Characterization of the electrophysiological properties of triple reuptake inhibitors on monoaminergic neurons

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Abstract
Triple reuptake inhibitors represent a potential new class of antidepressant drugs that block norepinephrine (NE), dopamine (DA) and serotonin [5-hydroxytryptamine (5-HT)] transporters. The present in-vivo electrophysiological study was undertaken to determine the effects of the triple reuptake inhibitors SEP-225289 and DOV216303 on the neuronal activities of locus coeruleus (LC) NE, ventral tegmental area (VTA) DA and dorsal raphe (DR) 5-HT neurons. Administered acutely, SEP-225289 and DOV216303 dose-dependently decreased the spontaneous firing rate of LC NE, VTA DA and DR 5-HT neurons through the activation of \( \alpha_2 \), \( D_2 \) and 5-HT\textsubscript{1A} autoreceptors, respectively. Both compounds predominantly inhibited the firing rate of LC NE neurons while producing only a partial decrease in VTA DA and DR 5-HT neuronal discharge. SEP-225289 was equipotent at inhibiting 5-HT and NE transporters since it prolonged to the same extent the time required for a 50\% recovery (RT\textsubscript{50}) of the firing activity of dorsal hippocampus CA3 pyramidal neurons from the inhibition induced by microiontophoretic application of 5-HT and NE. Finally, in the presence of WAY100635, a 5-HT\textsubscript{1A} receptor antagonist, SEP-225289 activated 5-HT neurons at doses that normally did not inhibit them. Taken together, the present results indicate that reciprocal interactions among NE, DA and 5-HT inputs need to be considered to anticipate the net effect of triple reuptake inhibitors on the enhancement of brain monoamine transmission. The results also suggest that the therapeutic action of triple reuptake inhibitors may be potentiated by antagonizing the cell body 5-HT\textsubscript{1A} autoreceptors.

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Introduction
Over the last 40 yr, many attempts to understand the pathophysiology of depression and the mechanisms of action of antidepressants have focused on brain monoamines. As a result, the majority of pharmacological agents that are now used in the treatment of mood disorders inhibit serotonin [5-hydroxytryptamine (5-HT)] and/or norepinephrine (NE) reuptake. Despite their effectiveness, single-action agents display some limits such as residual symptoms that do not allow complete remission in depressed patients (Frazer, 2001). A second generation of compounds targeting both monoamines (5-HT and NE) has therefore been developed with the aim of producing more robust effects (Chen & Skolnick, 2007). Among these antidepressants, venlafaxine was proposed to be significantly more effective than selective serotonin reuptake inhibitors (SSRIs) in depressed patients (Bauer et al, 2009; Mazeh et al, 2007; Montgomery et al, 2007; Fournier & Boyer, 1999; Smith et al, 2002). Although these findings suggest that the therapeutic efficacy of antidepressant drugs might depend on their capacity to simultaneously enhance brain 5-HT and NE transmission, this hypothesis remains controversial. Indeed, a recent meta-analysis emphasizes that venlafaxine, duloxetine, and milnacipran have a modest efficacy advantage compared to SSRIs in mood disorders (Papakostas et al, 2007).
Multiple lines of evidence indicate that dopamine (DA) also plays a role in the pathophysiology and treatment of depression. For example, reduced levels of DA and its metabolite were observed in the cerebrospinal fluid of depressed patients (Kapur & Mann, 1992; Roy et al. 1992; Willner, 1997). Moreover, pharmacological interventions that block central dopaminergic transmission or decrease brain DA levels produce depressive symptoms (Jimerson, 1984; Kapur & Mann, 1992) raising the possibility that a dopaminergic deficiency is an important factor in mood disorders. In agreement with this hypothesis, it has been reported that the prevalence of depression can reach up to 50% in individuals suffering from Parkinson’s disease (McDonald et al. 2003). In contrast, clinical studies indicate that it is possible to achieve an antidepressant action by enhancing DA neurotransmission. This is supported by several reports of the augmenting action of the D3 receptor agonists such as pramipexole in treatment-resistant patients (Cassano et al. 2004; Goldberg et al. 2004; Lattanzi et al. 2002; Perugi et al. 2001; Sporn et al. 2000; Zarate et al. 2004), and by the intrinsic antidepressant activity of these drugs in placebo-controlled trials (Bouras & Bridges, 1992; Corrigan et al. 2000).

