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Involvement of 5-HT$_3$ receptors in the action of vortioxetine in rat brain: Focus on glutamatergic and GABAergic neurotransmission

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ABSTRACT

The antidepressant vortioxetine is a 5-HT$_3$-R, 5-HT$_7$-R and 5-HT$_{1D}$-R antagonist, 5-HT$_{1B}$-R partial agonist, 5-HT$_{1A}$-R agonist, and serotonin (5-HT) transporter (SERT) inhibitor. Vortioxetine occupies all targets at high therapeutic doses and only SERT and 5-HT$_3$-R at low doses. Vortioxetine increases extracellular monoamine concentrations in rat forebrain more than selective serotonin reuptake inhibitors (SSRI) and shows pro-cognitive activity in preclinical models. Given its high affinity for 5-HT$_3$-R (Ki = 3.7 nM), selectively expressed in GABA interneurons, we hypothesized that vortioxetine may disinhibit glutamatergic and monoaminergic neurotransmission following 5-HT$_3$-R blockade.

Here we assessed vortioxetine effect on pyramidal neuron activity and extracellular 5-HT concentration using *in vivo* extracellular recordings of rat medial prefrontal cortex (mPFC) pyramidal neurons and microdialysis in mPFC and ventral hippocampus (vHPC). Vortioxetine, but not escitalopram, increased pyramidal neuron discharge in mPFC. This effect was prevented by SR57227A (5-HT$_3$-R agonist) and was mimicked by ondansetron (5-HT$_3$-R antagonist) and by escitalopram/ondansetron combinations. In microdialysis experiments, ondansetron augmented the 5-HT-enhancing effect of escitalopram in mPFC and vHPC. Local ondansetron in vHPC augmented escitalopram effect, indicating the participation of intrinsic mechanisms. Since 5-HT neurons express GABA$_B$ receptors, we examined their putative involvement in controlling 5-HT release after 5-HT$_3$-R blockade. Co-perfusion of baclofen (but not muscimol) reversed the increased 5-HT levels produced by vortioxetine and escitalopram/ondansetron combinations in vHPC.

The present results suggest that vortioxetine increases glutamatergic and serotonergic neurotransmission in rat forebrain by blocking 5-HT$_3$ receptors in GABA interneurons.

1. Introduction

Vortioxetine (Lu AA21004) is an antidepressant with a clinical efficacy similar to venlafaxine (Alvarez et al., 2012). Vortioxetine inhibits the serotonin (5-HT) transporter (SERT) and shows high *in vitro* affinity for several 5-HT receptors (5-HT-R), including 5-HT$_3$, 5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_7$ (Mork et al., 2012; Sanchez et al., 2015). Vortioxetine has pro-cognitive effects in rodent models (Bétry et al., 2015; Sanchez et al., 2015; Wallace et al., 2014) and its administration to rodents increases very markedly the extracellular concentration of monoamines in forebrain (Pehrson et al., 2013) to an extent greater than that produced by selective serotonin reuptake inhibitors (SSRI). This difference is most likely due to the multimodal pharmacological profile of vortioxetine, which may prevent local (e.g., mediated by 5-HT$_{1A}$ and 5-HT$_{1B}$ autoreceptors) and distal self-inhibitory mechanisms on monoamine cell groups.

Self-inhibitory mechanisms triggered by 5-HT autoreceptors in response to antidepressant drug administration were identified two decades ago (Artigas et al., 1996, 2001; Piñeyro and Blier, 1999). In addition to these local mechanisms, distal loops may also modulate serotonergic activity. Among them, long distance

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afferents from the medial prefrontal cortex (mPFC) tightly control serotonergic activity (Celada et al., 2001; Hajos et al., 1998; Martin-Ruiz et al., 2001) and play a major role in the response to behavioural challenges (Warden et al., 2012). The PFC also projects to the brainstem catecholamine nuclei (Gabbott et al., 2005) and thus, changes in PFC activity may also evoke downstream changes in the activity of the ascending dopaminergic and noradrenergic systems. The PFC contains a high density of neurons expressing 5-HT_{1A}-R (Santana et al., 2004), whose physiological activation inhibits pyramidal neuron activity (Amargós-Bosch et al., 2004; Puig et al., 2005). Therefore, 5-HT_{1A}-R activation by vortioxetine might modulate pyramidal output to brainstem catecholamine nuclei and increase forebrain catecholamine release, as reported for 5-HT_{1A}-R agonists (Díaz-Mataix et al., 2006; Hajos-Korcsok and Sharp, 1996).

In addition, given the high affinity (3.7 nM) of vortioxetine for 5-HT_3-R (Mork et al., 2012), they may also participate in the distinctive actions of vortioxetine, compared with SSRIs. 5-HT_3-R are present in forebrain GABAergic interneurons that control pyramidal neuron activity in hippocampus and PFC (Morales and Bloom, 1997; Puig et al., 2004). 5-HT_3-R are ion channels and their physiological activation by endogenous 5-HT markedly excites mPFC GABA interneurons (Puig et al., 2004).

Using single unit recordings in anesthetized rats, we examined the involvement of 5-HT_3-R in the mechanism of action of vortioxetine, under the working hypothesis that 5-HT_3 receptor blockade in GABA interneurons may disinhibit pyramidal neuron activity in mPFC. Likewise, using *in vivo* microdialysis in freely-moving rats, we examined the role of 5-HT_3-R blockade as a potential negative feed-back mechanism involved in the control of 5-HT release during SERT blockade by antidepressant drugs.

2. Material and methods

2.1. Animals and stereotaxic surgery

Male albino Wistar rats (Iffa Credo, Lyon, France) weighing 250–340 g were used. Animal care followed European Union regulations (directive 2010/63 of September 22, 2010) and was approved by the Institutional Animal Care and Use Committee.

2.2. Drugs and treatments

Vortioxetine (VOR) hydrobromide and escitalopram oxalate (ESCI) were provided by H. Lundbeck A/S (Copenhagen-Valby, Denmark). Ondansetron hydrochloride dihydrate (OND, 5-HT_3-R antagonist), SR57227A (SR, 5-HT_3-R agonist), baclofen (BAC, $GABA_B$-R agonist) and muscimol (MUS, $GABA_A$-R agonist) were purchased from Sigma-Aldrich (Buchs, Switzerland). Concentrated stock solutions were prepared in hydroxyl-propyl-ß-cyclodextrin 30% (VOR), saline (for systemic administrations) or artificial cerebrospinal fluid (aCSF, for local perfusions) and aliquots were stored at −20 °C. Working solutions were prepared daily by dilution. In electrophysiological experiments, drugs were administered intravenously (i.v.) through the femoral vein. In microdialysis experiments, drugs were administered subcutaneously (s.c.) or locally, by reverse microdialysis. The doses used were taken from the literature (Abellán et al., 2000a, 2000b; Bétry et al., 2013; Mork et al., 2012; Zhang et al., 2011) or from pilot experiments.

