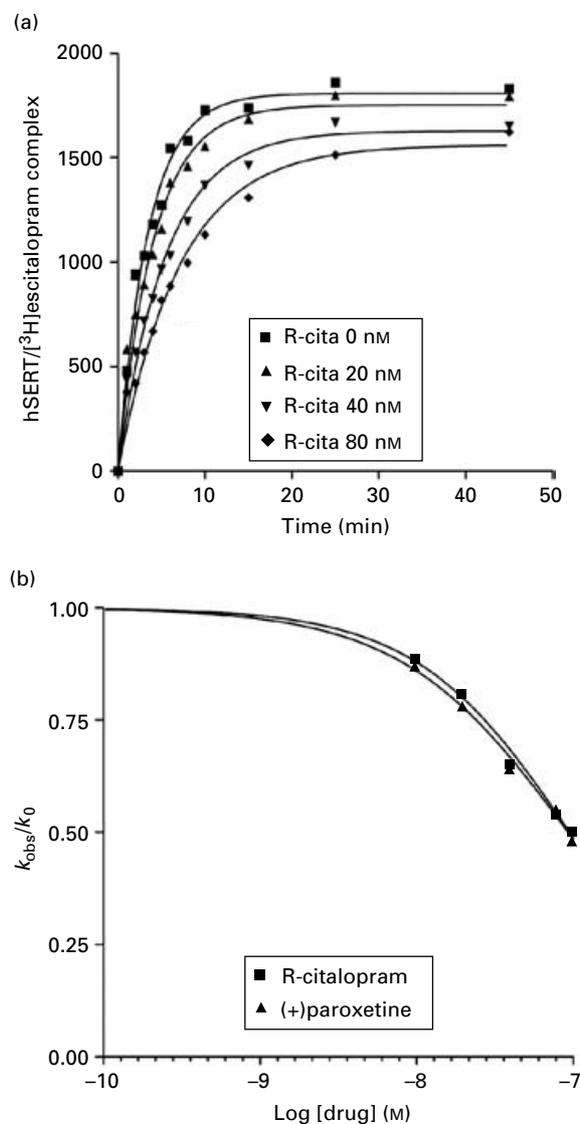
#### Drugs

The following drugs were supplied by Lundbeck (Valby, Denmark):  $[^3\text{H}]$ escitalopram,  $[^3\text{H}]$ R-citalopram,  $[^3\text{H}]$ fluoxetine (Lu 00-203-C), and  $[^3\text{H}]$ paroxetine (Lu 00-217-U).  $[^3\text{H}]$ sertraline and  $[^3\text{H}]$ duloxetine were purchased from Amersham Biosciences (Hørsholm, Denmark).  $[^3\text{H}]$ venlafaxine was supplied by Dr Mikael Bols (Aarhus University, Denmark). WAY 100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride) and 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin) were purchased from Sigma (Saint-Quentin Fallavier, France).

#### Results

##### Effect of R-citalopram and (+)paroxetine on hSERT association rates measured in vitro

Both R-citalopram and (+)paroxetine are low potent 5-HT reuptake inhibitors. R-citalopram attenuated



**Figure 1.** Concentration-effect curves for the allosteric effect on association rates for  $[^3\text{H}]$ escitalopram and hSERT. (a) Time kinetics for the association of  $[^3\text{H}]$ escitalopram and hSERT in the presence of increasing concentrations of R-citalopram. (b) Observed association rates,  $k_{\text{obs}}$ , are normalized with  $k_0$  and plotted vs. log drug concentrations to obtain  $\text{EC}_{50}$  values for R-citalopram and (+)paroxetine.  $k_0$  is the association rate measured in the absence of allosteric ligand.

the association of escitalopram and paroxetine with  $\text{EC}_{50}$  values of  $94 \pm 18$  nM and  $92 \pm 22$  nM respectively (Figure 1, Table 1). However, R-citalopram had no effect on the association rates of fluoxetine, sertraline, or venlafaxine. (+)Paroxetine attenuated the association of escitalopram with an  $\text{EC}_{50}$  value of  $76 \pm 15$  nM, but had no effect on the association rates of paroxetine, fluoxetine, sertraline or venlafaxine (Table 1).

