Chronic vortioxetine treatment improves the responsiveness to an acute stress acting through the ventral hippocampus in a glucocorticoid-dependent way.

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Abstract

Vortioxetine is a novel multimodal antidepressant approved in 2013 by the Food and Drug Administration and the European Medicines Agency for the treatment of major depressive disorder (MDD). It combines the modulation of serotonin receptors activity with the inhibition of serotonin transporter (SERT). In this study, we aim at establishing the effect of chronic vortioxetine treatment (5mg/kg twice/daily) in modulating neuroplastic mechanisms as well as hypothalamic pituitary adrenal axis (HPA) activity under basal condition and in response to an acute challenge. We found that prolonged vortioxetine administration significantly increased total Bdnf expression in the dorsal and ventral hippocampus of adult male rats and affected the stress-induced modulation of the immediate early genes Arc and Zif268, mainly in the ventral sub-region. Moreover, we also found that, within this brain area, chronic drug treatment was able to modulate glucocorticoid responsiveness at genomic level by enhancing the translocation of the glucocorticoid receptor (GR) in the nuclear compartment in response to the acute stress. Interestingly, this effect was mirrored by the up-regulation of different GR responsive-genes.

Taken together, our data suggest that repeated exposure to vortioxetine specifically targets the ventral hippocampus by improving the ability to cope with stressful conditions. Moreover, its ability to facilitate HPA axis function might provide an indication to use this drug in patients characterized by glucocorticoid resistance. [1]

[1]*Abbreviations*: VTX, vortioxetine; MDD, major depressive disorders; 5-HT, serotonin; SERT, serotonin transporter; GABA, gamma aminobutyric acid; Bdnf, brain derived neurotrophic factor; IEG, immediate early genes; Arc, activity regulated cytoskeleton associated protein; Zif268, early growth response 1; cFos, Fos proto-oncogene; PF, prefrontal cortex; dHip, dorsal hippocampus; vHip, ventral hippocampus; GR, glucocorticoid receptor; HPA, hypothalamic pituitary adrenal; VEH, vehicle; ASS, acute swim stress; RT-PCR, real-time polymerase chain reaction; Gadd45β, growth arrest and DNA damage-inducible protein; Fkbp5, FK506 binding protein 5; FoxO1, forkhead box protein O1; Nr4a1, nuclear receptor subfamily 4 group A member 1; Sgk-1, serum/glucocorticoid regulated kinase 1; Dusp1, mitogen-activated protein kinase phosphatase 1; P11, S100A10; ANOVA, analysis of variance; PLSD, Fisher's protected least significant difference.

1. **Introduction**

Vortioxetine (1-[2-(2,4-dimethyl-phenylsulfanyl)-phenyl]-piperazine, hydrobromide) (VTX) is a novel multimodal antidepressant approved for the treatment of the major depressive disorder (MDD). It combines the modulation of serotonin (5-HT) receptors activity with the inhibition of the 5-HT transporter (SERT). In particular, VTX is a 5-HT_{1A} receptor agonist, a 5-HT_{1B} receptor partial agonist, 5-HT_3, 5-HT_7, 5-HT_{1D} receptors antagonist and a SERT inhibitor [1]. Moreover, the drug may exert its activity through the modulation of brain circuits, due to its ability in regulating norepinephrinergic, dopaminergic, cholinergic, histaminergic, glutamatergic and gamma aminobutyric acid systems [2-7]. Clinical evidence showed the efficacy of the drug in improving mood and cognitive deficits [8-15] in depressed patients, and preclinical studies demonstrated that vortioxetine is effective in normalizing cognitive deficits, anxiety- and depressive-like behaviors, after both acute [3,4,16-19] and chronic administrations [19,20].

At molecular levels, the development of MDD has been associated with alterations of neurotrophic factors and related signaling [21-23], that also represent a target of the antidepressant therapies [23]. In particular, the role of the neurotrophin Brain Derived Neurotrophic Factor (BDNF) in the etiology and treatment of MDD has been widely investigated [24], with serum BDNF levels being lower in depressed patients [25] while an upregulation of its expression was found in post-mortem brains of treated patients in comparison with the untreated counterpart [26,27]. Moreover, also HPA axis dysregulation is a key feature of mood disorders [28,29] with studies showing that this alteration may persist despite recovery [30,31] and may predict recurrence of the disorder [32].

On these bases, it is important to investigate the impact of new antidepressant drugs on these systems that represent key players of MDD. Furthermore, we aimed to establish not only the effects of chronic treatment with vortioxetine *per sè* but also its influence on the responsiveness to an acute stress, which may be suggestive of an enhanced coping ability under challenging condition.

In particular, we evaluated the expression of Bdnf, of the immediate early genes (IEGs) Activity Regulated Cytoskeleton Associated Protein (Arc), early growth response 1 (Zif268) and Fos proto-oncogene (cFos) in rats chronically treated with vortioxetine and then exposed to an acute challenge. Moreover, in order to assess the drug's effect on the HPA axis, we focused on the genomic mechanism of the glucocorticoid receptor (GR) by measuring its protein levels in the nuclear compartment and the mRNA levels of genes regulated by GR.