2.3. Electrophysiological recordings

Electrophysiological recordings were performed as previously described (Kargieman et al., 2007; Puig et al., 2005) typically between 9 a.m. and 3 p.m. Rats were anesthetized with chloral hydrate (induction: 400 mg/kg i.p.]; maintenance: 50–70 mg/kg/h i.p. using a perfusion pump) to analyze the responses of layer V mPFC pyramidal neurons (AP from +3.4 to +3.2; ML from −0.5 to −0.9; DV from −1.5 to −4; mm) (Paxinos and Watson, 2005) to the i.v. administration of ESC, VOR, OND and SR, alone or in combination. Pyramidal neurons were identified by antidromic stimulation from projection areas of the mPFC such as the dorsal raphe ([DR]; AP -7,8; L −3,1; DV -6.6 electrode implanted with an angle of 30°) or the ventral tegmental area ([VTA]; AP -5,8; L −0,4; DV -8), followed by the collision test (Fuller and Schlag, 1976). Both stimulation sites identify the same population of midbrain-projecting PFC neurons (Vázquez-Borsetti et al., 2011). DR and VTA were stimulated at 0.5–2.0 mA, 0.2 ms square pulses, 0.9 Hz as previously reported (Puig et al., 2005). Of the 73 recorded neurons, 62 (85%) projected to DR, 6 (8%) projected to VTA and 5 (7%) projected to both areas. Neurons not responding to antidromic activation or without spontaneous firing activity were discarded.

The recording electrodes were filled with saline 2M (impedances 6–12 MΩ). Single unit recordings were amplified with a Neurodata IR283 (Cygnus Technology Inc., Delaware Water Gap, PA), post-amplified and filtered with a Cibertec amplifier (Cibertec, Madrid, Spain) and computed on-line using a DAT 1401plus interface system Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Pontamine Sky Blue was added for the identification of the recording site. After obtaining a stable baseline recording for 5 min, saline or SR was injected as pre-treatment. After 3–5 min, drugs were administered i.v. Cumulative doses were injected every 3 min. Typically, actual recording time was 23–40 min.

2.4. In vivo microdialysis

Microdialysis procedures were conducted as previously described (Amargós-Bosch et al., 2004). Briefly, anesthetized rats (sodium pentobarbital, 60 mg/kg i.p.) were stereotaxically implanted with concentric microdialysis probes equipped with a 4-mm Cuprophan membrane in the mPFC (AP +3.2, ML −0.8, DV −6.0 mm from skull) or ventral hippocampus (vHPC) (AP -5.3, ML -4.8, DV -8.8 mm from skull). Microdialysis experiments were carried out in freely-moving rats 20–24 h after surgery at the same time interval as the electrophysiological experiments. Probes were continuously perfused with aCSF pumped at 1.5 μL/min. Dialysate samples of 30 μL were collected every 20 min.

After an initial 60 min stabilization period, four baseline samples were collected before systemic or local pharmacological treatments. In a first set of experiments, ESC and OND were administrated both s.c. to study their effect on extracellular 5-HT in mPFC and vHPC. In experiments examining the role of hippocampal 5-HT_3-R in augmenting the effect of SERT inhibition on extracellular 5-HT, ESC or VOR was administered s.c. Once a stable effect was achieved, the perfusion fluid was replaced by one containing OND and/or BAC (or MUS). Dialysate 5-HT concentrations were analyzed by HPLC-amperometric detection (Hewlett Packard-1049, Palo Alto, CA, USA) at +0.60 V, with detection limit of 2 fmol/sample.

2.5. Histology

After experimental procedures were completed, animals were euthanized by an anaesthetic overdose. The brains were removed, frozen on dry ice and kept at −80 °C before being cut into coronal sections (50 μm) with a cryostat. Brain sections were stained to verify the recording and stimulation sites with neutral red or the correct placement of the probes with fast-green.

2.6. Data and statistical analysis

Firing rate was quantified by averaging the values in 2-min periods of each experimental period (2nd-3rd min after each drug cumulative dose administration or 4th–5th; 9th–10th; 14th–15th; 19th–20th; 24th–25th min after a single dose drug injection). Neurons were considered to be excited or inhibited when drugs induced a ±30% change of the discharge rate at least in two consecutive doses or time periods (Kargieman et al., 2007).

Microdialysis data are given as percentage of basal values, averaged from 4 pre-drug fractions and also as normalized areas under curve (AUC) corresponding to every treatment period. The treatment effects were assessed by one- or two-way repeated measures ANOVA or Student's *t*-test, as appropriate, using Statistica for Windows software (4.5 version) (StatSoft inc.). *Post-hoc* analysis was performed using Duncan's test. Data are expressed as means ± SEM. Statistical significance was set at $p < 0.05$ (two tailed).

3. Results

3.1. Vortioxetine effect on mPFC pyramidal activity. Prevention by the 5-HT$_3$-R agonist SR57227A

Vortioxetine (VOR; 0.1–0.2–0.4–0.8–1.6 mg/kg i.v., cumulative doses) dose-dependently increased the firing rate in 10 (77%) of the 13 neurons recorded. Two neurons (15%) were unaffected and another one (8%) decreased its activity. Maximal effect was observed at 0.4 mg/kg i.v. The increased discharge rate persisted for ≥13 min after the last VOR injection. Fig. 1A shows a representative example of VOR effect on the discharge of an mPFC pyramidal neuron. Fig. 1B and C shows the effect of VOR in all neurons (231 ± 44% of baseline, n = 13) and in activated neurons (273 ± 51% of baseline, n = 10). One-way ANOVA revealed a significant effect of VOR when considering all neurons ($F(6,72) = 3.00$; $p < 0.02$, n = 13) or only excited neurons ($F(6,54) = 3.21$; $p < 0.01$, n = 10) and *post-hoc* differences between VOR (0.4–0.8–1.6 mg/kg i.v.) and baseline-saline values.