These clinical studies support the development of a third generation of antidepressants: the triple reuptake inhibitors that simultaneously inhibit the serotonin transporter (SERT), NE transporter (NET) and DAT is as follows [IC50 values: 11, 6, 4 nM and 13, 20, 78 nM for SEP-225289 and DOV216303, respectively (Schreiber et al. 2009; Skolnick et al. 2003)].

Material and methods

Animals

Male Sprague–Dawley rats (Charles River, Canada) weighing 250–300 g, were used for the experiments. They were kept under standard laboratory conditions (12-h light/dark cycle, lights on 07:00 hours, with food and water available ad libitum) and handled according to the guidelines of the Canadian Council on Animal Care (CCAC). Protocols in this study were approved by the local Animal Care Committee (Ottawa Health Research Institute, Canada).

Test articles

SEP-225289 and DOV216303 were provided by Sepracor Inc. (USA). All other compounds used in the present study such as 5-HT creatinine sulfate, L-NE hydrochloride, quisqualic acid, WAY100635, idazoxan and haloperidol were purchased from Sigma (Canada).

In-vivo electrophysiological recordings

Rats were anaesthetized with chloral hydrate (400 mg/kg i.p.) and placed into a stereotaxic frame. The extracellular recordings of 5-HT, DA and NE neurons in the dorsal raphe (DR), the ventral tegmental area (VTA) and the locus coeruleus (LC), respectively, were performed using single-barrelled glass micropipettes (Stoelting, USA) preloaded with a 2 M NaCl solution. Their impedance typically ranged between 4–7 MΩ. The extracellular recordings of pyramidal neurons in the CA3 region of the hippocampus were performed using multi-barrelled glass micropipettes. The central barrel used for extracellular
unitary recording was filled with 2 M NaCl solution. One side barrel, filled with 2 M NaCl solution, was used for automatic current balancing. The three other side barrels were filled with NE [L-NE HCl, 20 mM in 0.2 M NaCl (pH 4)] or 5-HT creatinine sulfate [25 mM in 0.2 M NaCl (pH 4)]. NE and 5-HT were ejected as cations and retained with currents of 0.8 to 10 nA. Quis was ejected as an anion and retained with a current of +5 nA. The impedance of the central barrel was 2–5 MΩ and those of the balance barrel and side barrels were 20–30 MΩ and 50–100 MΩ, respectively.

Recording of DR 5-HT neurons

The single-barrelled glass micropipette was positioned using the following coordinates (in mm from lambda): AP, +1.0 to 1.2; L, 0 ± 0.1; V, 5 to 7. The presumed 5-HT neurons were then identified using the following criteria: a slow (0.5–2.5 Hz) and regular firing rate and long-duration (2–5 ms) bi- or triphasic extracellular waveform (Aghajanian & Vandermaelen, 1982b). As previously demonstrated, 5-HT neurons may display a bursting activity (Hajos et al., 2007). This occasional firing pattern of 5-HT neurons was analysed by spike interval burst analysis following the criteria set by Hajos et al. (2007).

Recording of VTA DA neurons

The single-barrelled glass micropipette was positioned using the following coordinates (in mm from lambda): AP, −1.0 to −1.2; L, 1.0 to 1.3; V, 5 to 7. Spontaneously active NE neurons were identified using the following criteria: regular firing rate (0.5–5.0 Hz) and positive action potential of long duration (>2.5 ms) exhibiting a brisk excitatory response to a nociceptive pinch of the contralateral hindpaw (Aghajanian & Vandermaelen, 1982a). Single-unit activity was recorded as discriminated action potentials amplified by a MDA3 amplifier (Bak Instrument, USA), post-amplified and filtered by an Audiosource equalizer, digitalized by a CED1401 interface system (Cambridge Electronic Design, UK), and processed online by Spike2 software for PC Windows. Data on single-spike firing rates and burst firing activity were analysed offline. Using a script developed for Spike2, a burst for VTA DA and LC NE neurons was identified as the occurrence of two spikes with an inter-spike interval <0.08 s. The termination of a burst was defined as an inter-spike interval of ≥0.16 s. As mentioned above, DR 5-HT neurons occasionally discharge in a brief burst of action potentials with a very short inter-spike time-interval (typically <10 ms). The electrophysiological characteristics of presumed monoaminergic neurons recorded in the rat DR, VTA and LC are depicted in Table 1.