The analyses were conducted in key brain areas implicated in mood disorders: the prefrontal cortex (PFC), the dorsal part of the hippocampus (dHip), involved in processing spatial and contextual information [33] and the ventral hippocampus (vHip), the subregion more related to the emotional behavior [34].

2. Methods

2.1. Animals

Adult male Sprague Dawley rats (Charles River, Italy), aged 10 weeks (320-340 gr), were brought into the laboratory two weeks before the start of the experiment. The animals were housed with food and water freely available and were maintained on a 12-h light/dark cycle in a constant temperature (22 ± 2°C) and humidity (50 ± 5%) conditions. All animal experiments were conducted according to the authorization from the Health Ministry n 1252/2015-PR in full accordance with the Italian legislation on animal experimentation (DL 26/2014) and adherent to EU recommendation (EEC Council Directive 2010/63). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Pharmacological treatment and stress procedure

Rats were treated with saline (VEH) or vortioxetine (16-18 animals for each group) (provided by H. Lundbeck, Copenhagen) at the dose of 5 mg/kg (ip) twice a day for 14 days [3]. As previously described [35-37], twenty-four hours after the last drug administration, half of the animals were left undisturbed in their home cages (sham) (7-9 rats for each group of treatment), while the other half were exposed to an acute stress (7-9 rats for each group of treatment) consisting of 5-min swim stress (ASS). To this aim, animals were individually placed in a bucket filled with water at room temperature (25°C) that was deep enough to not touch the bottom. After 5 min, they were dried with a towel and returned to their home cage. Rats were sacrificed 15 min after the end of the stress session and prefrontal cortices and both dorsal and ventral hippocampi were dissected, frozen on dry ice and stored - 80°C for later analyses.

Specifically, the prefrontal cortex (defined as Cg1, Cg3 and IL subregions corresponding to the plates 6-10 according to the atlas of Paxinos and Watson [38]) was dissected from 2-mm-thick slices, whereas the dorsal and the ventral hippocampus (respectively plates 25-33 and plates 34-43 according to the atlas of Paxinos and Watson) were dissected from the whole brain. The left hemisphere was taken for protein whereas the right was taken for RNA.

2.3. RNA preparation and gene expression analysis by quantitative Real-time PCR.

Total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions and quantified by spectrophotometric

analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (RT-PCR) to assess total Bdnf, Arc, cFos, Zif268, Growth Arrest and DNA Damage-inducible protein (Gadd45β), FK506 binding protein 5 (Fkbp5), Forkhead box protein O1 (FoxO1), Nuclear Receptor Subfamily 4 Group A Member 1 (Nr4a1), Serum/Glucocorticoid Regulated Kinase 1(Sgk-1), Mitogen-Activated Protein Kinase Phosphatase 1 (Dusp1) and S100A10 (P11) mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRTPCR instrument (CFX384 real time system, Bio-Rad Laboratories, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories, Italy). Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4). Primers sequences (Table 1) used were purchased from Eurofins MWG-Operon and Life Technologies.

Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reactions. A comparative cycle threshold method was used to calculate the relative target gene expression.

a) Gene	*Forward primer*	*Reverse primer*	*Probe*
Bdnf tot	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Arc	GGTGGGTGGCTCTGAAGAAT	ACTCCACCCAGTTCTTCACC	GATCCAGAACCACATGAATGGG
cFos	TCCTTACGGACTCCCCAC	CTCCGTTTCTCTTCCTCTTCAG	TGCTCTACTTTGCCCCTTCTGCC
Zif268	GAGCGAACAACCCTACGAG	GTATAGGTGATGGGAGGCAAC	TCTGAATAACGAGAAGGCGCTGGTG
Fkbp5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAAG	TGTCCATCTCCCAGGATTCTTTGGC
FoxO1	GAGTGGATGGTGAAGAGTGTG	GGACAGATTGTGGCGAATTG	TCAAGGATAAGGGCGACAGCAACAG
Sgk-1	GACTACATTAATGGCGGAGAGC	AGGGAGTGCAGATAACCCAAG	TGCTCGCTTCTACGCAGC
Dusp1	TGTGCCTGACAGTGCAGAAT	ATCTTTCCGGGAAGCATGGT	ATCCTGTCCTTCCTGTACCT
P11	AGAGTGCTCATGGAAAGGGA	AGCTCTGGAAGCCCACTTTT	ATAATGAAAGACCTGGACCAGTGC
36B4	TTCCCACTGGCTGAAAAGGT	CGCAGCCGCAAATGC	AAGGCCTTCCTGGCCGATCCATC

b) Gene	*Accession number*	*Assay ID*
Gadd45β	BC085337.1	Rn01452530_gl
Nr4a1	BC097313.1	Rn01533237_m1

Table 1: a) Sequences of Forward and Reverse Primers and Probes used in Real-time PCR Analyses and Purchased from Eurofins MWG-Operon.

b) Probes purchased from Life Technologies, which did not disclose the sequence.

