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Original Paper

In vivo and in vitro effects of vortioxetine on molecules associated with neuroplasticity

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Abstract

Neuroplasticity is fundamental for brain functions, abnormal changes of which are associated with mood disorders and cognitive impairment. Neuroplasticity can be affected by neuroactive medications and by aging. Vortioxetine, a multimodal antidepressant, has shown positive effects on cognitive functions in both pre-clinical and clinical studies. In rodent studies, vortioxetine increases glutamate neurotransmission, promotes dendritic branching and spine maturation, and elevates hippocampal expression of the activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) at the transcript level. The present study aims to assess the effects of vortioxetine on several neuroplasticity-related molecules in different experimental systems. Chronic (1 month) vortioxetine increased Arc/Arg3.1 protein levels in the cortical synaptosomes of young and middle-aged mice. In young mice, this was accompanied by an increase in actin-depolymerizing factor (ADF)/cofilin serine 3 phosphorylation without altering the total ADF/cofilin protein level, and an increase in the GluA1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor phosphorylation at serine 845 (S845) without altering serine 831 (S831) GluA1 phosphorylation nor the total GluA1 protein level. Similar effects were detected in cultured rat hippocampal neurons: Acute vortioxetine increased S845 GluA1 phosphorylation without changing S831 GluA1 phosphorylation or the total GluA1 protein level. These changes were accompanied by an increase in α subunit of Ca^{2+}/calmodulin-dependent kinase (CaMKIIα) phosphorylation (at threonine 286) without changing the total CaMKIIα protein level in cultured neurons. In addition, chronic (1 month) vortioxetine, but not fluoxetine, restored the age-associated reduction in Arc/Arg3.1 and c-Fos transcripts in the frontal cortex of middle-aged mice. Taken together, these results demonstrated that vortioxetine modulates molecular targets that are related to neuroplasticity.

Keywords

Activity-regulated cytoskeleton-associated protein, antidepressant, calmodulin-dependent kinase, cofilin, fluoxetine, GluA1, neuroplasticity, phosphorylation, protein expression, vortioxetine.

Introduction

Neuroplasticity is critical for brain functions. In addition to affecting learning and memory, neuroplasticity changes are also associated with the pathophysiology of affective disorders such as depression, and with the mechanisms of actions of antidepressants (Hayley and Litteljohn, 2013). Vortioxetine is an antidepressant with a multimodal mechanism of action, directly modulating several serotonin (5-HT) receptors (an agonist of the 5-HT_{1A} receptor, a partial agonist of the 5-HT_{1B} receptor; an antagonist of the 5-HT_{1D}, 5-HT_3, and 5-HT_7 receptors) in addition to inhibiting the serotonin transporter (Mork et al., 2012). Clinical studies in patients with major depressive disorder show that vortioxetine improves depressed mood, as well as some aspects of cognitive function (Katona et al., 2012; McIntyre et al., 2014; Mahableshwarkar et al., 2015; Sanchez et al., 2015). Similarly, in preclinical animal models vortioxetine has demonstrated antidepressant-like and pro-cognitive effects (Guilloux et al., 2013; Li et al., 2013, 2015a; Mork et al., 2012). Therefore, it is plausible that vortioxetine may induce neuroplastic changes.

Several preclinical studies provide evidence that modulation of neuroplasticity is part of the underlying mechanisms for vortioxetine. Direct receptor activities of vortioxetine (antagonism of the 5-HT_3 receptor in particular) inhibits gamma amino-butyric acid (GABA)ergic interneurons and thereby disinhibit neurotransmission, including that of glutamate (Riga et al., 2013, 2014). Vortioxetine also regulates functional plasticity (including long-term potentiation (LTP)) (Betry et al., 2015; Dale et al., 2014) and structural plasticity (such as dendritic branching and spine morphology) (Chen et al., 2016; Waller et al., 2015). Furthermore, in middle-aged mice and young adult rats, vortioxetine increases the expression of genes related to neuroplasticity at the transcript level in the hippocampus and prefrontal cortex, respectively (Du Jardin et al., 2013; Li et al., 2015a); however, the effects of vortioxetine on molecules involved in the regulation of neuroplasticity have so far not been examined in greater detail at the protein level.

Several factors were taken into consideration for the present study. Female mice were chosen for in vivo experiments, to address the need for preclinical studies in this under-studied

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population and to avoid stress-induced neuroplasticity changes (Li et al., 2015b) that result from fighting, which is more common among group-housed males. Considering that neuroplasticity is affected by aging, it is possible that age-associated differences may exist in neuroplastic responses to drug treatment. Therefore, we included young and middle-aged mice, as well as embryonic neuronal cultures, in the current study.

Given that the frontal cortex is essential for cognitive and affective functions, we investigated changes in neuroplasticity markers both in cortical tissue samples and in hippocampal neuronal cultures. Furthermore, we focused on activity-regulated cytoskeleton-associated protein (Arc/Arg3.1), actin-depolymerizing factor (ADF)/cofilin, the GluA1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and the Ca^{2+}/calmodulin-dependent kinase (CaMKII), as these molecules are indicted in functional and structural plasticity. To examine whether the effects of vortioxetine on neuroplasticity are related to the multimodal mechanism of action, we compared the effects of chronic vortioxetine and fluoxetine (a selective serotonin reuptake inhibitor (SSRI)) on the mRNA levels of genes in the frontal cortex of middle-aged mice. In addition to the same targets that were examined at the protein level (Arc/Arg3.1, Gria1 and Camk2a), we measured another molecular marker for neuronal activity, Fos. Because the expression of both Arc/Arg3.1 and Fos are under the control of the transcription factor cAMP response element-binding protein (CREB), similar changes in the expression of both genes may provide stronger evidence for activation of the relevant signaling cascades.