**Table 1.** Allosteric potencies for R-citalopram and (+)paroxetine at association rates for hSERT and the listed antidepressants

	R-citalopram	(+)Paroxetine
[ <sup>3</sup> H]Escitalopram	94 ± 18 nM	76 ± 15 nM
[ <sup>3</sup> H]Paroxetine	92 ± 21 nM	No effect
[ <sup>3</sup> H]Fluoxetine	No effect	n.d.
[ <sup>3</sup> H]Sertraline	No effect	n.d.
[ <sup>3</sup> H]Venlafaxine	No effect	n.d.
[ <sup>3</sup> H]Duloxetine	No effect	n.d.

Potencies are measured in vitro and given as EC<sub>50</sub> values ± s.d. n.d., Not determined.

#### *Effect of R-citalopram on the inhibition of dorsal raphe 5-HT neuronal activity induced by SSRIs*

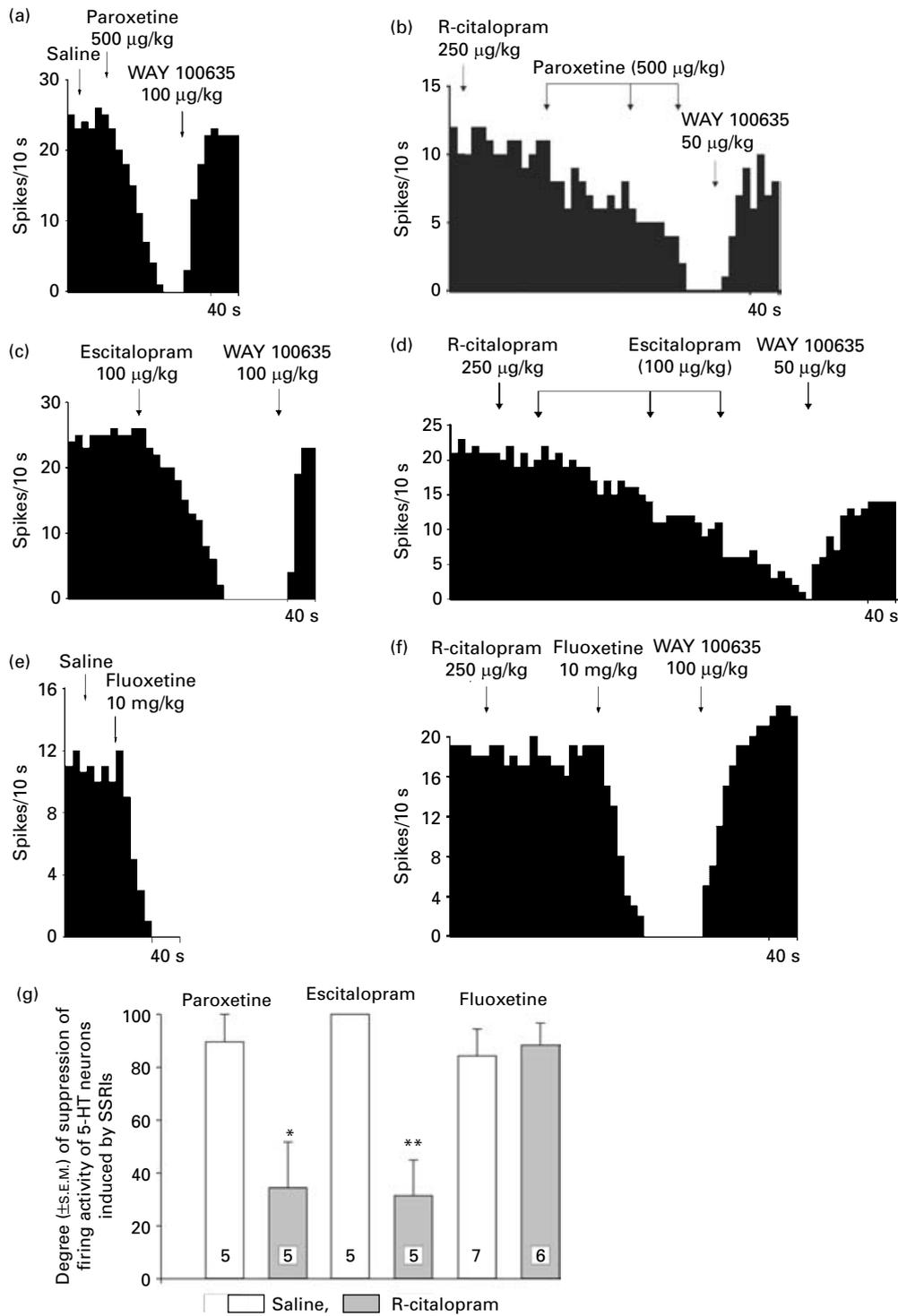
The Mann–Whitney *U* test showed no difference in basal neuronal firing between the different groups ( $p > 0.34$  for the paroxetine group,  $p > 0.91$  for the escitalopram group and  $p > 0.07$  for the fluoxetine group). As illustrated in Figure 2, R-citalopram (250 µg/kg i.v.) by itself did not modify the firing activity of dorsal raphe 5-HT neurons (Figure 2a). However, it prevented by 55% the inhibitory effect of paroxetine on dorsal raphe 5-HT neurons (Figure 2b). Without R-citalopram, the mean firing activity of 5-HT neurons was  $1.3 \pm 0.2$  Hz before the administration of paroxetine (500 µg/kg i.v.) and  $0.1 \pm 