2.4. Protein extraction and western blot analysis.

Western blot analysis was used to investigate GR protein levels. Tissues were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM EGTA, 1 mM HEPES solution in the presence of a complete set of proteases (Roche) and phosphatases (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 2,500 rpm for 10 min at 4°C to obtain the pellet corresponding to the nuclear fraction, which was re-suspended in a buffer (20 mM HEPES, 0.1 mM dithiothreitol, 0.1 mM EGTA) with protease and phosphatase inhibitors. The supernatant was further centrifuged at 10,000 g for 15 min at 4°C to obtain the pellet corresponding to the membrane fraction which was re-suspended in the buffer. The supernatant collected corresponded to the cytosolic fraction. The purity of the fractions obtained was showed in supplementary figure 1. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as a calibration standard. Equal amounts of protein were run under reducing conditions on 10% SDS-polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The blots were blocked (GR: 5% bovine serum albumin in TBS + sodioazyde 0,2%; βactin: 10% non-fat milk) and then incubated with the primary antibodies summarized in Table 2. Membranes were then incubated for 1 h at room temperature with the opportune secondary antibody (see Table 2).

Immunocomplexes were visualized by chemiluminescence using the Western Lightning Plus ECL (PerkinElmer) and the Chemidoc MP imaging system (Bio-Rad Laboratories). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa.

Protein	Primary antibody	Secondary antibody
GR	1:500 (Thermo Scientific), 4° O/N	Anti Rabbit, 1:2000, RT 1h
β-ACTIN	1:10000 (Sigma), RT 1h	Anti mouse, 1:10000, 1h RT

Table 2: Antibodies conditions used in western blot analyses. (O/N: over-night; RT: room temperature)

2.5. Statistical analysis

The effects of stress and/or the pharmacological treatment were analyzed with the two-way analysis of variance

(ANOVA) as independent factors. When appropriate, further differences were analyzed by Fisher's Protected Least Significant Difference (PLSD). The complete statistical analyses are summarized in supplementary Table 1. Significance for all tests was assumed for $p<0.05$. Data are presented as means standard error (SEM). For graphic clarity, results are presented as means percent of Vehicle/Sham.

3. Results

3.1. Chronic vortioxetine treatment up-regulates total Bdnf expression in the dorsal and ventral hippocampus.

In order to address the impact of chronic vortioxetine treatment on neuronal plasticity, we investigated the expression of Bdnf, a neurotrophic factor that plays a crucial role in brain plasticity and cellular resiliency [39-41]. The analyses were conducted under resting conditions or following an acute stress, in order to ascertain if vortioxetine is able to modulate the responsiveness of the neurotrophin to the acute challenge.

In the prefrontal cortex, we found a significant effect of stress exposure ($F_{1,32}$=62.089, p<0.001) with total Bdnf mRNA levels significantly increased by acute stress exposure independently from vortioxetine treatment (vehicle: +50%, p<0.001 vs VEH/Sham; vortioxetine: +43%, p<0.001 vs VTX/Sham) (Fig.1a).

On the contrary, the expression of total Bdnf was affected only by the treatment both in the dorsal (Fig.1b) and in the ventral hippocampus (Fig.1c) ($F_{1,34}$=10.213, p<0.01; $F_{1,31}$=10.636, p<0.01 respectively). Indeed, vortioxetine administration upregulated total Bdnf mRNA levels (dHip: +26%, vHip: +31%; p<0.05 vs VEH/Sham), while acute stress did not produce any significant effect in control rats as well as in animals chronically treated with vortioxetine.

3.2. Vortioxetine treatment modulates IEGs expression mainly in the ventral hippocampus

The influence of vortioxetine treatment on neuronal activation following the acute challenge was investigated by measuring the expression of the IEGs Arc, cFos, and Zif268, known to be implicated in the rapid and transient response to plasticity-evoked stimuli [42].

In the prefrontal cortex (Fig. 2a, b, c), we found a significant effect of stress exposure on all the genes considered (Arc: $F_{1,33}$=105.706, p<0.001; cFos: $F_{1,30}$=39.061, p<0.001; Zif268: $F_{1,33}$=97.383, p<0.001). We also found a significant effect of the treatment for Arc ($F_{1,33}$=7.351, p<0.05), but not for cFos and for Zif268, whereas treatment X stress interaction was not statistically significant for all the three IEGs. Accordingly, independently from vortioxetine treatment, the acute challenge significantly increased Arc (vehicle: +195%, p<0.001 vs VEH/Sham; vortioxetine: +221%, p<0.001 vs VTX/Sham) (Fig.2a), cFos (vehicle: +212%, p<0.001 vs VEH/Sham; vortioxetine +597%, p<0.001 vs VTX/Sham) (Fig.2b) and Zif268 (vehicle: +147%, p<0.001 vs VEH/Sham; vortioxetine: +177%, p<0.001 vs VTX/Sham) (Fig.2c) mRNA levels. Moreover, the up-regulation of Arc observed after the ASS in vehicle-pretreated groups is higher than the modulation found in VTX treated rats (-22%, p<0.05 vs VEH/ASS).

A similar effect was found in the dorsal hippocampus (Fig. 2d, e, f), where the two way ANOVA analysis reveals significant effect of stress exposure (Arc: $F_{1,34}$=9.534, p<0.01; cFos: $F_{1,34}$=104.649, p<0.001; Zif268: $F_{1,33}$=17.188, p<0.001). Indeed, the expression of all the IEGs was signicantly upregulated by the acute challenge in rats chronically treated with both vehicle and vortioxetine (Arc: vehicle: +48%, p<0.05 vs VEH/Sham, vortioxetine: +69%, p<0.05 vs VTX/Sham; cFos: vehicle: +254%, p<0.001 vs VEH/Sham, vortioxetine: +316%, p<0.001 VTX/Sham; Zif268: vehicle: +37%, p<0.05 vs VEH/Sham, vortioxetine: +50%, p<0.01 vs VTX/Sham).