Arc/Arg3.1 is an immediate early gene involved in the regulation of neuroplasticity, and in the consolidation of learning and memory (Bramham et al., 2010; Li et al., 2015b). Arc/Arg3.1 may regulate neuroplasticity at different levels, such as glutamate receptor trafficking, cytoskeleton remodeling and neuronal firing, as well as LTP and LTD (long-term depression) (Bramham et al., 2010; Ren et al., 2014). Vortioxetine reverses the age-associated reduction in hippocampal Arc/Arg3.1 mRNA level in middle-aged mice, which is accompanied by improved performance in a hippocampal-dependent behavioral task (Li et al., 2015a). It is not clear whether this effect can also be detected at the protein level, in other brain regions, or in animals of different ages. Hence, the present study assessed the effects of chronic vortioxetine treatment on the levels of Arc/Arg3.1 mRNA and protein in the frontal cortex of young and middle-aged mice.

Structural neuroplasticity can be regulated by ADF/cofilin, a family of actin depolymerizing factors. ADF/cofilin controls actin turnover, which is a process important for dendrites and spine remodeling (Rust, 2015), as well as for LTP and LTD (Fukazawa et al., 2003; Zhou et al., 2004). Dysregulation of ADF/cofilin has been linked to disruptions of memory (Wang et al., 2013; Zimmermann et al., 2015) and to neurodegenerative or neurodevelopmental disorders (Bamburg et al., 2010; Barone et al., 2014; Zimmermann et al., 2015). Phosphorylation at serine 3 (S3) inactivates ADF/cofilin (Yang et al., 1998) and promotes actin polymerization, which can be regulated by Arc/Arg3.1 (Messaoudi et al., 2007). Here we assess the effects of chronic vortioxetine treatment on the protein levels of total ADF/cofilin and its phosphorylation in cortical tissue from young and middle-aged mice. The antibodies used in this study recognize all members of the ADF/cofilin family.

The AMPA receptor (AMPAR) is essential for glutamate neurotransmission-induced neuroplasticity (Bassani et al., 2013) and for learning and memory (Kessels and Malinow, 2009). Phosphorylation of the AMPAR subunit GluA1 at serine 831 (S831), by CaMKII or protein kinase C and serine 845 (S845, by PKA) residues can alter the localization, conductance and open probability of GluA1-containing AMPAR (Banke et al., 2000; Derkach et al., 1999; Hayashi et al., 2000), thereby regulating neuroplasticity. In addition, Arc/Arg3.1 modulates the trafficking of AMPARs by upregulating their endocytosis (Byers et al., 2015; Chowdhury et al., 2006). Even though vortioxetine increases LTP in the in vitro electrophysiology studies done in hippocampus slices (Dale et al., 2014), it is not clear whether it modulates the expression or phosphorylation of GluA1. In the present study, we therefore investigated the effects of chronic vortioxetine treatment on the levels of total GluA1, S831 and S845 GluA1 phosphorylation in the cortex of young and middle-aged mice, as well as the effects of acute vortioxetine treatment on these measures in cultured neurons.

Kinase CaMKII is critical for neuroplasticity processes such as LTP (Lisman et al., 2012; Shonesy et al., 2014). A significant increase in CaMKII autophosphorylation was detected following induction of LTP in the hippocampal CA1 region (Fukunaga, 1993), and may be considered as a marker for CaMKII activation (Shonesy et al., 2014). Activation of CaMKII is essential for the cognitive enhancer nefiracetam to potentiate AMPAR-mediated LTP (Moriguchi et al., 2008). In the present study, we therefore investigated the effects of vortioxetine on CaMKII autophosphorylation at threonine 286 (T286) and the total CaMKII levels in a primary hippocampal neuronal culture system.

Methods

Animals

Chronic drug effects were measured in young adult (3 months of age) or middle-aged (12 months of age) female C57BL/6 mice (Charles River, Wilmington, MA, USA) housed three per cage. In addition, acute drug effects were measured in cultured hippocampal neurons obtained from singly housed pregnant Sprague-Dawley rat dams (Charles River, Wilmington, MA, USA) on embryonic day 18 (E18). These animals were acclimatized under controlled environmental conditions (22 ± 2 °C; with lights on from 06:00 to 18:00 h) with *ad libitum* access to water and food for 1 week prior to the drug treatments or tissue collection. All animal experiments were carried out in accordance to the Institutional Animal Care and Use Committee of Lundbeck Research, following the US National Institute of Health (NIH) and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

Study design

The chronic effects of vortioxetine on Arc/Arg3.1, cofilin and GluA1 were first examined in the frontal cortical homogenates from both young (3 months old) and middle-aged (12 months old) female mice, after 1 month of treatment (n = 5 to 6 mice per group). The effects of acute vortioxetine (1 h exposure) on GluA1 and CaMKII proteins were examined in cultured rat hippocampal

neurons (n = 3 independent experiments). Finally, the effects of chronic vortioxetine and fluoxetine on the transcript levels of Arc/Arg3.1, Fos, Gria1 (coding for GluA1) and Camk2a (coding for CaMKIIα) were compared in frontal cortical homogenates from middle-aged (12 months old) female mice after 1 month of treatment. Vehicle-treated young adult (3 months old) female mice were included as an age control (n = 10 mice per group).