Given the high affinity of VOR for 5-HT$_3$-R, we studied the potential prevention of its effect by the 5-HT$_3$-R agonist SR57227A by comparing SR57227A + VOR *versus* SAL + VOR. A single dose (1.28 mg/kg i.v.) of SR57227A fully prevented the elevation of pyramidal cell firing induced by VOR. Two-way ANOVA revealed significant effects of pre-treatment ($F(1,22) = 4.41$; $p < 0.05$), treatment ($F(6,132) = 4.03$; $p < 0.002$) and pre-treatment·treatment interaction ($F(6,132) = 2.83$; $p < 0.02$ n = 13 and 11 for SAL + VOR, and SR57227A + VOR groups, respectively), with significant *post-hoc* differences between SAL and SR57227A pre-treatments at VOR doses of 0.4, 0.8 and 1.6 mg/kg i.v. Fig. 1D shows a representative example of the lack of an effect of cumulative doses of VOR in SR57227A-pre-treated rats. Fig. 1E shows the prevention by SR57227A of VOR effect in all neurons. The prevention of VOR effect by SR57227A was observed in 10 of the 11 pyramidal neurons. Fig. 1F shows the dose-effect relationships for both experimental groups.

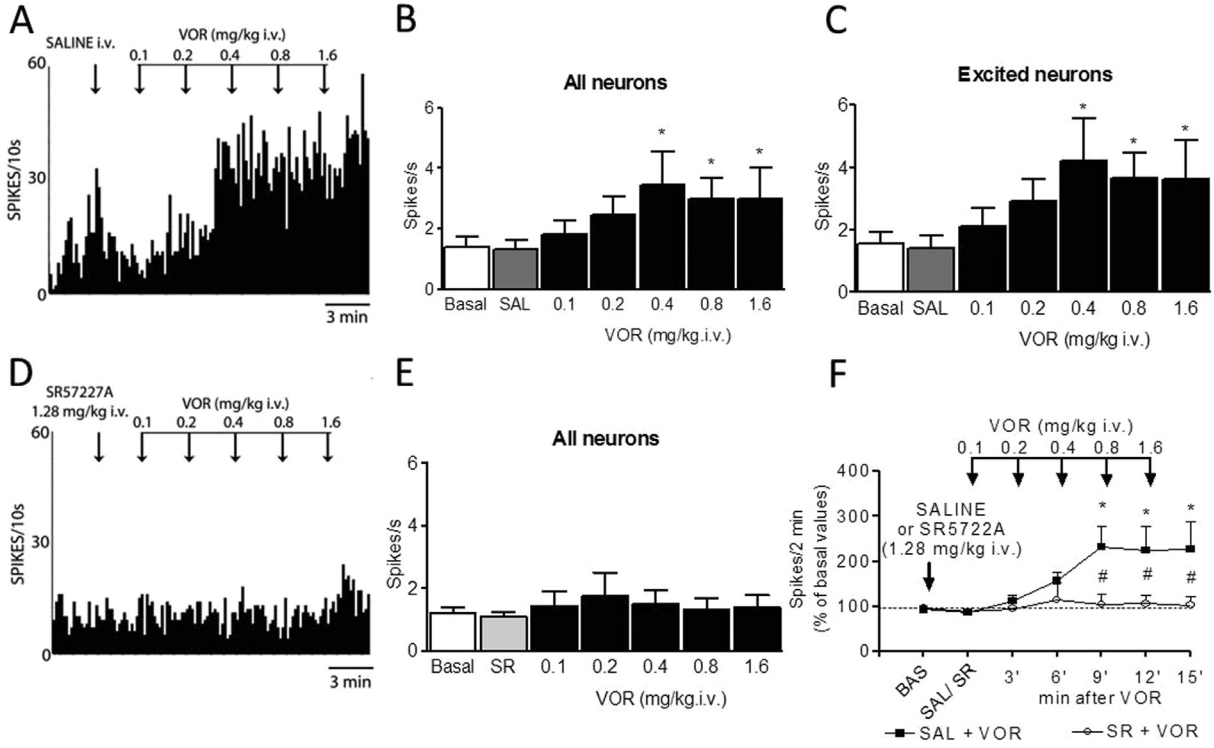

Fig. 1. Effect of vortioxetine (VOR) on the firing rate of layer V (midbrain-projecting) pyramidal neurons in rat medial prefrontal cortex (mPFC) and its prevention by the 5-HT$_3$-R agonist SR57227A (SR). **A)** Representative example of a pyramidal neuron whose discharge was increased by intravenous VOR administration (0.1–0.2–0.4–0.8–1.6 mg/kg i.v., cumulative doses). **B)** and **C)** Bar graphs showing the average effect of VOR on firing rate in all neurons (**B**, n = 13) and in the subpopulation of excited neurons (**C**, n = 10). **D)** Representative example of the prevention of VOR effect on mPFC pyramidal discharge by pre-treatment with SR57227A (1.28 mg/kg, i.v.). Note the lack of effect of the 5-HT$_3$-R agonist SR57227A on pyramidal discharge. **E)** Bar graph showing the lack of VOR effect in SR57227A pre-treated rats in all recorded neurons (n = 11). **F)** Graph showing the comparison between saline (SAL) + vortioxetine (SAL + VOR) and SR57227A + vortioxetine (SR + VOR) groups. *$p < 0.05$ *versus* basal (BAS) and SAL values in SAL + VOR group; #$p < 0.003$ SAL *versus* SR57227A pre-treatments at VOR doses of 0.4, 0.8 and 1.6 mg/kg i.v.

3.2. Effect of ESC, OND and ESC + OND on pyramidal neuron activity in mPFC

The administration of escitalopram (ESC, 0.1–0.2–0.4–0.8–1.6 mg/kg i.v., cumulative doses) did not alter the discharge rate of mPFC pyramidal neurons (n = 9). Five neurons (55.5%) were not significantly activated, 3 were unaffected (33.3%) and 1 was inhibited (11.2%). Fig. 2A and B shows a representative example and the effect of ESC in all recorded neurons, respectively. Similarly, a single dose of ESC (0.1 mg/kg i.v.) did not affect the firing rate of mPFC pyramidal neurons (n = 8). Five neurons (62.5%) were not significantly inhibited by ESC, 1 (12.5%) was unaffected and 2 (25%) were not significanty activated. Fig. 2C shows the effect of a single dose of ESC in all recorded neurons.