In the ventral hippocampus (Fig. 2g, h, i), the modulation of IEGs appears to be more heterogeneous. In particular, acute stress exposure significantly increased Arc mRNA levels only in vehicle-treated rats (+168%, p<0.001 vs VEH/Sham), as indicated by the significant effect of stress ($F_{1,32}$=14.076, p<0.01) and of the interaction ($F_{1,32}$=6.516, p<0.05) while Zif268 was specifically up-regulated (+55%, p<0.001 vs VTX/Sham) after the acute challenge only in vortioxetine-treated rats (stress: $F_{1,33}$=13.974, p<0.01; treatment X stress interaction: $F_{1,33}$=8.079, p<0.01). Consequently, the mRNA levels of Zif268 in vortioxetine groups exposed to ASS was significantly different from that measured in VEH/ASS rats (+28%, p<0.05 vs VEH/ASS).

As shown in Fig. 2h, and similarly to what was found in PFC and dHip, cFos mRNA levels were significantly affected by stress ($F_{1,34}$=100.369, p<0.001). In agreement with these results, exposure to the acute stress increased cFos expression both in vehicle (+442%, p<0.001 vs VEH/Sham) and in vortioxetine treated rats (+277%, p<0.001 vs VTX/Sham), with the effect found in the first groups being greater than the one observed in the second (-21%, p<0.05 vs VEH/ASS).

3.3. Chronic vortioxetine facilitates the translocation of the glucocorticoid receptor into the nucleus after an acute challenge.

Since, as described above, we found that vortioxetine modulates Bdnf expression and the stress-induction of the IEGs mainly in the ventral hippocampus, we investigated glucocorticoid-dependent mechanisms specifically in this brain region. It is known that activation of the HPA axis may affect neuronal function by modulating the translocation of GR to the nuclear compartment, where the receptor modulates the gene transcription through the binding with selected GR responsive elements on the DNA sequence [43]. Hence, in order to investigate if chronic VTX treatment may influence the localization and/or translocation of GR after acute stress exposure, we evaluated the protein levels of the receptor in the whole homogenate as well as in the nuclear and cytosolic compartments. While we didn't observe any statistically significant change of GR levels in the homogenate (Fig.3a), GR protein levels were significantly affected by stress in the

nuclear compartment ($F_{1,28}$=4.735, p<0.05). The effect of the acute stress on GR translocation appears to be primarily driven by vortioxetine since only this group showes a significant increase of nuclear GR levels after the challenge (+48%, p<0.05 vs VTX/Sham), an effect that was paralleled by a trend toward a reduction of GR levels in the cytosol (-29%, p>0.05) (Fig.3c). In Fig. 3d, the changes mediated by the acute challenge in the nuclear and cytosolic compartment were expressed as percent of sham rats, highlighting that chronic vortioxetine treatment appears to facilitate the translocation of GR in the nucleus in response to the acute stress.

To test the possibility that the different translocation may be due to the availability of hormone we also measured the expression of the 11β-hydroxysteroid dehydrogenases isozymes (11β-HSD) 1 and 2, but we did not find any differences (data not shown).

3.4. Vortioxetine promotes the transcription of GR responsive-genes following the AS-stress

Since in the nuclear compartment GR regulates gene transcription, we measured the mRNA levels of different GR-responsive genes, namely Gadd45β, FoxO1, Fkbp5, Sgk1, Dusp-1, Nr4a1 and P11 in the vHip (Fig4), dHip and PFC (supplementary table 2 a/b).

Interestingly, we found that the enhanced translocation of GR in the nucleus in rat ventral hippocampus, observed after stress exposure in vortioxetine-treated rats, was mirrored by an up regulation of the expression of specific genes. In particular, Gadd45β (Fig4a), FoxO1 (Fig.4b) and Fkbp5 (Fig4c) mRNA levels were increased by the acute challenge only in animals chronically pre-treated with vortioxetine (+21%, p<0.05 vs VTX/Sham; +19%, p<0.05 vs VTX/Sham; +62%, p<0.01 vs VTX/Sham respectively). These effects were confirmed by the two-way ANOVA analysis. Indeed, we found a significant effect of the stress ($F_{1,31}$=6.381, p<0.05) for Gadd45β; a significant treatment X stress interaction ($F_{1,34}$=10.738, p<0.01) for FoxO1, while with respect to Fkbp5 a significant effect of stress ($F_{1, 34}$=10.473, p<0.01) and of the treatment X stress interaction ($F_{1,34}$=4.485, p<0.05).

Differently, stress affected the expression of Sgk1 ($F_{1,34}$=151.120, p<0.001) (Fig.4d) and Dusp-1 ($F_{1,34}$=313.549, p<0.001) (Fig.4e). In fact, we found that stress exposure produced a significant increase in both vehicle (Sgk1: +156%, p<0.001 vs VEH/Sham; Dusp1: +321%, p<0.001 vs VEH/Sham) and vortioxetine treated rats (Sgk1: +164%, p<0,001 vs VTX/Sham; Dusp1 +292%, p<0,001 vs VTX/Sham).