Table 1. Electrophysiological characteristics of presumed monoaminergic neurons recorded in the rat DR, VTA and LC

<table>
<thead>
<tr>
<th>Monoaminergic neurons</th>
<th>DR 5-HT neurons (n = 19)</th>
<th>VTA DA neurons (n = 22)</th>
<th>LC NE neurons (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous firing rate (Hz)</td>
<td>1.5 ± 0.1</td>
<td>5.1 ± 0.4</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Waveform duration (ms)</td>
<td>2.5 ± 0.1</td>
<td>4.2 ± 0.5</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>% of neurons exhibiting bursting activity</td>
<td>15 (n = 3)</td>
<td>100 (n = 22)</td>
<td>31 (n = 5)</td>
</tr>
<tr>
<td>Number of bursts/min</td>
<td>12 ± 3</td>
<td>38 ± 4</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Number of single spikes within burst</td>
<td>2 ± 0.0</td>
<td>3.5 ± 0.6</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

DR, dorsal raphe; VTA, ventral tegmental area; LC, locus coeruleus. n = number of neurons recorded.
Recording of pyramidal neurons of the CA3 region of the dorsal hippocampus

The five-barrelled glass micropipettes were positioned using the following coordinates (in mm from bregma): AP, 3.8 to 4.5; L, 4 to 4.2; V, 3 to 4.5. Quis-stimulated pyramidal neurons were identified by their high amplitude (0.5–1.2 mV), high frequency (8–12 Hz) and long duration (0.6–1.0 ms) action potential and by their characteristic complex spike discharge (Kandel et al. 1961).

Assessment of neuronal responsiveness

In the DR, VTA and LC, the percent of baseline firing rate was measured 60 s after systemic administration of the triple reuptake inhibitors. In the DR various parameters were determined to examine the electrophysiological effects of co-administration of SEP-225289 with WAY100635. These parameters included the number of single spikes, bursts, cells per track and the firing rate of 5-HT neurons. In the dorsal hippocampus another parameter was used to assess neuronal responsiveness to microiontophoretic application: the RT<sub>50</sub> value (in seconds) which represents the time required for the firing activity to recover by 50% from the cessation of the microiontophoretic application of 5-HT or NE (Pineyro et al. 1994). In the present study, the RT<sub>50</sub> value was used to provide an index of the capacity of NE and 5-HT terminals in the dorsal hippocampus to remove NE or 5-HT from the synaptic cleft through their respective reuptake transporter in the presence or absence of the triple reuptake inhibitor SEP-225289.

Statistical analysis

Statistical analyses were performed using the computer software StatView 5.0. (Abacus Concepts Inc., USA). Electrophysiological data expressed as means ± S.E.M. of percent of baseline firing rate, as means ± S.E.M. of single spikes or bursts or as means ± S.E.M. of the RT<sub>50</sub> parameter measured from the same neurons. Data were analysed by using a one- or two-way analysis of variance (ANOVA), with treatment, treatment and pre-treatment or treatment and brain region as main factors. Fisher’s protected least significant difference (PLSD) test was used to analyse the statistical significance between groups. The paired Student’s t test was used in experiments comparing two groups. In each experiment, a level of p < 0.05 was accepted as evidence for a statistically significant effect.

Results

Assessment of the effects of the triple reuptake inhibitors SEP-225289 and DOV216303 on the spontaneous firing rate of LC NE, VTA DA and DR 5-HT neurons

SEP-225289

A two-way ANOVA for the percent of basal firing rate of monoamine neurons indicated an overall significant inhibitory effect of treatment [F(4, 78) = 45.5, p < 0.001] and region [F(2, 78) = 25.7, p < 0.001] factors. A significant interaction between those two independent variables was also detected [F(8, 78) = 3.3, p < 0.01].

In LC, cumulative doses of SEP-225289 decreased the spontaneous firing rate of NE neurons with a significant effect observed at all doses tested (1–8 mg/kg; Fig. 1a–d). This suppressant effect was reversed by the α<sub>2</sub>-adrenoceptor antagonist idazoxan confirming the adrenergic nature of the inhibition.

In the VTA and DR, SEP-225289 also produced a significant inhibition of DA and 5-HT neurons, respectively, and this effect became apparent from the dose of 3 mg/kg (Fig. 1d). As depicted in Fig. 1b, c, SEP-225289-induced inhibition of DA and 5-HT neuronal activities was completely reversed by the selective D<sub>1</sub> and 5-HT<sub>1A</sub> receptor antagonists, haloperidol and WAY100635, respectively. It is noteworthy that for all doses tested, the inhibitory effects of SEP-225289 in the LC were significantly more pronounced than those observed in the VTA or the DR (Fig. 1d).