The administration of ondansetron (OND; 0.16–0.32–1.28 mg/kg i.v., cumulative doses) evoked a dose-dependent increase in pyramidal discharge in 7 out of 11 neurons (63.6%) recorded (4 were unaffected; 36.4%). The maximal effect was achieved at

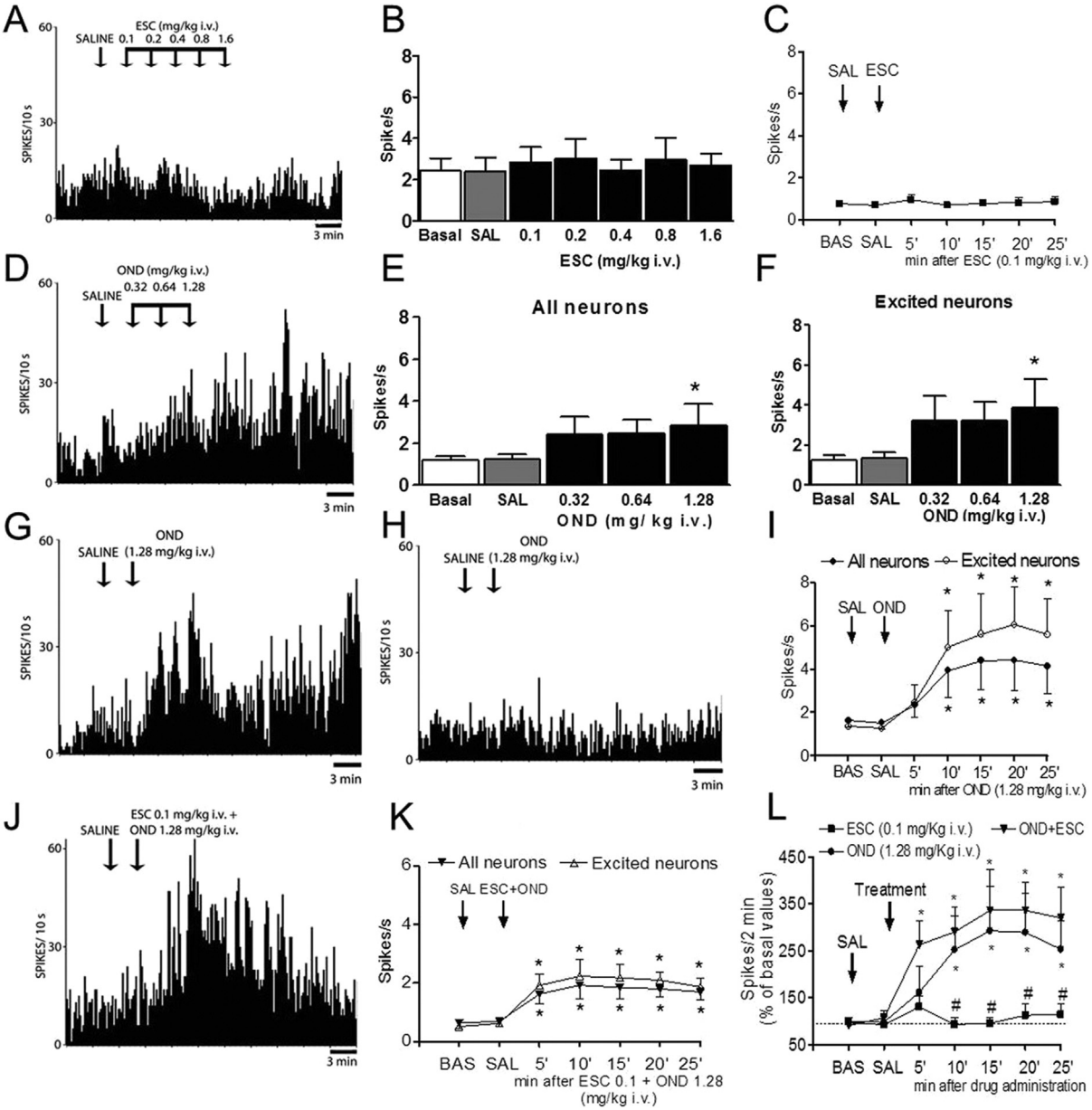

Fig. 2. Effects of escitalopram (ESC, a selective serotonin reuptake inhibitor, SSRI), ondansetron (OND, a 5-HT_3-R antagonist) and the combination of escitalopram + ondansetron (ESC + OND) on pyramidal neuron activity in mPFC. **A)** Example of a pyramidal neuron whose discharge was unaffected by ESC cumulative doses administration (0.1–0.2–0.4–0.8–1.6 mg/kg i.v.). **B)** Bar graph showing the average effect of ESC on firing rate in all neurons (n = 9). **C)** Graph showing the lack of effect of the administration of a single dose of ESC (0.1 mg/kg i.v., n = 8) during a 25-min period after drug administration. **D)** Example of a pyramidal neuron whose discharge was augmented by OND cumulative doses administration (0.32–0.64–1.28 mg/kg i.v.). **E)** and **F)** Bar graphs showing the average effect of OND on firing rate in all neurons (**E**, n = 11) and in excited neuron sub-population (**F**, n = 7). **G)** and **H)** Examples of two pyramidal neurons showing an increase **G)** or a lack of effect **H)** after OND administration (1.28 mg/kg i.v., single dose). **I)** Graph showing the increasing effect on pyramidal activity of single dose of OND in all neurons (n = 9) and in excited neuron subpopulation (n = 6). **J)** Representative example of a pyramidal neuron response to the administration of the drug combination ESC (0.1 mg/kg i.v.) + OND (1.28 mg/kg i.v.). **K)** Graph showing the increasing effect of a single dose of OND on pyramidal activity in all neurons (n = 9) and in excited neuron subpopulation (n = 6). **L)** Graph showing the comparison between escitalopram (ESC), ondansetron (OND) and escitalopram + ondansetron (ESC + OND) groups. *p < 0.05 vs Basal and SAL values in OND (single and cumulative doses) and ESC + OND groups; #p < 0.05 ESC at 10′–15′–20′–25′ min after administration *versus* OND (10–15–20–25 min after administration) and ESC + OND (5–10–15–20–25 min after administration).

1.28 mg/kg i.v. Fig. 2D shows a representative example of an activated neuron. Fig. 2E and F shows the effect of OND in all neurons (239 ± 55% of baseline, n = 11) and in activated neurons (311 ± 74% of baseline, n = 7). One-way ANOVA revealed a significant effect of OND when considering all neurons ($F(4,40) = 2.91$; $p < 0.05$, n = 11) or only excited neurons ($F(4,24) = 3.31$; $p < 0.03$, n = 7) with *post-hoc* differences between OND (1.28 mg/kg i.v.) and baseline-saline values. The increased discharge rate persisted for ≥20 min after the last OND injection. Similarly, a single dose of OND (1.28 mg/kg i.v.) increased the firing rate in 6 of the 9 (66.7%) pyramidal neurons recorded. Two neurons (22.2%) were inhibited and 1 (11.1%) was unaffected. Maximal effect was observed at 10 min post-administration and persisted for the duration of recordings (typically 25 min post-administration). Fig. 2 shows representative examples of activated (2G) and unaffected (2H) neurons. Fig. 2I shows the effect of OND in all neurons (253 ± 73% of baseline, n = 9) and in the subgroup of excited neurons (333 ± 94% of baseline, n = 6). One-way ANOVA indicated a significant effect of the treatment for all neurons ($F(6,48) = 2.28$; $p < 0.03$; n = 9) or only excited neurons ($F(6,30) = 4.18$; $p < 0.005$; n = 6), with *post-hoc* differences between OND (1.28 mg/kg i.v. at 10–15–20–25 min after administration) and baseline-saline values.