As shown in Fig. 4f, Nr4a1 mRNA levels were significantly up-regulated by the acute challenge (VEH: +72%, p<0.001 vs VEH/Sham; VTX: +29% p<0.05 vs VTX/Sham) independently from the pretreatment, as indicated by the significant

stress effect ($F_{1,34}$=18.023, p<0.001). Moreover, as indicated by the two-way ANOVA analysis (treatment: $F_{1,34}$=4.201, p<0.05), Nr4a1 mRNA levels were significantly up-regulated as a consequence of vortioxetine treatment (+43%, p<0.05 vs VEH/Sham). No significant effect of the treatment and of the acute challenge was found in the expression of P11 (Fig.4g).

On the contrary, vortioxetine pretreatment did not influence the enhancement of Gadd45β, [redacted] gene expression due to stress exposure in dHip and PFC (supplementary table 2 a/b).

4. **Discussion**

In this study, we provide evidence on the ability of chronic treatment with the novel antidepressant vortioxetine to modulate neuronal plasticity and the responsiveness to an acute stress in a region-specific manner.

We found that chronic vortioxetine treatment enhanced Bdnf gene expression levels in both the ventral and the dorsal hippocampus, in line with the well-accepted evidence showing that, when chronically administered, antidepressants, characterized by a different pharmacological profiles, have a positive impact on neuronal plasticity [41,44-48] through the modulation of key molecules, such as the neurotrophin Bdnf, with an anatomical specificity [41,44,45,49-51]. As an example, agomelatine acts on Bdnf mainly in the hippocampus [49], fluoxetine enhances its expression in the ventral tegmental area and in the prefrontal cortex [50], whereas duloxetine preferentially modulates the neurotrophin expression in the frontal and prefrontal cortex [51]. Moreover, it has been demonstrated that vortioxetine normalized the depressive-like behaviour and the reduction of BDNF observed in animals exposed to the chronic mild stress paradigm [52] and increased plasma BDNF concentration in depressed patients [53].

According to previous observation showing that neurotrophins, as modulators of synaptic transmission and plasticity, may play a key role also in the response of the brain in a dynamic situation after a pharmacological treatment [35-37], we show that chronic vortioxetine is also able to affect neuronal responsiveness in stressful situations, which may hold important translational implications through an enhancement of brain coping and resilience. In particular, our results suggest that the influence of vortioxetine on stress-response is specifically observed in the ventral hippocampus, a structure that plays a relevant role in anxiety and mood disorders [34].

Indeed, in line with previous data showing an upregulation of Arc in the PFC of rats treated with duloxetine and exposed to the swim stress [54], here we found that the exposure to the acute challenge significantly increased the expression all the IEGs considered independently from the pretreatment in the dorsal hippocampus and in the prefrontal cortex. Conversely, in ventral hippocampus VTX modulated the response to the stress by preventing the increase of Arc and by inducing the expression of Zif 268. Actually, the slight up-regulation of Arc mRNA levels induced by VTX per sè, that has been observed also after the treatment with the antipsychotic lurasidone in the same brain region [55] and in the hippocampus of rats chronically fed with chow containing VTX [56], probably prevented a possible further increase due to the acute stress exposure. On the contrary, we highlighted the ability of the drug to enhance the response to the challenge on Zif268 expression, indicating that this transcription factor, regulated by neurotransmitters and trophic

substances [57], may arrange the reaction to stimuli following the chronic antidepressant administration in this distinct brain subregion.

The anatomical selectivity of VTX effects may be due, at least to some extent, to 5-HT_{1A} receptors that are modulated by VTX and are highly enriched in the CA1 [58] and CA3 [59,60] subregions of the ventral hippocampus as well as to the enhancement of extracellular levels of serotonin due to VTX treatment observed specifically in vHip [3,4].

Next, in order to assess whether the HPA stress-responsiveness may be modulated by the pharmacological treatment, we investigated genomic glucocorticoid receptor mechanisms specifically in the vHip. Interestingly, we found that vortioxetine induced the translocation of GR into the nucleus in response to stress, suggesting that the activation of the genomic pathway may be facilitated in animals chronically treated with the antidepressant. This effect was paralleled by a concomitant decrease of the receptor in the cytosolic compartment, possibly through the dissociation from the chaperone proteins. These results are in line with previous studies showing that antidepressant drugs modulate glucocorticoid receptors activity by interfering at different levels [61] and that, in particular, desimipramine and duloxetine treatment are able to increase nuclear glucocorticoid receptors levels [35,62]. Indeed, it has been demonstrated that desimipramine, tested in cell culture, increased GR into the nucleus and potentiated dexamethasone-induced GR translocation [62], while chronic duloxetine treatment is able to up-regulate nuclear GR in response to stress in rat whole hippocampus [35]. Interestingly, the increased translocation is not due to changes in corticosterone metabolism since we did not observe alterations in the expression of 11β-HSD1-2.