DOV216303

A two-way ANOVA for the percent of basal firing rate of monoamine neurons indicated an overall significant inhibitory effect of treatment [F(3, 87) = 52.6, p < 0.001] and region [F(2, 87) = 32.2, p < 0.001] factors. A significant interaction between those two independent variables was also detected [F(6, 87) = 4.7, p < 0.001].

DOV216303 significantly reduced the spontaneous firing rate of LC NE, VTA DA and DR 5-HT neurons at all doses tested except for the VTA and DR whose decreases were detected from the dose of 5 mg/kg (Fig. 2).

As observed with SEP-225289, the inhibition of neuronal activities induced by DOV216303 for all doses tested was significantly higher in LC than in VTA or DR (Fig. 2).

The present results showed that relatively high doses of the triple reuptake inhibitors SEP-225289 and DOV216303 are required to inhibit the electrical activity of 5-HT and NE neurons, in comparison to some other single- or dual-reuptake inhibitors (El Mansari
Assessment of the action of SEP-225289 on the firing rate of CA3 pyramidal neurons of the dorsal hippocampus and the reuptake of 5-HT and NE

The recovery time (RT), from the suppression of hippocampus pyramidal neuron firing activity following microiontophoresis application of 5-HT and NE, was assessed by determining the RT values before and after the acute intravenous administration of cumulative doses of SEP-225289 (1–8 mg/kg). Although SEP-225289 (1 and 2 mg/kg) did not modify the firing activity of CA3 pyramidal neurons, a significant reduction (~50%) was detected with the highest dose (8 mg/kg; Fig. 3a). As previously described, microiontophoretic applications of 5-HT and NE resulted in a suppression of the firing activity of CA3 pyramidal neurons which gradually recovered upon the cessation of the application. Two-way ANOVA analysis on RT values normalized for microiontophoretically applied 5-HT or NE indicated a significant effect of SEP-225289 (3.1 = 3.1, p < 0.05; Fig. 3a, b), but no significant difference between monoamines [F(3, 32) = 1.1, p = 0.3]. Indeed, SEP-225289 similarly decreased 5-HT and NE uptake to a similar extent independently of the dose.

These findings suggest that in vivo, SEP-225289 was equipotent at inhibiting 5-HT and NE transporters. These observations ruled out the possibility that the weak inhibition of DR 5-HT firing rate induced by SEP-225289 was related to a low potency at blocking SERT. An alternative hypothesis would be that an increase in extracellular DA and/or NE levels, which are excitatory in the DR (Katz et al. 2009), had counterbalanced the inhibitory effect of SEP-225289 on 5-HT neuronal activity.

Excitatory effects of SEP-225289 on 5-HT neuronal activity in presence of the 5-HT1A receptor antagonist WAY100635

In order to determine whether the enhancement of DA and/or NE limited the inhibitory effects of SEP-225289 on the firing rate of 5-HT neurons, its electrophysiological effects in the DR were evaluated in rats significantly different from the spontaneous firing rate of 5-HT neurons; + indicates that at each dose tested the inhibition of the firing rate of NE neurons was significantly higher than that of DA or 5-HT neurons.
pre-treated with the 5-HT<sub>1A</sub> receptor antagonist WAY100635. A one-way ANOVA for the percent of basal firing rate for DR 5-HT neurons indicated an overall significant excitatory effect of treatment factor \[F(3, 20) = 8.2, p < 0.001\].

Fig. 2. Comparison of the effects of DOV216303 on the firing activity of LC NE, VTA DA and DR 5-HT neurons. (a-c) Examples of integrated firing histograms showing the effects of cumulative intravenous doses of DOV216303 on the spontaneous activity of NE, DA and 5-HT neurons. At the end of each recording, the reversal effects of the \(a_2\), \(D_2\) or 5-HT<sub>1A</sub> receptor antagonists on DOV216303-induced inhibition of monoaminergic firing rate was also indicated. In these firing rate histograms, the arrows indicate the compounds administered and the time at which the injection of the specified doses was complete. (d) Symbols represent the mean (± S.E.M.) of percent of basal firing rate observed at each dose in the LC (●, \(n = 9\)), VTA (●, \(n = 15\)) and DR (○, \(n = 9\)). These means were measured on the 60-s period preceding each DOV216303 administration. *** \(p < 0.001\), significantly different from the spontaneous firing rate of NE neurons; ** \(p < 0.01\), # \(p < 0.05\), significantly different from the spontaneous firing rate of DA neurons; **** \(p < 0.001\), significantly different from the spontaneous firing rate of 5-HT neurons; + indicates that at each dose tested the inhibition of the firing rate of NE neurons was significantly higher than that of DA or 5-HT neurons.