In vivo drug treatment and sample collection

For the chronic experiments, mice were randomly assigned to different groups and received 1 month of treatment. For the vehicle group, mice were fed with Purina 5001 rodent chow and plain tap water. The vortioxetine group was fed with Purina 5001 chow containing 600 mg vortioxetine/kg of food, and plain tap water. The fluoxetine group received Purina 5001 chow and tap water containing 147 mg fluoxetine/L water. Food pellets were prepared by Research Diets Inc. (New Brunswick, NJ). Compounds were synthesized by Lundbeck A/S (Valby, Denmark). The doses of vortioxetine and fluoxetine used had previously been shown to fully occupy the serotonin transporter (using *ex vivo* autoradiography) and had demonstrated behavioral and molecular effects (Li et al., 2015a). After 1 month of drug administration, the animals were euthanized and their frontal cortices quickly dissected. The left cortices were stored in Ambion RNAlater® reagent (Thermo Fisher Scientific, Waltham, MA, USA) at −20 °C for mRNA analysis, and the right cortices were snap frozen and stored at −80 °C for protein analysis.

Isolation, primary culture and drug treatment of neurons

On embryonic day 18 (E18), pregnant dams were anesthetized with CO_2 and approximately 12 fetuses were obtained from each rat. Whole brains were rapidly isolated from each fetus and their hippocampi were dissected under a light microscope. Fetuses, whole brains and dissected hippocampi were maintained in chilled Hank's balanced salt solution (HBSS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10 mM HEPES (SigmaAldrich, Saint Louise, MO, USA) for the duration of the dissection. Cells were dissociated from hippocampi using a papain dissociation system (Worthington Biochemical Corporation, Lakewood, NJ, USA) and resuspended in Neurobasal Media (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 2% Invitrogen B27 supplement (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5 mM glutamine (100 × Glutamax, Thermo Fisher Scientific, Waltham, MA, USA). Cell quantification was performed using a Cellometer Auto ×4 cell counter (Nexcelom Biosciences, Lawrence, MA, USA), after which the cells were plated into Corning 12-well poly-D-lysine-coated plates (Sigma-Aldrich) at a density of 7.5×10^5 cells per well, in a volume of 2 mL per well. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2/95% air, and their culture media was refreshed every 4–5 days, for 21 days.

For the acute *in vitro* study, dissociated hippocampal cells were treated with culture media containing 1 μM vortioxetine, 100 nM nefiracetam, or vehicle control (0.1% dimethyl sulfoxide (DMSO)) freshly prepared from a 10 mM stock solution for 1 h. Nefiracetam was chosen as a positive control, because it activates CaMKII and increases CaMKIIα autophosphorylation. The dosage of vortioxetine is similar to what was used in spine morphology studies (Waller et al., 2015), and the dose of nefiracetam was chosen based on a previous report (Moriguchi et al., 2008).

Synaptosome preparation from frontal cortices

Crude synaptosomes were prepared as previously published (Li et al., 2010), and synaptic proteins were confirmed to be enriched in this preparation. Tissues were homogenized in lysis buffer (0.32 M sucrose, 20 mM HEPES at pH 7.4, 1 mM EDTA, 1× protease inhibitor cocktail (Sigma-Aldrich), and 1× phosphatase inhibitor cocktail (Sigma-Aldrich)), using a Dounce homogenizer. Homogenates were separated by centrifugation for 15 min at 2800 rcf at 4°C. The supernatant was further centrifuged at 12,000 rcf for 10 min at 4°C. The pellet from the second centrifugation was resuspended in buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 1× protease inhibitor cocktail and 1× phosphatase inhibitor cocktail), and then sonicated using a VirSonic 60 ultrasonic cell disruptor (The Virtus Company, Inc. Gardiner, NY, USA). The protein concentration of the resultant crude synaptosome was determined using the Micro BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

Cell lysate preparation

Hippocampal cells from at least two wells that received the same treatment were combined and lysed using an adjusted RIPA lysis buffer (62.5 mM Tris-HCl at pH 6.8, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 200 mM DTT, 1 μg/mL phosphatase inhibitor (Sigma-Aldrich), 1 μg/mL protease inhibitor cocktail (Sigma-Aldrich), 2% SDS, 0.5% Triton X-100 and 10% glycerol). Following centrifugation for 30 min at 16,000 rcf at 4°C, the supernatant was removed and aliquoted for further analysis. We measured the concentration of extracted proteins using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Western blotting

Synaptosomal (40 μg) or cell lysate (20 μg) samples were separated by electrophoresis using Invitrogen NuPAGE® Novex® 4–12% Bis-Tris protein gels (Thermo Fisher Scientific, Waltham, MA, USA) and transferred to nitrocellulose membranes, which were first incubated with blocking buffer (Rockland Immunochemicals Inc., Limerick, PA, USA) for 1 h at room temperature, followed by an overnight incubation with blocking buffer containing primary antibodies at 4°C. Afterwards, the membranes were thoroughly washed with PBS plus 0.1% Tween 20, and then incubated with blocking buffer containing fluorescent-labeled secondary antibodies, for 1 h at room temperature. After additional washes in PBS buffer, the membranes were scanned using the Li-COR Odyssey system (Li-COR Biosciences, Lincoln, NE, USA). The densities of the target bands were measured using the accompanying software (Image Studio Lite 4.0, Li-COR Biosciences, Lincoln, NE, USA). Membranes were

Table 1. Information on antibodies used in Western blotting experiments.