0.1$  Hz after paroxetine injection. After R-citalopram administration, the firing rate was  $1 \pm 0.5$  Hz before and  $0.7 \pm 0.2$  Hz after paroxetine injection ( $p < 0.03$ , Mann–Whitney *U* test,  $n = 5$ , Figure 2a, b, g). Similarly, the effect of escitalopram was also blocked by 68%, after the injection of R-citalopram. In the absence of R-citalopram, the mean firing activity of 5-HT neurons was  $2.1 \pm 0.2$  Hz before the administration of 100 µg/kg escitalopram and 0 Hz after escitalopram injection. Interestingly, after R-citalopram administration, the firing rate was  $1.8 \pm 0.2$  Hz before and  $0.9 \pm 0.3$  Hz after escitalopram injection ( $p < 0.005$ , Mann–Whitney *U* test,  $n = 5$ , Figure 2c, d, g), confirming our previous results (El Mansari et al., 2005). However, the inhibitory effect of fluoxetine on dorsal raphe 5-HT neurons was not blocked by R-citalopram (change of 4%; Mann–Whitney *U* test,  $p > 0.9$ , Figure 2e–g). Indeed, without R-citalopram, the mean firing activity of 5-HT neurons was  $1.3 \pm 0.2$  Hz before the administration of 10 mg/kg fluoxetine and  $0.2 \pm 0.2$  Hz after fluoxetine injection ( $n = 7$ ). After R-citalopram administration, the firing rate was  $2 \pm 0.3$  Hz before and  $0.3 \pm 0.3$  Hz after fluoxetine injection ( $n = 6$ ).