In order to further clarify the role of 5-HT_3-R in the enhancing effect on pyramidal activity by VOR, we evaluated the action of ESC + OND (to mimic the mechanism of action of VOR). This drug combination evoked an increase in the firing rate in 9 out of 12 (75%) recorded neurons. Three neurons (25%) were unaffected. The combination of OND + ESC increased pyramidal neuron discharge to an extent similar to OND alone. Maximal effect was observed at 10 min post-administration and persisted for the duration of recordings (25 min post-administration). Fig. 2J shows a representative example of an activated neuron. Fig. 2K shows the effect of the drug combination in all neurons (290 ± 52% of baseline, n = 12) and in the subgroup of excited neurons (357 ± 53% of baseline, n = 9). One-way ANOVA indicated a significant effect of treatment considering all neurons ($F(6,66) = 5.88$; $p < 0.0001$; n = 12) or only excited neurons ($F(6,48) = 7.41$; $p < 0.00001$; n = 9), with *post-hoc* differences between ESC + OND at 5–10–15–20–25 min post-administration and baseline-saline values.

Finally, we compared the effects on the mPFC pyramidal discharge induced by the three treatments (ESC, OND, ESC + OND). Two-way ANOVA (with time and treatment as factors) revealed a significant effect of time ($F(6,156) = 7.73$; $p < 0.00001$), treatment ($F(2,26) = 3.53$; $p < 0.05$) and treatment × time interaction ($F(12,156) = 1.90$; $p < 0.05$) (n = 8, 9 and 12 for ESC, OND and ESC + OND groups, respectively), with *post-hoc* differences between OND or ESC + OND and ESC (Fig. 2L).

3.3. Effect of systemic administration of ESC and OND on extracellular 5-HT concentration in mPFC and vHPC

OND administration (10 mg/kg s.c.) had no effect alone but augmented the effect of ESC (3.2 mg/kg s.c.) on extracellular 5-HT in mPFC (Fig. 3A–B) and vHPC (Fig. 3C–D). In mPFC, two-way ANOVA for repeated measures of AUC values revealed significant effects of group ($F(3,25) = 11.38$; $p < 0.00001$), treatment ($F(2,50) = 17.01$; $p < 0.00001$) and treatment·group interaction ($F(6,50) = 8.69$; $p < 0.00001$, n = 4, 6, 10 and 9 for SAL + SAL, SAL + OND, ESC + SAL and ESC + OND groups, respectively) with significant *post-hoc* differences between ESC + OND and ESC. In vHPC, two-way ANOVA for repeated measures of AUC values also revealed significant effects of group ($F(3,27) = 10.76$; $p < 0.0001$), treatment ($F(2,54) = 11.26$; $p < 0.0001$) and treatment·group interaction ($F(6,54) = 9.10$; $p < 0.00001$, n = 5, 5, 9 and 12 for SAL + SAL, SAL + OND, ESC + SAL and ESC + OND groups, respectively) with significant *post-hoc* differences between ESC + OND and ESC.

3.4. Relevance of local 5-HT_3-R in vHPC on vortioxetine effect on extracellular 5-HT: involvement of $GABA_B$-R

To examine the potential involvement of local 5-HT3-R –containing microcircuits in vHPC in the augmentation of ESC effects by systemic OND, we examined the ability of local OND infusion in vHPC (300 μM in aCSF) to augment the effect of ESC (3.2 mg/kg s.c.) on extracellular 5-HT (Fig. 4A and B). The s.c. ESC administration increased extracellular 5-HT in vHPC to ≈3-fold the basal levels. This effect was sustained, lasting for ≈15 fractions (300 min). The local application of 300 μM OND in vHPC produced a robust potentiation of ESC effect. Two-way ANOVA for repeated measures of AUC values revealed significant effects of group ($F(1,12) = 7.75$; $p < 0.02$), treatment ($F(2,24) = 42.13$; $p < 0.00001$) and treatment·group interaction ($F(2,24) = 9.97$; $p < 0.001$ n = 7 for both ESC and ESC + OND groups), with significant *post-hoc* differences between ESC and ESC + OND. Local OND application alone increased the extracellular 5-HT concentration in vHPC (380 ± 8% of basal values; $p < 0.03$; n = 5) (Fig. 4C).

Unlike mPFC, the vHPC lacks afferent pathways to the raphe nuclei that may explain the OND augmentation of ESC effect, thus suggesting the involvement of local microcircuits in this effect. We then hypothesized that 5-HT_3-R blockade by OND and VOR would reduce GABA release and consequently, the GABA tone on local $GABA_B$-R controlling 5-HT release. We then examined whether the restoration of the $GABA_B$-R tone with baclofen (BAC) could attenuate the augmenting effect of OND on extracellular 5-HT. The local co-perfusion of BAC (100 μM) by reverse dialysis in vHPC fully reversed the increase of extracellular 5-HT produced by the local application of OND after systemic ESC administration, and normalised 5-HT values (Fig. 4D and E). One-way ANOVA for repeated measures of AUC values revealed significant effect of treatment ($F(3,15) = 10.79$; $p < 0.0005$; n = 6), with significant *post-hoc* differences between ESC, ESC + OND and ESC + OND + BAC treatments. Moreover, the local perfusion of BAC did not alter ESC-induced increase of extracellular 5-HT (Fig. 4F). One-way ANOVA for repeated measures of AUC values revealed significant effect of treatment ($F(2,10) = 4.77$; $p < 0.05$; n = 6), with no *post-hoc* differences between ESC and ESC + BAC treatments.

Similarly, local BAC infusion (100 μM in aCSF) by reverse dialysis in vHPC attenuated the effect of VOR (5 mg/kg s.c.) on extracellular 5-HT. Unlike BAC, local administration of the $GABA_A$-R agonist muscimol (MUS) in vHPC (100 μM in aCSF) failed in reversing VOR effect on extracellular 5-HT (Fig. 4G and H). Two-way ANOVA for repeated measures of AUC values revealed significant effects of group ($F(2,18) = 9.15$; $p < 0.002$), treatment ($F(2,36) = 53.29$; $p < 0.00001$) and the treatment·group interaction ($F(4,36) = 14.72$; $p < 0.00001$ n = 9, n = 7 and n = 5 for VOR, VOR + BAC and VOR + MUS, respectively) with significant post-hoc differences between VOR or VOR + MUS and VOR + BAC treatments.