The effect of vortioxetine on nuclear GR translocation was associated with an increased expression of some GR-responsive genes, such as Gadd45β, Nr4a1, FoxO1 and Fkbp5, which may contribute to the rapid coping response set in motion by the acute stress. Interestingly, not all GR-responsive genes investigated appeared to be modulated in the same manner, suggesting that GR translocation is not sufficient to activate their transcription and that other cofactors, such as for example BDNF [63], may participate in their functional modulation in response to external stimuli [64]. Moreover, it has been shown that the modulations of the different GR-target genes are variable depending on the nature and duration of the stimulus, cell type, sex, age (reviewed in [65]).

Among the genes whose stress-induced expression was significantly modulated by chronic vortioxetine, Gadd45β is a neuronal activity immediate early gene that promote the proliferation of neural progenitors in adult hippocampus and may control epigenetic mechanisms by acting as DNA demethylase [66], whereas the transcription factor N4ra1 is implicated in the hippocampal activity-dependent neuronal plasticity and memory formation [67]. With respect to FoxO1,

it is involved in the regulation of cellular homeostasis and resistance to oxidative stress [68] and it was found to be modulated in rat prefrontal cortex by chronic duloxetine administration, whereas Fkbp5 is an important co-chaperone for GR and its mRNA levels are upregulated by chronic stress and restored by duloxetine in the ventral hippocampus [69]. Interestingly, the plethora of processes regulated by these factors potentially magnifies the effect that vortioxetine may produce by modulating them, increasing the potential benefit they may in turn result.

In summary, our data indicate that the modulation observed in vortioxetine treated animals after stress might have an impact on the rapid responses to a challenging experience within brain regions involved in emotional processes, such as the ventral hippocampus, which is compromised in depression [70]. Moreover, our findings suggest that vortioxetine treatment by facilitating HPA axis function may be particularly effective in patients characterized by the so-called glucocorticoid resistance [61,71].

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Figure Legend:

Fig 1: Analysis of total Bdnf mRNA levels in the prefrontal cortex (PFC) (panel b), dorsal (dHip) (panel b) and ventral hippocampus (vHip) (panel c) of rats chronically treated with vortioxetine and exposed to the acute swim stress (ASS).

The data are expressed as a percentage of Vehicle/Sham (set at 100%) and represent the mean ± SEM of 7-9 independent determinations. * p<0.05, *** p<0.001 vs VEH/Sham; ### p<0.001 vs VTX/Sham (two-way ANOVA with Fisher's PLSD).

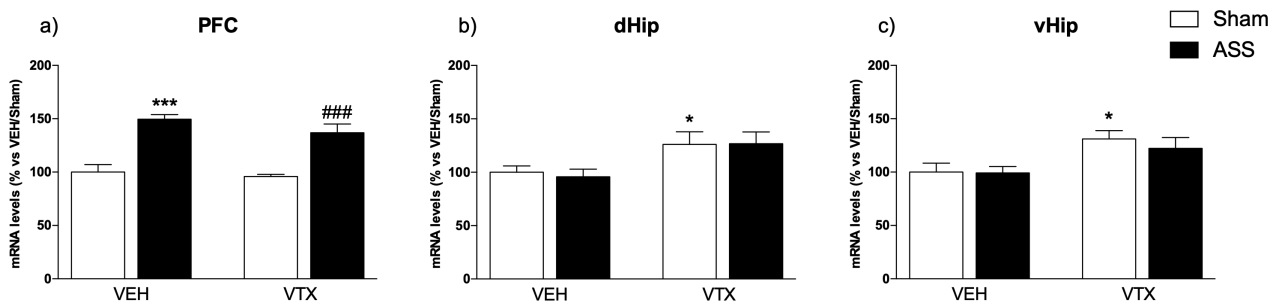


Fig 2: Analysis of Arc (panel a-d-g), cFos (panel b-e-h), and Zif268 (panel c-f-i) mRNA levels in the prefrontal cortex (PFC), dorsal (dHip) and ventral hippocampus (vHip) of rats chronically treated with vortioxetine and exposed to the acute swim stress (ASS).

The data are expressed as a percentage of Vehicle/Sham (set at 100%) and represent the mean ± SEM of 6-9 independent determinations. * p<0.05, *** p<0.001 vs VEH/Sham; ## p<0.01, ### p<0.001 vs VTX/Sham; § p<0.05 vs VEH/ASS (two-way ANOVA with Fisher's PLSD).