Fig. 3. In-vivo blocking activity of SEP-225289 towards 5-HT and NE transporters. (a) Example of integrated firing histograms showing the effects of microiontophoretically applied 5-HT and NE before and after intravenous cumulative doses of SEP-225289 on the spontaneous activity of CA3 pyramidal neurons. White and black squares above the peaks indicate the duration of iontophoretic ejection of 5-HT and NE, respectively. In these firing rate histograms, the arrows indicate the time at which the injection of SEP-225289 was completed. (b) Data are mean (± S.E.M.) of normalized RT<sub>50</sub> values calculated from microiontophoretic applications of 5-HT (●) and NE (■) after SEP-225289. * \(p < 0.05\), significantly different from SEP-225289 (1 mg/kg i.v.); n.s., not statistically significant (\(n = 5–6\) rats per group).
Fig. 4. Effect of 5-HT<sub>1A</sub> receptor inactivation on the electrophysiological effects of SEP-225289 on DR 5-HT firing rate. (a) Example of integrated firing histograms showing the effects of cumulative intravenous doses of SEP-225289 on the spontaneous activity of DR 5-HT neurons in rats pre-treated with the 5-HT<sub>1A</sub> receptor antagonist WAY100635. In these firing rate histograms, the arrows indicate the compounds administered and the time at which the injection of the specified doses was completed. (b) Symbols represent the mean (± S.E.M.) of percent of increase in basal firing rate observed at each dose in the DR (●, ●, ●; n = 7). These means were measured on the 60-s period preceding each SEP-225289 administration. * p < 0.05, ** p < 0.01, *** p < 0.001, significantly different from the spontaneous firing rate of 5-HT neurons. (c, d) Data are mean (± S.E.M.) of the number of single spikes (c) and number of bursts (d) of 5-HT neurons. * p < 0.05, ** p < 0.01, significantly different from SEP-225289 pre-injection value using a one-way ANOVA followed by Fisher’s PLSD test.

In rats pre-treated with WAY100635, SEP-225289 (0.5–2 mg/kg i.v.) elicited a significant increase in DR 5-HT firing rate (Fig. 4a, b). To address the possibility that the excitatory effect of SEP-225289 was due to an alteration of single spike and/or burst activity, a more detailed analysis was performed. Two separate one-way ANOVAs indicated an overall significant effect of treatment factor for the number of single spikes [F(3, 20) = 4.5, p < 0.01] and the number of bursts [F(3, 20) = 3.1, p < 0.05]. Thus, in rats pre-treated with WAY100635, SEP-225289 significantly increased the number of single spikes and bursts (Fig. 4c, d). In agreement with previous findings (Haddjeri et al. 2004), neither the spontaneous firing rate of DR 5-HT neurons (1.4 ± 0.1 vs. 1.3 ± 0.1; n = 8), nor the number of single spikes (80.4 ± 7.2 vs. 76.9 ± 10.1; n = 8) and bursts (3.1 ± 0.4 vs. 2.8 ± 0.5; n = 8) were altered by WAY100635 (100 µg/kg i.v.) when given alone, in comparison to baseline.

Supporting the excitatory effects of SEP-225289 on DR 5-HT neurons in conditions of 5-HT<sub>1A</sub> autoreceptor blockade, there was a significant increase in the number of neurons recorded per track and their mean firing rate after administration of SEP-225289 (Table 2). As previously reported in drug-naive rats, a subpopulation of DR 5-HT neurons discharged in single-spike mode and bursting activity with two (doublets) or occasionally three spikes (Ghanbari et al. 2007). In the present study, about 15% of 5-HT neurons (6/38) displayed a bursting activity. This percentage was doubled by the combination of WAY100635 and SEP-225289 (12/36 neurons) (Table 2).