Primary antibodies	Species	Dilutions	Sources
Arc/Arg3.1	Rabbit	1:2000	Custom-made polyclonal antibody against full-length murine protein (Biogenes, Berlin, Germany)
GAPDH	Rabbit	1:20000	Sigma-Aldrich (Saint Louise, MO, USA) G9545
Phospho-GluR1 (S831)	Rabbit	1:1000	Millipore (Temecula, CA, USA) 04-823
Phospho-GluR1 (S845)	Rabbit	1:1000	Millipore (Temecula, CA, USA) 04-1073
GluR1	Mouse	1:10000	Millipore (Temecula, CA, USA) MAB2263
Phospho-Cofilin (S3)	Rabbit	1:750	Cell Signaling Technology (Danvers, MA, USA) 3311
Cofilin	Mouse	1:750	Cell Signaling Technology (Danvers, MA, USA) 3318
Phospho-CaMKIIα (T286)	Rabbit	1:2000	Abcam (Cambridge, MA, USA) AB5683
CaMKIIα	Mouse	1:2000	Sigma-Aldrich (St. Louis, MO, USA) C265
β-Actin	Mouse	1:100000	Sigma-Aldrich (St. Louis, MO, USA) A2228
Secondary antibodies	**Species**	**Dilutions**	**Sources**
Anti-rabbit IRDye 680RD	Goat	1:20000	Li-Cor Biosciences (Lincoln, NE, USA) 925-68071
Anti-mouse IRDye 680LT	Goat	1:20000	Li-Cor Biosciences (Lincoln, NE, USA) 925-68020
Anti-rabbit IRDye 800CW	Goat	1:20000	Li-Cor Biosciences (Lincoln, NE, USA) 925-32211
Anti-mouse IRDye 800CW	Goat	1:20000	Li-Cor Biosciences (Lincoln, NE, USA) 925-32210

stripped (Revitablot™ Western Blot Stripping Buffer, Rockland Immunochemicals Inc., Limerick, PA, USA) and re-probed for different targets. The antibody information is listed in Table 1. We calculated the levels of phosphorylated GluA1 (p-S845 GluA1 and p-S831 GluA1), ADF/cofilin (p-S3 ADF/cofilin) and CaMKIIα (p-T286 CaMKIIα) as proportions of total GluA1, ADF/cofilin, or CaMKIIα, respectively. The levels of total protein of GluA1, ADF/cofilin and CaMKIIα were calculated relative to GAPDH or β-actin levels. In order to compare samples run on different gels, a common synaptosomal sample was included in every gel and used as a quantitative control. The raw signals were first standardized to the quantitative control, and then expressed as fold changes, relative to vehicle-treated young animals (Figures 1B, 2 and 3) or vehicle-treated neuronal cultures (Figure 4).

Gene expression analysis using quantitative RT-PCR

Transcript level gene expression was measured using quantitative RT-PCR, as previously described (Li et al., 2015a). RNA was isolated from tissue homogenates using a RNAqueous® kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) and mRNA was enriched by removing genomic DNA with a DNase digestion step. RNA concentration and quality were determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription of mRNA to cDNA was accomplished using Superscript II™ and random hexamers (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were performed in duplicate using 600 ng of RNA in each reaction and then the products were pooled. Concentrations of cDNA were quantified using a dye intercalation assay (Quant-iT™ OliGreen® ssDNA Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). SensiFAST™ Probe Hi-Rox mix (Bioline, Taunton, MA), gene specific probe and primers (Thermo Fisher Scientific, Waltham, MA, USA), and 2 ng of cDNA were mixed and total volumes were adjusted to 20 μL. All qPCR reactions were performed in duplicate on 384-well plates using an Applied Biosystems 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Normalization of raw qPCR signals was performed using the geometric mean of a panel of house-keeping genes (B2m, Ppia, Gapdh, Rplp0, Rpl13a, Tbp, Ubc) that were selected for their stable expression based on the geNorm algorithm (Vandesompele et al., 2002). No significant change in the expression levels of housekeeping genes was found between different groups. The relative expression level of each target was calculated using the comparative threshold cycle (C_T) method, with the expression level of vehicle-treated young mice (Figure 1A) or vehicle-treated middle-aged mice (Figure 5) defined as 100.