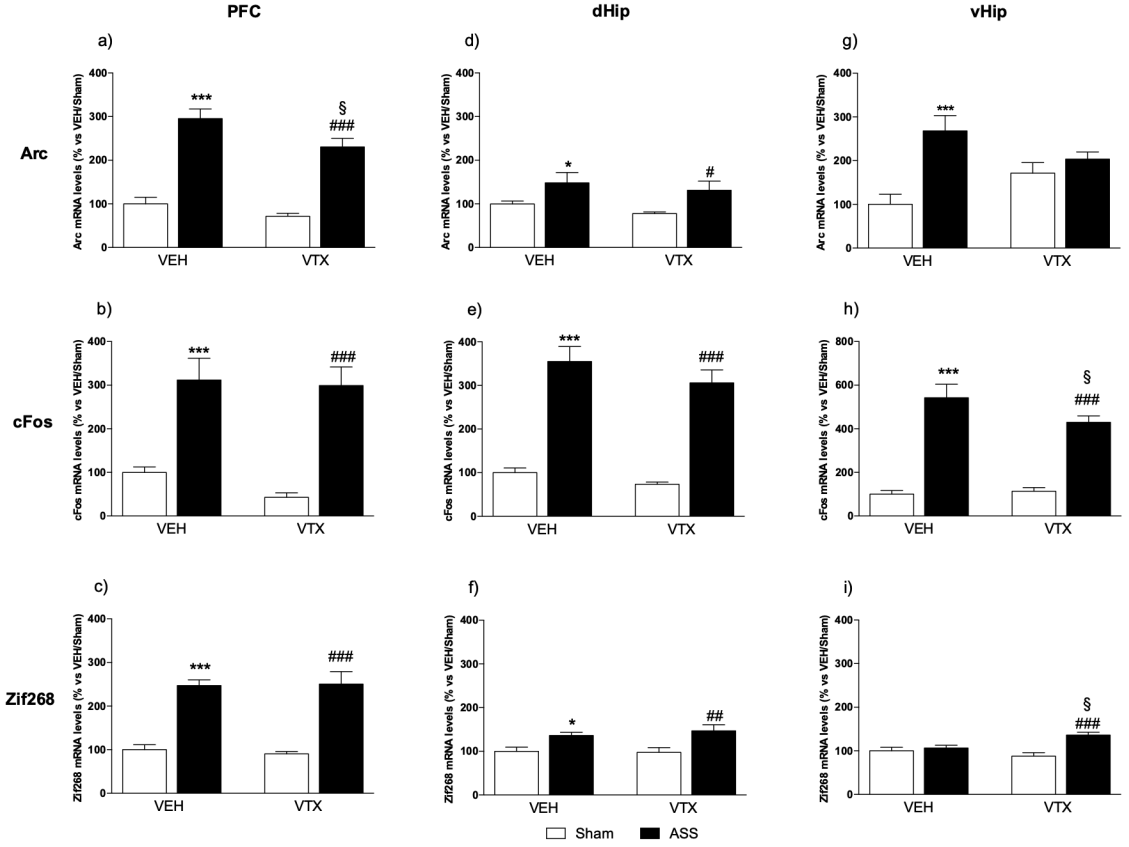


Fig 3: Analysis of GR protein levels in the whole homogenate (panel a), in the nuclear (panel b) and cytosolic fractions of ventral hippocampus (vHip) of rats chronically treated with vortioxetine and exposed to the acute swim stress (ASS).
The data are expressed as a percentage of Vehicle/Sham (set at 100%) (panel a-b-c) or of Sham animals (panel d) and represent the mean ± SEM of 5-9 independent determinations. # p<0.05 vs VTX/Sham (two-way ANOVA with Fisher's PLSD).