Discussion

The results of the present study showed that the acute administration of the triple reuptake inhibitors SEP-225289 and DOV216303 reduced the firing rate of NE, DA and 5-HT neurons through the activation of α<sub>2</sub>, D<sub>4</sub> and 5-HT<sub>1A</sub> autoreceptors, respectively. Both compounds exerted a predominant effect in the LC since they almost completely inhibited the firing rate of NE neurons, while producing only a partial decrease in VTA DA and DR 5-HT neuronal activities. The unexpected moderate inhibitory effect of SEP-225289 in the
latter regions was not due to a distinct blockade of 5-HT and NE transporters since this triple reuptake inhibitor similarly prolonged both catecholamine-induced decreases in CA3 pyramidal neurons’ firing rate. This is consistent with in-vitro data showing that in CHO cells, SEP-225289 inhibits the 5-HT and NE transporters with an IC50 of 11 and 6 nM, respectively (Schreiber et al. 2009).

SEP-225289 and DOV216303 are two novel molecules that simultaneously inhibit the reuptake of NE, DA and 5-HT, thus enhancing brain monoamine levels and prolonging their duration of action at pre- and post-synaptic levels (Schreiber et al. 2009). The observation that both compounds dose-dependently attenuated the firing activity of monoaminergic neurons emphasizes their ability to penetrate the blood-brain barrier and effectively block, in vivo, all three monoaminergic transporters. Given the pharmacological profiles of SEP-225289 and DOV216303, it was anticipated that their inhibitory effects in the LC, VTA and DR would be indirectly mediated in each nucleus by stimulating somatodendritic autoreceptors, as observed with selective reuptake inhibitors [El Mansari et al. (2005); norepinephrine reuptake inhibitor (NRIs), Szabo & Blier (2001); dopamine reuptake inhibitor (DRI), Einhorn et al. (1988)]. This contention was supported by the fact that SEP-225289- and DOV216303-induced attenuation of LC NE, VTA DA and DR 5-HT firing rates was reversed by the selective α2, D4 and 5-HT1A receptor antagonists, respectively. SEP-225289 and DOV216303 preferentially reduced the firing rate of LC NE neurons, despite the similar affinities of the former drug for all three transporters. The weak potency of SEP-225289 and DOV216303 at inhibiting DA neurons in the VTA is not really surprising. Indeed, there is considerable evidence from electrophysiological studies indicating that the apparent lack of complete inhibition is a feature of DA neurons.

As an example, the systemic administration of the selective DRI GBR12909 (Einhorn et al. 1988) or the local application D4 receptor agonists in the VTA (Aghajanian & Bunney, 1977a, b; White & Wang, 1984a, b) produce a partial decrease in the firing rate of DA neurons. It is well established that the VTA receives a dense noradrenergic and serotonergic innervation (Adell & Artigas, 2004; Esposito, 2006) and that the local activation of α2-adrenergic (Grenhoff et al. 1995; Steffensen et al. 1998) or 5-HT2C receptors increases the firing rate of DA neurons (Di Giovanni et al. 2000; Di Matteo et al. 2000; Pessia et al. 1994). Indirect excitatory mechanisms might also be involved to explain the dampened effects of SEP-225289 and DOV216303 on DA neuronal activity involving, for example, the laterodorsal tegmentum (LDT) and the medial prefrontal cortex (mPFC) which project heavily to the VTA (Bortolozzi et al. 2005; Diaz-Mataix et al. 2005; Omelchenko & Sesack, 2005). Taken together, these results support the possibility that an increase in NE and/or 5-HT release in the rat brain could limit the electrophysiological effect of acutely administered SEP-225289 and DOV216303 on VTA DA neurons. Inhibitory α2-adrenergic and 5-HT2A receptors have, however, been detected in the VTA, and evidence suggests that the enhancement of NE and/or 5-HT transmission produces an overall net inhibitory influence on the firing rate of VTA DA neurons (Guiard et al. 2008; Ugedo et al. 1989). Consequently, it is likely that the moderate effect of these triple reuptake inhibitors on VTA DA neurons resulted from a balance between excitatory and inhibitory monoaminergic inputs. It is important to note that in the present study rats were anaesthetized with chloral hydrate. This condition might have altered the activity of DA neurons and their response to SEP-225289 or DOV216303.

### Table 2. Effect of SEP-225289 on the activity of the serotonergic system in presence of the 5-HT1A receptor antagonist WAY100635

<table>
<thead>
<tr>
<th>DR 5-HT neurons</th>
<th>Before WAY100635 + SEP-225289</th>
<th>After WAY100635 + SEP-225289</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons per tract</td>
<td>6.3 ± 1.1 (n = 38)</td>
<td>11.6 ± 1.2* (n = 36)</td>
</tr>
<tr>
<td>Firing rate (Hz)</td>
<td>1.2 ± 0.1</td>
<td>1.8 ± 0.1***</td>
</tr>
<tr>
<td>Neurons exhibiting bursting activity per track</td>
<td>1 ± 0.2 (n = 6)</td>
<td>4 ± 0.5*** (n = 8)</td>
</tr>
</tbody>
</table>

Data are mean (±S.E.M.) of the number of neurons recorded per track, of the number of neurons discharging with a bursting mode and of all neurons firing rate (in Hz).