#### *Effect of R-citalopram on escitalopram, paroxetine and fluoxetine action on [5-HT]<sub>ext</sub> levels in the ventral hippocampus*

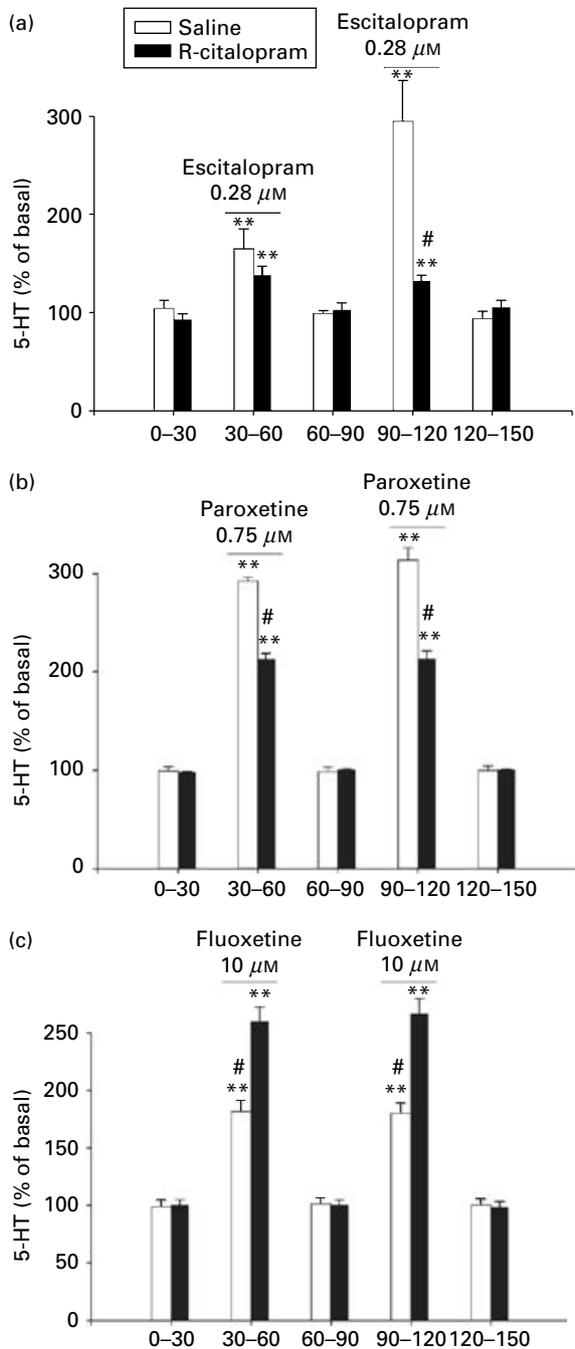
Basal levels of 5-HT in dialysate collected from the ventral hippocampus were  $4.2 \pm 0.3$  fmol/10 µl. Before any treatment, basal hippocampal [5-HT]<sub>ext</sub> levels in groups of rats locally infused with either aCSF, escitalopram, paroxetine or fluoxetine did not significantly differ between groups (one-way ANOVA for repeated measures,  $p > 0.4$ ). Neither the antagonist R-citalopram administered intraperitoneally modified the basal hippocampal [5-HT]<sub>ext</sub> during the post-treatment period. In the present experiments, escitalopram, paroxetine, and fluoxetine were locally infused by reverse dialysis with perfusate concentrations of 0.28 µM, 0.75 µM and 10 µM respectively. R-citalopram was administered intraperitoneally at a dose of 8 mg/kg. Two-way ANOVA revealed significant main effects of escitalopram ( $F_{2,16} = 15$ ,  $p < 0.01$ ) and R-citalopram ( $F_{1,8} = 12$ ,  $p < 0.01$ ), as well as a significant interaction between these treatments ( $F_{2,16} = 7.7$ ,  $p < 0.01$ ). Post-hoc comparisons with the PLSD Fisher test indicated significant increases (maximum 295% over baseline) of [5-HT]<sub>ext</sub> levels during first and second applications of escitalopram ( $p < 0.01$ ) that were significantly reduced by R-citalopram ( $p < 0.01$ , Figure 3a). Similarly, there was a significant main effect of paroxetine ( $F_{2,16} = 131$ ,  $p < 0.01$ ) and R-citalopram ( $F_{1,8} = 34$ ,  $p < 0.01$ ), as well as a significant interaction between these two treatments ( $F_{2,16} = 9.9$ ,  $p < 0.01$ ). Post-hoc comparisons with the PLSD Fisher test indicated significant increases (maximum 313% over baseline) of [5-HT]<sub>ext</sub> levels during first and second applications of paroxetine ( $p < 0.01$ ) that were significantly reduced by R-citalopram ( $p < 0.01$ , Figure 3b). Finally, two-way ANOVA showed a significant main effect of fluoxetine ( $F_{2,16} = 156$ ,  $p < 0.01$ ) and R-citalopram ( $F_{1,8} = 28$ ,  $p < 0.01$ ), as well as a significant interaction between these treatments ( $F_{2,16} = 17$ ,  $p < 0.01$ ). Post-hoc comparisons with the PLSD Fisher test indicated significant increases (maximum 181% over baseline) of [5-HT]<sub>ext</sub> levels during first and second applications of fluoxetine ( $p < 0.01$ ) that were significantly enhanced by R-citalopram ( $P < 0.01$ , Figure 3c).

#### **Discussion**

These results confirm that the suppressant effect on dorsal raphe 5-HT neurons by escitalopram is prevented by the prior administration of R-citalopram, indicating that R-citalopram counteracts the effect



**Figure 2.** Integrated firing rate histograms of dorsal raphe 5-HT neurons in naive rats showing their response to (a) paroxetine and (b) paroxetine co-administered with R-citalopram; (c) to escitalopram and (d) escitalopram co-administered with R-citalopram; (e) to fluoxetine and (f) fluoxetine co-administered with R-citalopram. The inhibitory effect of SSRIs was reversed by WAY 100635. (g) Mean values ( $\pm$ S.E.M.) of the suppressant effect of paroxetine (500 µg/kg i.v.), escitalopram (100 µg/kg i.v.), and fluoxetine (10 mg/kg i.v.) on the firing activity of 5-HT neurons in rats with or without R-citalopram (250 mg/kg i.v.). The data are expressed as percentage of basal values. The numbers at the bottom of the columns indicate the number of rats tested (in each rat only one neuron was tested). \*  $p < 0.05$  and \*\*  $p < 0.01$ , using the Mann-Whitney *U* test.