4. Discussion

The present results indicate that acute vortioxetine administration dose-dependently increased the discharge rate of mPFC pyramidal neurons projecting to midbrain, with a maximal effect at 0.4 mg/kg i.v. In contrast, escitalopram did not significantly alter the same neuronal population at doses that fully block SERT (vortioxetine: Bétry et al., 2013; escitalopram: unpublished observations). Likewise, vortioxetine increased extracellular 5-HT concentration in mPFC and vHPC to an extent greater than that evoked by escitalopram. This difference is likely due to the simultaneous blockade of SERT and 5-HT_3-R by vortioxetine, since its effects were

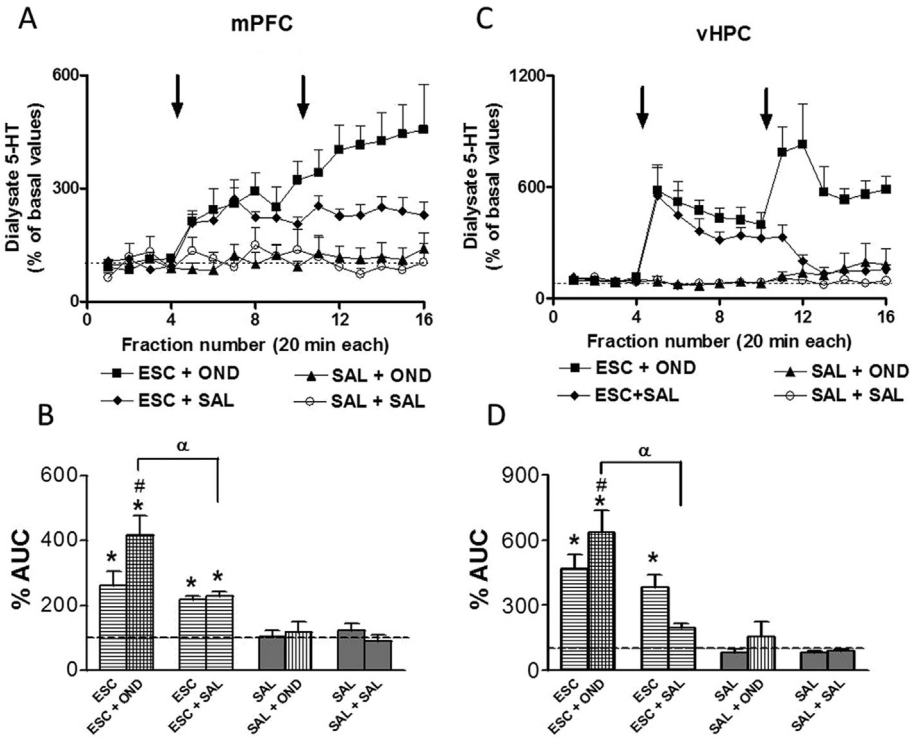

Fig. 3. Effect of systemic administration of escitalopram (ESC) and ondansetron (OND) on extracellular 5-HT concentration in medial prefrontal cortex (mPFC) and ventral hippocampus (vHPC). The subcutaneous (10 mg/kg) administration of OND augments the enhancing effect of ESC (3.2 mg/kg s.c.) on extracellular 5-HT in mPFC (**A** and **B**) and vHPC (**C** and **D**). Note the lack of effect of OND by itself in both areas. **A)** and **C)** Graphs show data as % of basal values averaged from 4 pre-drug fractions during two 120-min post-drug administration periods in mPFC **(A)** and vHPC **(C)**. **B)** and **D)** Results are also represented as normalized areas under curve (AUC) corresponding to each treatment period for mPFC **B)** and vHPC **C)**. The experimental groups performed for each area are: escitalopram + ondansetron (ESC + OND), escitalopram + saline (ESC + SAL), saline + ondansetron (SAL + OND) and saline + saline (SAL + SAL). Arrows mark first and second drug administrations. *p < 0.01 *versus* Basal values; #p < 0.05 *versus* ESC in ESC + OND group; α p < 0.0001 ESC + OND *versus* ESC + SAL periods.

prevented by the 5-HT_3-R agonist SR57227A and were mimicked by escitalopram and ondansetron combinations (and by ondansetron alone for pyramidal discharge elevations).

We suggest that vortioxetine blockade of 5-HT_3-R disinhibits pyramidal neuron activity and 5-HT release following the removal of the GABAergic tone on pyramidal neurons (mainly mediated by $GABA_A$-R) and on 5-HT nerve terminals (mediated by $GABA_B$-R) (Fig. 5). Hence, unlike SSRI, vortioxetine increased the glutamatergic/GABAergic neurotransmission ratio in rat brain. These effects may explain the superior effects of vortioxetine in various experimental models, such as neurotransmitter release (Pehrson et al., 2013) and pro-cognitive effects in rodents (Bétry et al., 2015; Sanchez et al., 2015; Wallace et al., 2014).

5-HT_3-Rs are exclusively expressed in GABAergic interneurons in cortical and hippocampal areas (Lee et al., 2010; Morales and Bloom, 1997; Puig et al., 2004). 5-HT_3-R have also been identified in GABAergic interstitial cells of the corpus callosum, which may control cortical and subcortical networks (von Engelhardt et al., 2011). Cortical GABAergic interneurons activate $GABA_A$-R to rapidly control electrical and biochemical signalling in pyramidal neurons. GABAergic interneurons are classified according to their morphology, connectivity, electrophysiological properties and expression profile of neuropeptides and calcium binding proteins, such as parvalbumin (PV), calbindin or calretinin (DeFelipe et al., 2013). PV-positive neurons are a subpopulation of GABAergic cells comprising chandelier and large basket cells (DeFelipe, 1997) that tightly control neuronal activity via axo-somatic (basket cells) or axo-axonic (chandelier cells) synapses on deep layer pyramidal neurons (DeFelipe and Fariñas, 1992). In the mPFC, 5-HT_3-R are expressed in non-PV, non-somatostatin GABAergic interneurons, located in upper cortical layers (Lee et al., 2010; Morales and Bloom, 1997; Puig et al., 2004), which include neurogliaform cells (Oláh et al., 2009; Tamás et al., 2003) and VIP-expressing interneurons (Lee et al., 2013; Pi et al., 2013). The percentage of mPFC GABAergic neurons expressing 5-HT_3-R is 40% in layer I, 18% in layers II-III, 6% in layer V and 8% in layer VI (Puig et al., 2004). Given their predominant location in superficial layers, we expected that 5-HT_3-R blockade would have a moderate impact on the activity of midbrain-projecting pyramidal neurons, located in layers V-VI (Gabbott et al., 2005). However, ≈ 70% of the recorded pyramidal neurons showed a marked increase of their discharge rate after 5-HT_3-R blockade with vortioxetine or ondansetron.