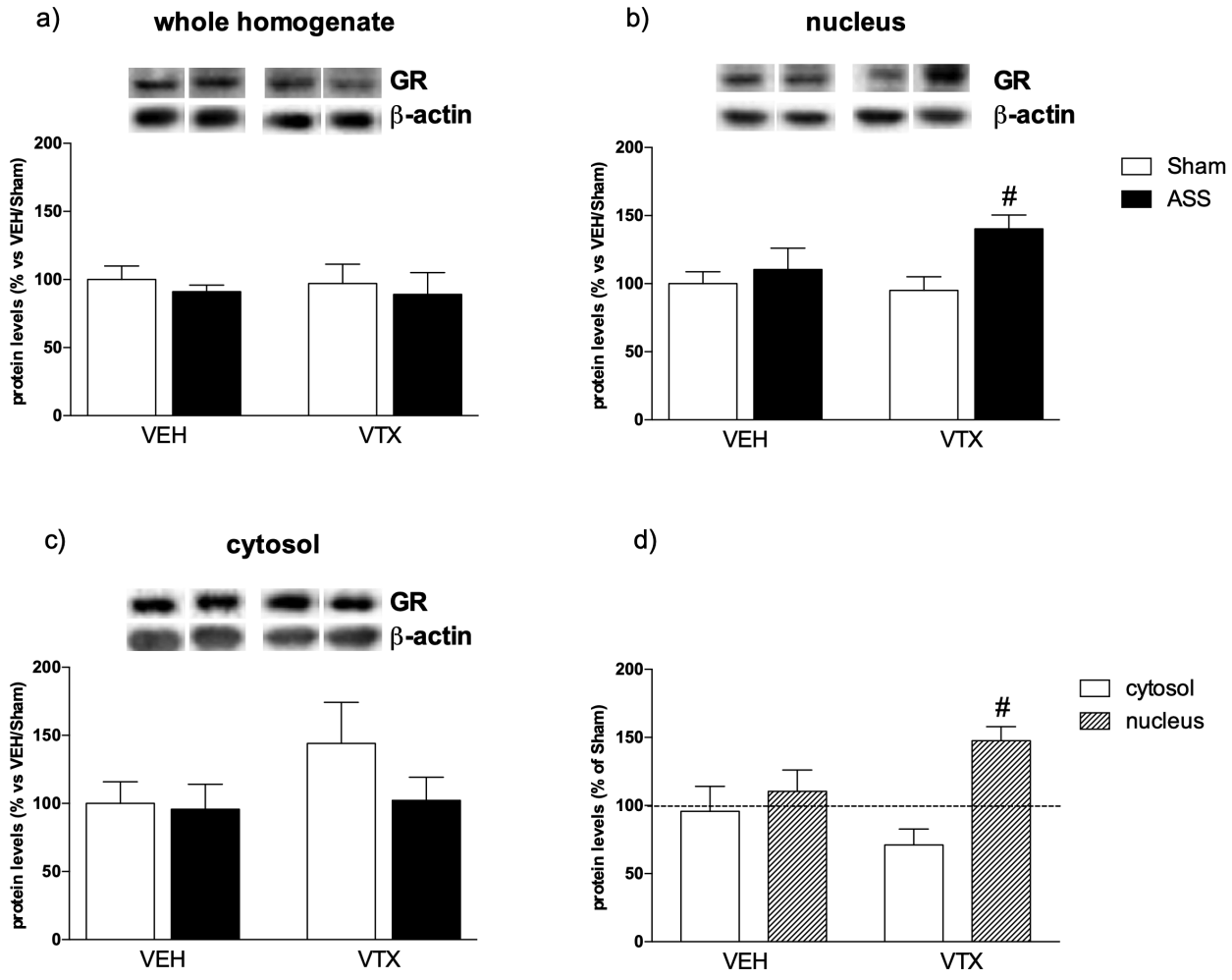
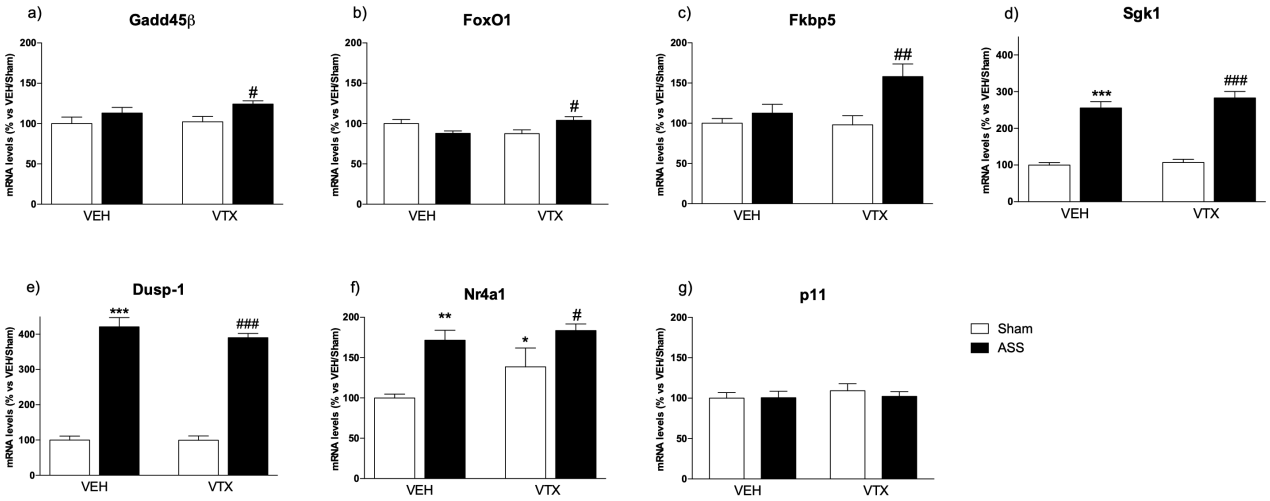


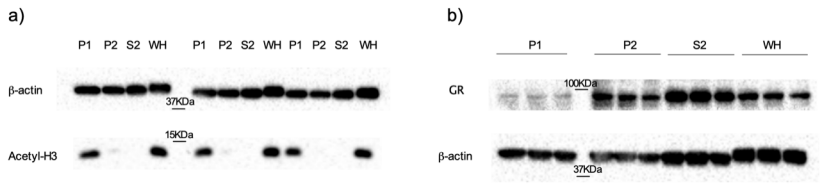
Fig 4: Analysis of GR responsive-genes Gadd45β (panel a), FoxO1 (panel b), Fkbp5 (panel c), Sgk-1 (panel d), Dusp1 (panel e), Nr4a1 (panel f) and P11 (panel g) *in the ventral hippocampus (vHip) of rats chronically treated with vortioxetine and exposed to the acute swim stress (ASS).*

The data are expressed as a percentage of Vehicle/Sham (set at 100%) and represent the mean ± SEM of 7-9 independent determinations. * p<0.05, *** p<0.001 vs VEH/Sham; # p<0.05, ## p<0.01, ### p<0.001 vs VTX/Sham (two-way ANOVA with Fisher's PLSD).



Supplementary Legend:

Fig.1: Western blot images of the purity of fractions (nuclei: P1; membranes: P2; cytosol S2; whole homogenate: WH) obtained with the protein extraction (panel a) and GR relative abundance in the subcellular fractions (panel b) in control animals (VEH/Sham). β-actin was used as internal standard.



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Author's contributions

FC and MAR were responsible for the study concept and design

PB and GC performed and analyzed the molecular analysis.

PB performed the pharmacological treatment and exposed the animal to the stress protocol.

Data analysis and interpretation were done by PB, FC, and MAR.

PB drafted the manuscript and FC and MAR critically revised the manuscript.

All authors critically reviewed the content and approved the final version for publication.

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