Statistical analysis

Results are expressed as the mean ± the standard error (SE). Statistical analyses were performed using JMP (SAS, Cary, NC, USA) or Prism (GraphPad, La Jolla, CA, USA) software. Two-way ANOVA (with age and treatment as two independent factors) followed by *post-hoc* Tukey-Kramer tests (Figure 1, Figure 2 and Figure 3), or one-way ANOVA followed by *post-hoc* Dunnett's methods (Figure 4 and Figure 5) were used. Significant levels were defined as $p < 0.05$.

Results

In vivo effects of chronic vortioxetine in young and middle-aged mice

Young and middle-aged mice were treated with vortioxetine for 1 month. The expression of the immediate early gene Arc/Arg3.1 (both at the transcript and protein levels) and several other molecular targets associated with Arc/Arg3.1 and neuroplasticity (both at total protein levels and phosphorylated protein levels) were measured in cortical tissues.

Arc/Arg3.1 expression was measured at the transcript level using quantitative RT-PCR (Figure 1A); and at the protein level, using Western blotting (Figure 1B). There was a significant overall difference in Arc/Arg3.1 transcript levels (Figure 1A; $F_{(3,20)} = 10.70$; $n = 6$ per group; $p < 0.05$); with significant age

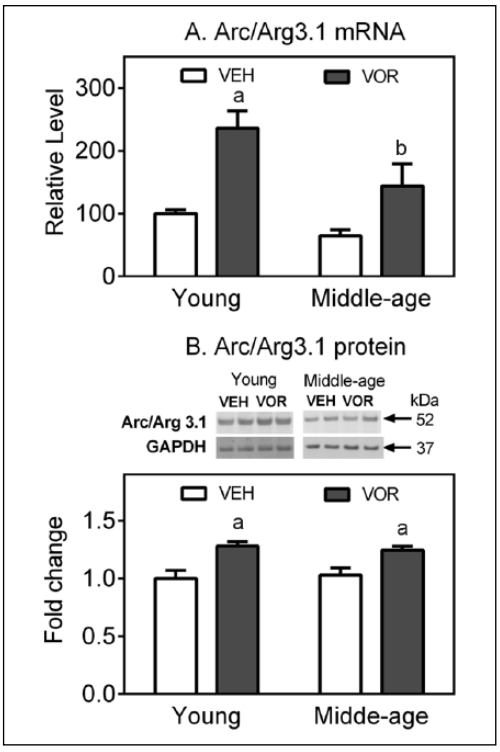

Figure 1. Arc/Arg3.1 expression in the mouse frontal cortex. Arc/Arg3.1 expression at the transcript level was determined using quantitative RT-PCR (A). Vortioxetine significantly increased Arc/Arg3.1 mRNA levels in the frontal cortex of young mice. The increase after vortioxetine treatment did not reach significant levels in the middle-aged mice. Arc/Arg3.1 expression was also examined at the protein level, using Western blotting of synaptosome preparations (B). Vortioxetine significantly increased Arc/Arg3.1 protein levels in the frontal cortex of both young and middle-aged mice (n = 6 per group), 2-way ANOVA followed by Tukey-Kramer *post-hoc* tests.
[a]$p < 0.05$ compared to vehicle treatment.
[b]$p < 0.05$ compared to young mice that received the same treatment.
ANOVA: analysis of variance; mRNA: messenger ribonucleic acid; RT-PCR: reverse transcriptase polymerase chain reaction; VEH: vehicle; VOR: vortioxetine.

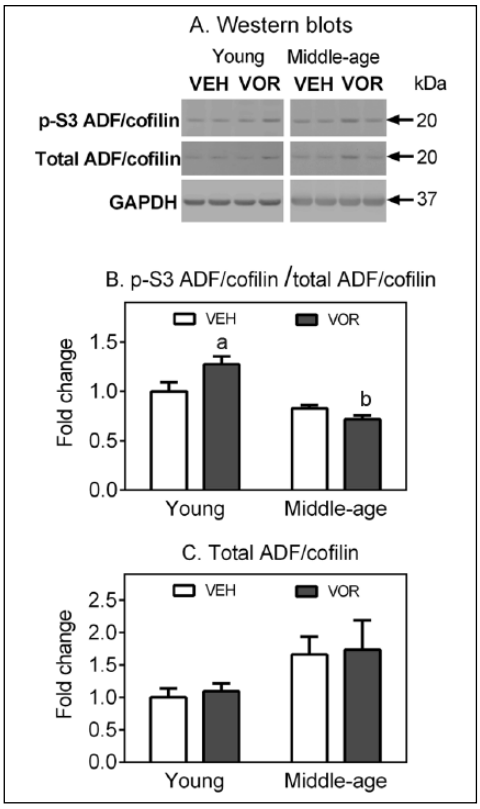

Figure 2. ADF/cofilin protein level and phosphorylation status in synaptosomal preparations from mouse frontal cortex. Total ADF/cofilin and phosphorylated cofilin were examined using Western blotting. Representative blot images are shown (A). VOR significantly increased the phosphorylation of ADF/cofilin (p-S3 ADF/cofilin-1/total ADF/cofilin-1) in young, but not in middle-aged, mice (B). There was no significant difference in total ADF/cofilin levels (C). n = 5–6 per group. Two-way ANOVA followed by Tukey-Kramer *post-hoc* tests.
[a]$p < 0.05$ compared to VEH treatment.
[b]$p < 0.05$ compared to young mice that received the same treatment.
ADF: actin depolymerizing factor; ANOVA: analysis of variance; VEH: vehicle; VOR: vortioxetine.