**Figure 3.** Effect of (a) escitalopram (0.28 μM), (b) paroxetine (0.75 μM) and (c) fluoxetine (10 μM) on  $[5\text{-HT}]_{\text{ext}}$  levels in microdialysates from the ventral hippocampus of anaesthetized rats. Note the significant increase in  $[5\text{-HT}]_{\text{ext}}$  compared to baseline (\*\*  $p < 0.01$ ). Systemic administration of R-citalopram significantly reduced the enhancing action of escitalopram, paroxetine but not fluoxetine, on  $[5\text{-HT}]_{\text{ext}}$  levels (#  $p < 0.01$ ). All sample values calculated as the amount of 5-HT outflow collected from the ventral hippocampus are expressed as a percentage of basal values ( $\pm$ S.E.M.),  $n = 5$  rats in all groups.

of escitalopram in the racemate (El Mansari et al., 2005). These results are extended in the present study by showing that R-citalopram antagonizes the inhibitory effect of paroxetine, but not fluoxetine, on dorsal raphe neuronal activity. The same antagonistic activity of R-citalopram was also shown in the present study at 5-HT nerve terminals in the 5-HT projecting area, namely the ventral hippocampus. R-citalopram alone produces no increase in 5-HT above baseline levels, but upon co-administration attenuates the increase in  $[5\text{-HT}]_{\text{ext}}$  levels induced by escitalopram and paroxetine, but not fluoxetine. These results confirm and extend results showing the blockade by R-citalopram of the increase in 5-HT levels induced by escitalopram in other post-synaptic 5-HT projecting areas, such as the frontal cortex (Mørk et al., 2003).

The blocking effect of R-citalopram is consistent with its action reported in validated animal models of depression and anxiety. For instance, R-citalopram attenuated the anxiolytic-like effect of escitalopram in rat chronic mild stress model (Sánchez et al., 2003a), in the rat ultrasonic vocalization model (Sánchez, 2003), and in maternally separated mouse pups (Fish et al., 2004). Moreover, in the CA1 region of the dorsal hippocampus, we showed also that R-citalopram did not modify in-vivo basal synaptic transmission by itself, but counteracted the escitalopram-induced decrease of long-term potentiation (LTP; Mnie-Filali et al., 2005). Finally, inhibition of escitalopram activity by R-citalopram has also been observed in rodents using a model based on the potentiation of serotonin syndrome by 5-HTP (Sánchez and Kreilgaard, 2004), in fact, R-citalopram antagonized the potentiation of 5-HTP-induced behaviour by escitalopram, but not fluoxetine (Cremers et al., 2004; Stórustovu et al., 2004). Altogether, these results indicate that R-citalopram may interfere with the inhibitory activity of escitalopram and paroxetine, but not fluoxetine, both presynaptically in the dorsal raphe nucleus and in the 5-HT-innervated structures.

Although some possibilities have been suggested, the mechanism by which R-citalopram prevents the effect of escitalopram on 5-HT transmission is not yet fully elucidated. However, previous findings suggested that R-citalopram may interfere with the inhibitory activity of escitalopram at the 5-HT transporter (Mørk et al., 2003; for review see Sánchez et al., 2004). In-vitro studies, using brain neuronal and platelet membranes, have revealed the existence of at least two binding sites for citalopram on hSERT; a primary high-affinity binding site that mediates the inhibition of 5-HT reuptake, and an allosteric