* p < 0.05, *** p < 0.001 significantly different from pre-treatment value using Student’s t test.
since electrophysiological and neurochemical evidence suggest that chloral hydrate interferes with the dopaminergic system (Chen & Kandasamy, 1996; Fa et al. 2003; Kelland et al. 1989; Sabeti et al. 2003). In particular, it has been shown that chloral hydrate could reduce DA reuptake (Sabeti et al. 2003) or increase the potency of D₃ receptor agonists such as apomorphine or quinpirole (Quin) in inhibiting the firing rate of DA neurons (Kelland et al. 1989). However, these effects of chloral hydrate should have contributed to enhance the inhibitory action of SEP-225289 or DOV216303 on the firing rate of VTA DA neurons.

The observation that even high doses of SEP-225289 or DOV216303 failed to completely inhibit 5-HT neurons is more puzzling, in particular because the majority of SSRIs, such as escitalopram and citalopram (El Mansari et al. 2005), paroxetine (Gartside et al. 1997) or fluoxetine (Smith & Lakoski, 1997) as well as the serotonin NR1s (SNR1s) venlafaxine (Béique et al. 1999) and duloxetine (Kasamo et al. 1996) produce a complete suppression of firing. The blunted effect of the triple reuptake inhibitors on DR 5-HT neurons reported herein might thus be due to their activity at both catecholaminergic transporters. In-vitro studies performed in rat synaptosomes showed that SEP-225289 and DOV216303 are potent and fairly balanced 5-HT/NE/DA reuptake inhibitors (Schreiber et al. 2009; Skolnick et al. 2003). In particular, in-vitro functional studies have reported that SEP-225289 and DOV216303 inhibit the [³H]5-HT and [³H]NE uptake in HEK-293 cells expressing the corresponding human recombinant transporters with IC₅₀ values of 11 and 10 nm for SERT and 6 and 23 nm for NET, respectively. To confirm these results, the present experiments compared the direct effect of SEP-225289 on 5-HT and NE reuptake in the hippocampus. It has indeed been established that the RT₅₀ value provides a reliable index to assess, in vitro, the uptake activity following microiontophoretic applications of both 5-HT (Pineyro et al. 1994) and NE (de Montigny et al. 1980) on CA3 pyramidal neurons. In the present study, despite the relatively small number of rats (n=5–6), it was observed that SEP-225289 prolonged such RT₅₀ values to a similar extent thus confirming its dual reuptake blocking properties and its putative equipotency at inhibiting 5-HT and NE transporters. It is thus conceivable that the lesser than expected activity of SEP-225289 or DOV216303 on the firing activity of 5-HT neurons resulted, at least in part, from the accumulation of DA and/or NE which could be excitatory in the DR. In the present study, this putative property of catecholamines on 5-HT neuronal activity was investigated by determining the electrophysiological effects of SEP-225289 on DR 5-HT neurons in the presence of the 5-HT₁A receptor antagonist WAY100635. As shown in Fig. 4b, when SEP-225289 was administered to rats following an acute intravenous administration of WAY100635, the discharge of 5-HT neurons was markedly increased. In addition, the mean number of 5-HT neurons recorded per track and their firing rate was significantly enhanced after the combination of WAY100635 with SEP-225289, therefore suggesting that a subpopulation of 5-HT neurons can be activated in these pharmacological conditions. Moreover, the excitatory effect of SEP-225289, unveiled in the presence of WAY100635, was characterized by an increase in the number of 5-HT neurons discharging in a doublet spiking activity. Such effects may enhance 5-HT release at nerve terminals to an extent greater than if only single spiking activity had been increased (Gartside et al. 2000). In accord with this hypothesis, it has recently been demonstrated that the increase in cortical extracellular 5-HT concentrations induced by the concomitant blockade of 5-HT, NE and DA transporters is potentiated by the addition of WAY100635 to the mix (Weikop et al. 2007a, b). Altogether, these results put into evidence that the enhancement of noradrenergic and/or dopaminergic transmission exerted a major excitatory role in the regulation of DR, which limited the inhibitory effect of the serotonergic component of SEP-225289 on 5-HT neurons. This is consistent with previous studies having shown that both application of DA and the D₃/D₄ agonist Quin in DR slices produces a concentration-dependent membrane depolarization of 5-HT neurons (Aman et al. 2007; Haj-Dahmane, 2001). Nevertheless, although the systemic administration of D₃ receptor agonist apomorphine increased the firing rate of 5-HT neurons, when applied in the DR, it failed to increase 5-HT output (Martin-Ruiz et al. 2001). The latter result suggests that D₃ receptors located outside the DR may also control serotonergic activity. Complementing this DA-induced excitatory effect, it is well established that the DR is driven by a noradrenergic input that stimulates DR 5-HT neurons through a₁-adrenoceptors (Hopwood & Stamford, 2001; Pudovkina et al. 2003; Svensson et al. 1975; Vandermaelen & Aghajanian, 1983). Finally, the fact that bupropion, a DA and NE releaser (Dong & Blier, 2001), not only prevents but also reverses escitalopram-induced decrease in 5-HT neuronal activity (Ghanbari et al. 2008), supports the idea that the simultaneous increase in catecholaminergic transmission may counteract the electrophysiological effect of SERT inhibition in the DR.
The putative excitatory role of catecholamine in the DR has been recently further investigated. It has been shown that neither pre-treatment with the NRI reboxetine nor pre-treatment with the DRI GBR12909, at doses previously shown to elevate extracellular NE (Page & Lucki, 2002) or DA levels (Baumann et al., 1994), attenuated SSRI (escitalopram)-induced decrease in DR 5-HT firing rate. However, when NE and DA reuptake was simultaneously blocked by the dual-acting DA and NE reuptake inhibitor nomifensine, the effect of escitalopram was markedly attenuated (Katz et al., 2009). Since the DR receives noradrenergic and dopaminergic innervation and expresses both NET and DAT, the functional status of monoaminergic autoreceptors clearly exposes a functional discrepancy between triple reuptake inhibitors and single- or dual-acting agents. Whether this feature would favourably affect any antidepressant response in humans remains to be demonstrated. Preclinical studies have demonstrated antidepressant-like activity of SEP-225289 (Sepracor Inc., unpublished data) and DOV216303 (Chen & Skolnick, 2007; Skolnick & Basile, 2007; Skolnick et al., 2003, 2006) in the tail suspension test and forced swim test. Although there are no published results comparing triple reuptake inhibitors to single- or dual-acting agents in chronic depression models, this study strongly suggests that the accumulation of catecholamines in the DR in response to the administration of triple reuptake inhibitors could play a major role in preventing the deleterious offset, at least partially, in the initial inhibitory effect of the SERT blockade on 5-HT transmission. Finally, in view of the development of new antidepressant drug strategies aimed at producing medications with greater efficacy, the association of a triple reuptake inhibitor with a selective 5-HT1A autoreceptor antagonist should be envisaged. In this regard, it would now be interesting to examine the electrophysiological effect of triple reuptake inhibitors after sustained administration and the functional status of monoaminergic autoreceptors.