($F_{(1,20)} = 7.95$; $p < 0.05$) and vortioxetine treatment ($F_{(1,20)} = 22.58$; $p < 0.01$) effects. There was no significant interaction between these two factors, indicating the vortioxetine effect was similar in both age groups. *Post-hoc* analysis demonstrated that vortioxetine significantly increased the Arc/Arg3.1 mRNA levels in young mice, while the increase in middle-aged mice did not reach significance ($p = 0.09$). The Arc/Arg3.1 level in young mice treated with vortioxetine was significantly higher than in middle-aged mice that received vortioxetine treatment. At the protein level (Figure 1B), there was a significant overall difference ($F_{(3,20)} = 7.29$; n = 6 per group; $p < 0.05$), with treatment effect being significant ($F_{(1,20)} = 21.49$; $p < 0.05$). There was no significant age effect or significant interaction between these two factors. *Post-hoc* analysis indicated that vortioxetine significantly increased Arc/Arg3.1 protein levels in the cortical synaptosomes of both young and middle-aged mice.

Total and phosphorylated ADF/cofilin (at S3 residue, p-S3 ADF/cofilin) levels in cortical synaptosomes were measured using Western blotting (Figure 2). There was a significant overall difference in the proportion of ADF/cofilin that was phosphorylated at residue S3 (p-S3 ADF/cofilin/total ADF/cofilin; $F_{(3,19)} = 13.31$; n = 5 to 6 per group; $p < 0.05$; Figure 2B). Both the age effect ($F_{(1,19)} = 31.95$; $p < 0.05$) and the interaction between age and treatment ($F_{(1,19)} = 8.69$; $p < 0.05$) were significant. *Post-hoc* analysis indicated that vortioxetine significantly increased ADF/cofilin phosphorylation in young mice. Young mice that received vortioxetine treatment also had a significantly higher proportion of ADF/cofilin that was phosphorylated than the middle-aged mice treated with vortioxetine. On the other hand, there was no significant difference in total ADF/cofilin levels ($F_{(3,19)} = 1.69$; n = 5 to 6 per group; Figure 2C).

The total and phosphorylated GluA1 protein levels in cortical synaptosomes were also measured using Western blotting (Figure 3). There was a significant difference in the proportion of the GluA1 protein that was phosphorylated at S845 (p-S845 GluA1/total GluA1; $F_{(3,20)} = 23.03$; n = 6 per group; $p < 0.05$; Figure 3B), with significant effects of age ($F_{(1,20)} = 36.47$; $p < 0.05$), vortioxetine treatment ($F_{(1,20)} = 24.83$; $p < 0.05$), and the interaction between

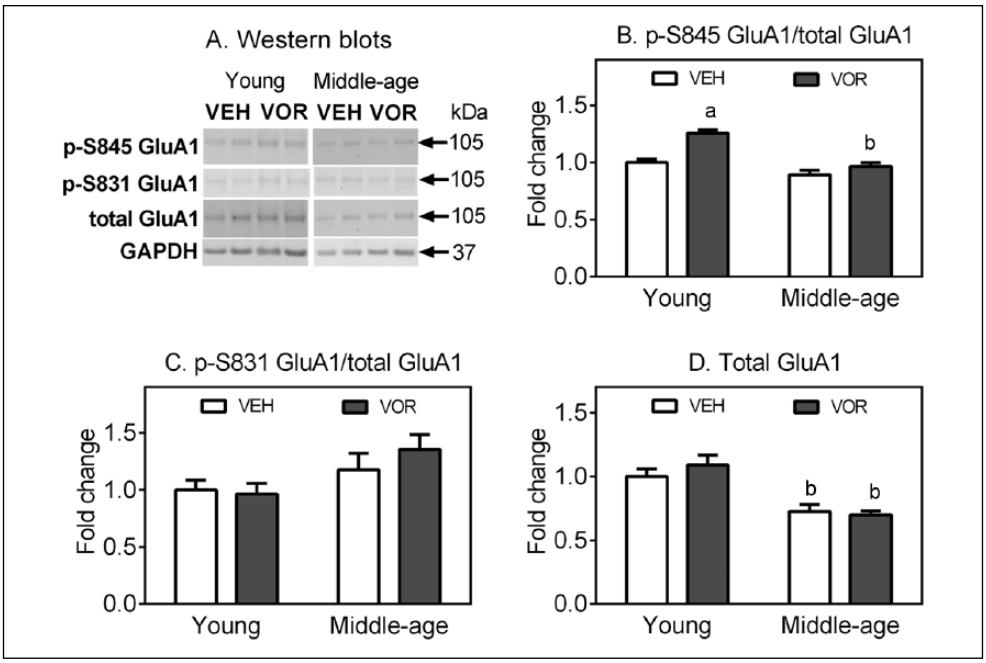

Figure 3. AMPA receptor subunit GluA1 protein level and phosphorylation status in synaptosome preparations from mouse frontal cortex. Total and phosphorylated GluA1 levels were measured using Western blotting of synaptosome preparations (A). Vortioxetine induced an increase in S845 GluA1 phosphorylation (p-S845 GluA1/total GluA1) in young, but not in middle-aged, mice (B). Neither age nor vortioxetine treatment significantly changed the level of S831 GluA1 phosphorylation (p-S831 GluA1/total GluA1, C). Middle-aged mice had significantly lower levels of the GluA1 subunit, and vortioxetine treatment had no effect (D). $N = 6$ per group; 2-way ANOVA followed by Tukey-Kramer *post-hoc* tests.
[a]$p < 0.05$ compared to VEH treatment;
[b]$p < 0.05$ compared to young mice that received the same treatment.
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA: analysis of variance; VEH: vehicle; VOR: vortioxetine.