low-affinity binding site that modulates the binding of ligands at the primary site (Plenge and Møllerup, 1985; Plenge et al., 1991; Wennogle and Meyerson, 1985). Moreover, recent studies in COS-1 cells expressing hSERT using radioligand binding have shown that both citalopram enantiomers bind to an allosteric site, and both decreased the dissociation rate of [<sup>3</sup>H]escitalopram from the primary site on the transporter. However, the allosteric potency of escitalopram is about five times higher than that of R-citalopram (Chen et al., 2005a,b). The present in-vitro experiments extend these results by showing that R-citalopram attenuates the association of [<sup>3</sup>H]escitalopram with hSERT, thus counteracting the action of escitalopram. A similar effect of R-citalopram is observed for the association of [<sup>3</sup>H]paroxetine with hSERT, in contrast, no effect was observed for the association rates for fluoxetine, sertraline and venlafaxine. The inactive enantiomer of paroxetine, (+)paroxetine, also attenuates the association rate of [<sup>3</sup>H]escitalopram, but has no effect on the association of [<sup>3</sup>H]paroxetine. The effects of R-citalopram and (+)paroxetine on association rates correlate well with their observed antagonistic properties reported in the present electrophysiological and microdialysis studies, mentioned above. It seems that the allosteric action of citalopram might be dual. During association, when the primary hSERT binding site is unoccupied, the negative allosteric effect of R-citalopram is high, with an EC<sub>50</sub> value of around 100 nM, which is within the range measured during treatment with citalopram. On the other hand, when the primary site is occupied by escitalopram, the allosteric inhibition is positive by nature; however, the EC<sub>50</sub> values for the effects on dissociation rates are in the micromolar range, that is, ~4 μM for escitalopram and ~20 μM for R-citalopram (Chen et al., 2005b). If the allosteric effect on dissociation is of therapeutic importance, this is likely to be the case only for escitalopram. Thus, in terms of hSERT occupancy measurements, this raises the possibility that during association with hSERT, R-citalopram counteracts the 5-HT reuptake inhibitory potential of escitalopram, which in this study is observed as a delay in the suppression of dorsal raphe firing rates and a reduction in [5-HT]<sub>ext</sub> levels compared to the effects of escitalopram administered separately. During steady state, escitalopram has a potentially stabilizing effect on the [<sup>3</sup>H]escitalopram/hSERT complex (Chen et al. 2005a,b), which at a functional level might translate into a self-potentiating effect.

The attenuating effect of R-citalopram, also reported in the present study, was suggested to underlie

the delayed recovery of 5-HT neuron firing activity following long-term treatment with citalopram vs. escitalopram (El Mansari et al., 2005; Haddjeri et al., 2005). The higher level of 5-HT that escitalopram can induce vs. citalopram could mean that somatodendritic 5-HT<sub>1A</sub> autoreceptors involved in counteracting the acute SSRI-induced increase of 5-HT levels are more rapidly desensitized. Indeed, in our previous in-vivo electrophysiological study (El Mansari et al., 2005), it was shown that there was a complete recovery of firing activity of 5-HT neurons following a 2-wk escitalopram treatment, but only after 3-wk with citalopram. Furthermore, it was reported that R-citalopram significantly attenuated the recovery of firing observed after 2 wk of treatment with escitalopram (Haddjeri et al., 2005). It is possible that in absence of R-citalopram, escitalopram can potentiate its own effect by binding to the allosteric site of the transporter, whereas fluoxetine has no measurable effect on binding of [<sup>3</sup>H]fluoxetine at an allosteric site on the transporter (Chen et al., 2005a). Interestingly, 5-HT neuron firing recovery and 5-HT<sub>1A</sub> autoreceptor desensitization was also first observed after a 3-wk treatment with fluoxetine (Czachura and Rasmussen, 2000). Fluoxetine is practically devoid of a stabilizing effect on the radioligand/hSERT complex with any radioligand complex, including [<sup>3</sup>H]fluoxetine. In competition experiments, fluoxetine does not inhibit the allosteric effect of escitalopram, thus strengthening the hypothesis that the effect of R-citalopram on escitalopram, unlike its effect on fluoxetine, can be via an allosteric mechanism (Chen et al., 2005a,b). In contrast to a previous study in the ventral hippocampus (Cremers et al., 2004), our microdialysis study showed an increase in 5-HT levels in response to co-administration of fluoxetine and R-citalopram. This discrepancy can stem from the fact that different methods were used to measure [5-HT]<sub>ext</sub> (on-column laser-induced fluorescence detection vs. high-performance liquid chromatography with electrochemical detection) and/or difference in drug administration route (local vs. i.p.). However, 5-HTP-induced behavioural response was augmented by the addition of R-citalopram to fluoxetine indicating that the antagonistic effect of R-citalopram does not extend to fluoxetine, but appears to be specific for escitalopram. Furthermore, experiments measuring the inhibition of 5-HT-elicited currents in *Xenopus* oocytes expressing hSERT protein, suggested that R-citalopram antagonizes the inhibition of hSERT by escitalopram but not fluoxetine, suggesting an additive (or synergistic) action with this SSRI (Stórustovu et al., 2004).

In conclusion, the present in-vivo studies show that R-citalopram counteracts the activity of escitalopram and paroxetine, but not fluoxetine, probably via the allosteric binding site of the 5-HT transporter, both in the dorsal raphe nucleus and ventral hippocampus. These results are in agreement with the observation that R-citalopram attenuated the association rates of escitalopram and paroxetine but not fluoxetine, to the 5-HT transporter. Further experiments are needed to elucidate the mechanisms by which the allosteric modulation takes place.

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### Statement of Interest

None.

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