Conclusions

The relative weak potency of SEP-225289 and DOV216303 at inhibiting the firing rate of monoamine neurons after their acute systemic administration clearly exposes a functional discrepancy between triple reuptake inhibitors and single- or dual-acting agents. Whether this feature would favourably affect any antidepressant response in humans remains to be demonstrated. Preclinical studies have demonstrated antidepressant-like activity of SEP-225289 (Sepracor Inc., unpublished data) and DOV216303 (Chen & Skolnick, 2007; Skolnick & Basile, 2007; Skolnick et al., 2003, 2006) in the tail suspension test and forced swim test. Although there are no published results comparing triple reuptake inhibitors to single- or dual-acting agents in chronic depression models, this study strongly suggests that the accumulation of catecholamines in the DR in response to the administration of triple reuptake inhibitors could play a major role in preventing the deleterious offset, at least partially, in the initial inhibitory effect of the SERT blockade on 5-HT transmission. Finally, in view of the development of new antidepressant drug strategies aimed at producing medications with greater efficacy, the association of a triple reuptake inhibitor with a selective 5-HT1A autoreceptor antagonist should be envisaged. In this regard, it would now be interesting to examine the electrophysiological effect of triple reuptake inhibitors after sustained administration and the functional status of monoaminergic autoreceptors.

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Statement of Interest

None.

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