these two factors ($F_{(1,20)} = 7.78$; $p < 0.05$). *Post-hoc* analysis indicated that vortioxetine induced a significant increase in the S845 GluA1 phosphorylation in young mice. Young mice treated with vortioxetine also had significantly higher S845 GluA1 phosphorylation than middle-aged mice that received vortioxetine treatment. In contrast, there was no overall significant difference in the proportion of GluA1 that was phosphorylated at the S831 residue (p-S831 GluA1/total GluA1; $n = 6$ per group; $F_{(3,20)} = 2.39$; $p = 0.1$; Figure 3C). Lastly, there was a significant difference in total GluA1 levels ($F_{(3,20)} = 11.42$; $n = 6$ per group; $p < 0.05$; Figure 3D), with a significant age effect ($F_{(1,20)} = 32.97$; $p < 0.05$), but no vortioxetine effect or interaction between these two factors. *Post-hoc* analysis demonstrated that middle-aged mice had significantly lower levels of total GluA1 protein in both the vehicle-treated and vortioxetine-treated groups.

In vitro effects of acute vortioxetine in hippocampal neuronal cultures

To further address whether the effects of vortioxetine are mediated by CaMKII, we examined the acute effect of vortioxetine along with a positive control, nefiracetam, on the phosphorylation and total levels of CaMKIIα and GluA1 in an *in vitro* system (Figure 4).

There was a significant overall difference in the proportion of CaMKIIα that is phosphorylated at the T286 residue (p-T286 CaMKIIα/total CaMKIIα) following the drug treatments ($F_{(2,6)} = 8.871$; n = 3; $p < 0.05$; Figure 4B). *Post-hoc* analysis indicated that both vortioxetine (1 μM) and nefiracetam (100 nM) significantly increased T286 CaMKIIα phosphorylation relative to vehicle (0.1% DMSO) treatment. On the other hand, there was no significant difference in total CaMKIIα protein level after the drug treatments (Figure 4C).

There was a significant overall difference in S845 GluA1 phosphorylation (p-S845 GluA1/total GluA1; $F_{(2,6)} = 16.41$; $n = 3$; $p < 0.05$; Figure 4D). *Post-hoc* analysis indicated that vortioxetine, but not nefiracetam, significantly increased S845 GluA1 phosphorylation. On the other hand, S831 GluA1 phosphorylation (p-S831 GluA1/total GluA1; Figure 4E); and total GluA1 (Figure 4F) were not affected by these drug treatments.

Comparison of chronic vortioxetine and fluoxetine with respect to cortical gene expression in middle-aged mice

In a separate, larger cohort (10 mice per group) of middle-aged female mice, we measured the effects of chronic vortioxetine and fluoxetine on mRNA levels of several of the targets examined in the present study (Arc/Arg3.1, Camk2a coding for CamKIIα, Gria1 coding for GluA1) and Fos (coding for c-Fos, a molecular marker for neuronal activity). Vehicle-treated young mice were included as an age control. There was a significant overall difference in Arc/Arg3.1 transcript levels ($F_{(3,36)} = 6.25$; $p < 0.05$; Figure 5A). *Post-hoc* analysis indicated that middle-aged mice had significantly lower levels of Arc/Arg3.1 mRNA