Several reasons may account for this apparent discrepancy. On the one hand, 5-HT_3-R are ion channels and their physiological stimulation by 5-HT markedly increased the discharge of 5-HT_3-R-expressing GABAergic interneurons in mPFC (Puig et al., 2004). In agreement, the microiontophoretic and systemic administration of 5-HT_3-R agonists decreased the firing activity of putative pyramidal neurons in mPFC (Ashby et al., 1991; Bachy et al., 1993; Edwards et al., 1996). Similar effects have been reported in the hippocampus after the physiological release of 5-HT or local application of 5-HT_3-R agonists (Varga et al., 2009). Hence, 5-HT_3-R-expressing interneurons may inhibit pyramidal activity more than anticipated from their location in superficial and middle layers due to the ionic nature of 5-HT_3-R. On the other hand, 5-HT_3-R may be present in GABAergic interneurons controlling pyramidal neuron activity via axo-somatic contacts (Freund, 2003; Marlin and Carter, 2014) and/or in cortical networks of synaptically- and electrically-coupled interneurons (Galarreta and Hestrin, 2001; Tamás et al., 2003; Traub et al., 2001).

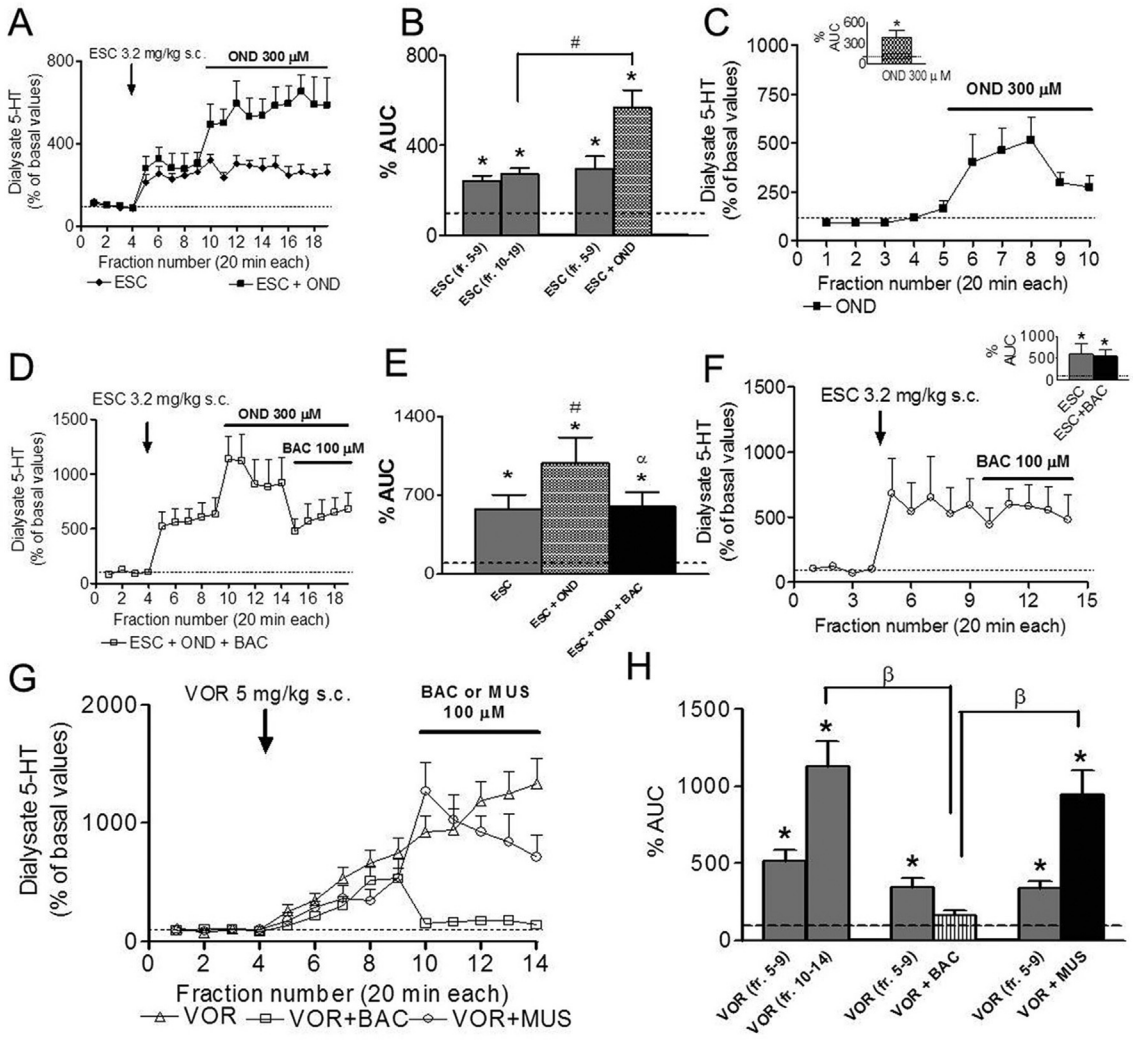

Fig. 4. Relevance of local hippocampal microcircuits containing 5-HT_3-R in vortioxetine effect on extracellular 5-HT. Involvement of $GABA_B$-R. **A)** and **B)** Graphs (**A** % of basal values; **B** AUCs) showing the potentiating effect of local infusion in vHPC of the 5-HT_3-R antagonist ondansetron (OND, 300 µM) on the augmenting in extracellular 5-HT concentration induced by systemic administration of escitalopram (ESC, 3.2 mg/kg s.c.) in freely-moving rats. Note that the local administration of OND alone increases the extracellular 5-HT concentration in vHPC **(C). D)** and **E)** The local co-perfusion of baclofen (BAC, 100 µM) by reverse dialysis in vHPC reverses the increase of extracellular 5-HT produced by the local application of OND after systemic ESC administration. Data are represented as % of basal values **(D)** or as AUC.**(E)**. Note that the local administration of BAC alone does not alter the effect induced by the systemic administration of ESC **(F). G)** (% of basal values) and **H)** (AUCs) Local BAC infusion (100 µM in aCSF) in vHPC (but not muscimol, MUS, 100 µM in aCSF) attenuates the effect of systemic VOR (5 mg/kg s.c.) on extracellular 5-HT levels. *$p < 0.05$ vs Basal values; #$p < 0.03$ ESC *versus* ESC + OND; α $p < 0.03$ ESC + OND *versus* ESC + OND + BAC; β $p < 0.0001$ VOR + BAC *versus* VOR and VOR + MUS.

Overall, the present results are consistent with a strong and rapid ionotropic action of 5-HT on a subpopulation of cortical and hippocampal interneurons through the activation of 5-HT_3-R. The present observations suggest that 5-HT_3-R are tonically activated by 5-HT in the anesthetized rat since both vortioxetine and ondansetron elevated pyramidal neuron discharge. Given the presence of an excitatory control of the mPFC on 5-HT neuronal activity (Celada et al., 2001; Hajos et al., 1998), it is likely that the actions of vortioxetine in mPFC may contribute to the rapid recovery of serotonergic cell firing observed after repeated vortioxetine treatment (Bétry et al., 2013; Sanchez et al., 2015).

The increased pyramidal discharge induced by vortioxetine in mPFC may also be involved in its pro-cognitive effect in rodents (Bétry et al., 2015; Sanchez et al., 2015; Wallace et al., 2014). The PFC is critically involved in cognition, including working memory and executive functions (Miller and Cohen, 2001). Primate studies have revealed that the neurobiological substrate of working memory is the maintenance of patterns of persistent neuronal activity in the dorsolateral PFC (equivalent to the prelimbic mPFC in rodents) during mnemonic processes (Curtis and D'Esposito, 2003; Fuster and Alexander, 1971). Hence, the increased pyramidal discharge induced by vortioxetine may facilitate the emergence and maintenance of these activity patterns during working memory tasks.

Another distinctive feature of vortioxetine is its ability to increase extracellular monoamine concentrations above the level produced by SSRI (Pehrson et al., 2013). Here we provide evidence that, at least for 5-HT, this effect is likely due to the removal of a $GABA_B$-R tone on 5-HT terminals following 5-HT_3-R blockade in local interneurons. Ondansetron augmented the effect of escitalopram on extracellular 5-HT in mPFC and vHPC, indicating that 5-HT_3-R blockade can facilitate 5-HT release following SERT inhibition in both areas. Further experiments were carried out in vHPC, which -unlike mPFC- lacks direct afferent pathways to midbrain raphe, thus avoiding potential confounding effects due to the distal modulation of serotonergic neuron activity. Local ondansetron

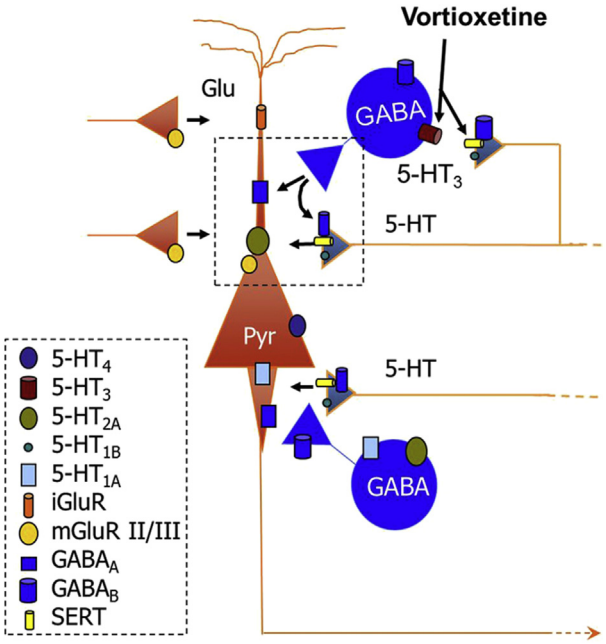

Fig. 5. Schematic representation of the putative actions of vortioxetine on pyramidal neuron discharge and on 5-HT release. The blockade of 5-HT_3-R located on GABA interneurons reduces GABA input onto $GABA_A$-R controlling the activity of mPFC pyramidal neurons, thus enhancing their discharge rate. Likewise, blockade of 5-HT_3-R reduces the GABA tone on terminal $GABA_B$-R controlling 5-HT release by nerve terminals, therefore augmenting the effect of SERT blockade on extracellular 5-HT (see text for an extended discussion).

application in vHPC also augmented escitalopram effect on extracellular 5-HT, suggesting the existence of local microcircuits that attenuate 5-HT release via 5-HT_3-R activation. Raphe 5-HT neurons express $GABA_B$-R (Abellán et al., 2000a), which exert a biphasic control of 5-HT neuronal activity and 5-HT release (Abellán et al., 2000b; Tao et al., 1996). In control conditions, stimulation of terminal $GABA_B$-R did not affect striatal 5-HT release (Abellán et al., 2000), possibly due to the existence of a tonic control by endogenous GABA. However, the present results suggest that the removal of the GABA tone on hippocampal $GABA_B$-R disinhibits 5-HT release, and that the restoration of the tone by the prototypical $GABA_B$-R agonist baclofen -but not the $GABA_A$-R agonist muscimol- attenuates 5-HT release (see Fig. 4H).

5. Conclusions

In summary, the present study indicates that vortioxetine has strong antagonist effects on 5-HT_3-R which add on top of SERT inhibition to evoke a robust disinhibition of pyramidal neuron activity in mPFC and of 5-HT release in forebrain. Since vortioxetine shows affinity for various 5-HT-R, an additional role of other receptors (e.g., 5-HT_{1A}-R, 5-HT_{1B}-R, 5-HT_7-R) cannot be discarded. However, it is likely that the simultaneous blockade of SERT and 5-HT_3-R accounts for the differential actions of vortioxetine in rodents, compared to SSRIs, such as the greater increase in extracellular monoamine concentrations and the pro-cognitive actions. The present results also show that, in addition to 5-HT_{1A} and 5-HT_{1B} autoreceptors, forebrain serotonergic activity is tonically limited by the activation of 5-HT_3-R in GABAergic interneurons. The latter inhibitory effect can be exerted at the local level (e.g., by negatively modulating 5-HT release) or distally, via the attenuation of mPFC-midbrain excitatory pathways after the stimulation of 5-HT_3-R in GABAergic interneurons. The attenuation of these negative feedmechanisms may improve serotonergic activity and therefore, the treatment of major depression.

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