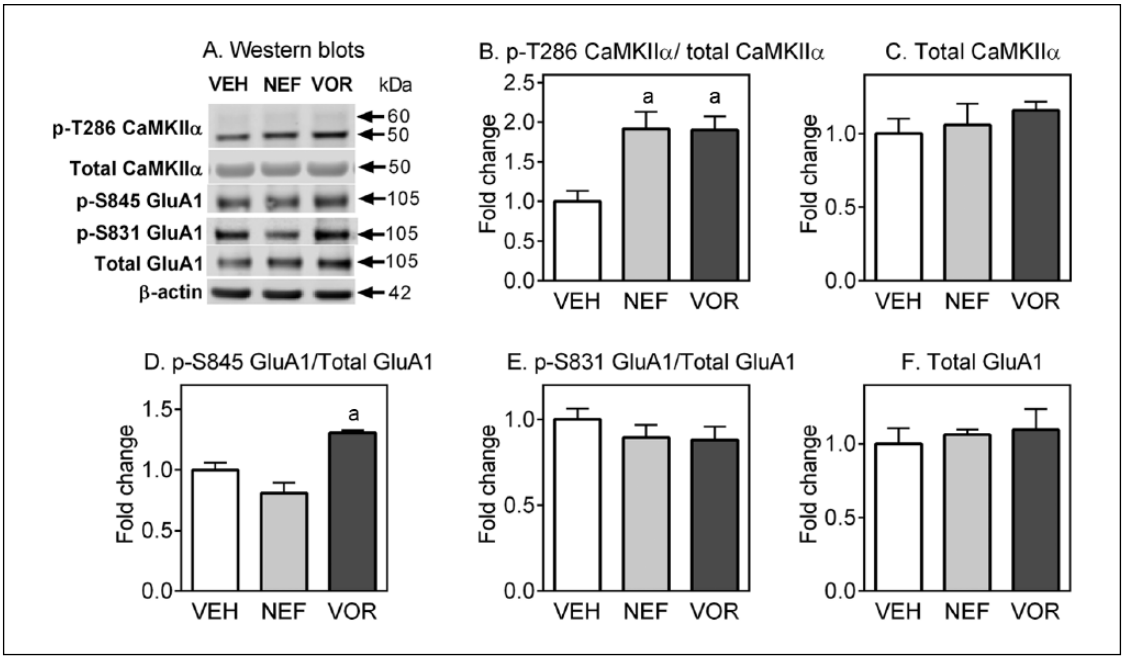

Figure 4. Total levels and phosphorylation status of CaMKIIα and GluA1 in dissociated embryonic hippocampal neurons. Total and phosphorylated CaMKIIα and GluA1 levels were measured in cell lysates obtained from cultured hippocampal neurons using Western blotting (A). Both NEF and vortioxetine significantly increased CaMKIIα phosphorylation at T286 (p-T286 CaMKIIα/total CaMKIIα, B). There was no change in total CaMKIIα protein level (C). VOR, but not NEF, significantly increased S845 GluA1 phosphorylation (p-S845 GluA1/total GluA1, D). S831 GluA1 phosphorylation (p-S831 GluA1/total GluA1, E) and total GluA1 (F) were not changed by drug treatments. Results are summarized from three independent experiments. One-way ANOVA followed by Dunnett's comparison.
[a]$p < 0.05$ compared to vehicle treatment.
ANOVA: analysis of variance; NEF: nefiracetam; VEH: vehicle; VOR: vortioxetine.

when compared to young mice. Vortioxetine, but not fluoxetine, significantly increased Arc/Arg3.1 mRNA levels in the middle-aged mice. There was also a significant overall difference in the mRNA levels of another immediate early gene, Fos ($F_{(3,36)} = 7.83$; $p < 0.05$; Figure 5B). *Post-hoc* analysis indicated that middle-aged mice had significantly lower levels of Fos mRNA, compared to young mice. Vortioxetine, but not fluoxetine, reversed this age-associated reduction in Fos mRNA levels. On the other hand, there was no significant difference in mRNA levels of the GluA1 subunit of the AMPA receptor (Gria1, Figure 5C), nor CaMKIIα (Camk2a, Figure 5D).

Discussion

In the present study, molecular changes following vortioxetine treatment were examined in murine cortical tissue *in vivo* and in rat embryonic hippocampal neuronal cultures, *in vitro*. Vortioxetine increased cortical expression of the immediate early genes Arc/Arg3.1 (at both the transcript and protein levels) and Fos (at the transcript level). Vortioxetine also increased phosphorylation of CaMKIIα (*in vitro*), ADF/cofilin (*in vivo*), and the GluA1 subunit of the AMPA receptor (both *in vivo* and *in vitro*). Taken together, these findings support the hypothesis that vortioxetine increases moieties and processes involved in synaptic plasticity and suggest several possible directions for future studies.

Increased Arc/Arg3.1 expression following chronic vortioxetine treatment was detected in different brain regions in mice of different ages (current and earlier studies by Li et al., 2015a). This may result from increased glutamate neurotransmission (Bramham et al., 2010; Morin et al., 2015), which is achieved by vortioxetine (Betry et al., 2015; Dale et al., 2014; Riga et al., 2013). In addition, direct modulation of other G-protein coupled 5-HT receptors (Li et al., 2015b) may also contribute to this effect of vortioxetine. Consistent with its key role in neuroplasticity (Bramham et al., 2008), upregulation of Arc/Arg3.1 expression may be associated with other molecular changes induced by vortioxetine.

Increasing ADF/cofilin phosphorylation may be part of the underlying mechanism for the effects of vortioxetine on structural plasticity (Chen et al., 2016; Waller et al., 2015). To our knowledge, this is the first report on the effects of an antidepressant on ADF/cofilin. Previous studies demonstrate that modulating cofilin phosphorylation can regulate the size and number of dendritic spines (Calabrese et al, 2014; Carlisle et al., 2008; Chen et al., 2007; Foote et al., 2015; Gu et al., 2010). This is consistent with previous reports that increased Arc/Arg3.1 expression is associated with increased cofilin phosphorylation (Messaoudi et al., 2007; Suzuki et al., 2011). How vortioxetine modulates the phosphorylation of ADF/cofilin is yet to be examined. It is known that the main kinase phosphorylates ADF/cofilin (LIM domain kinase 1, LIMK1) and that the main phosphatase that dephosphorylates ADF/cofilin (protein phosphatase slingshot-1 (SSH-1)) can be regulated by PKA (Nadella et al., 2009; Romarowski et al., 2015) and/or CaMKII (Zhao et al., 2012). It will be interesting to examine which of these possible mechanisms underlies the effect of vortioxetine on ADF/cofilin phosphorylation.

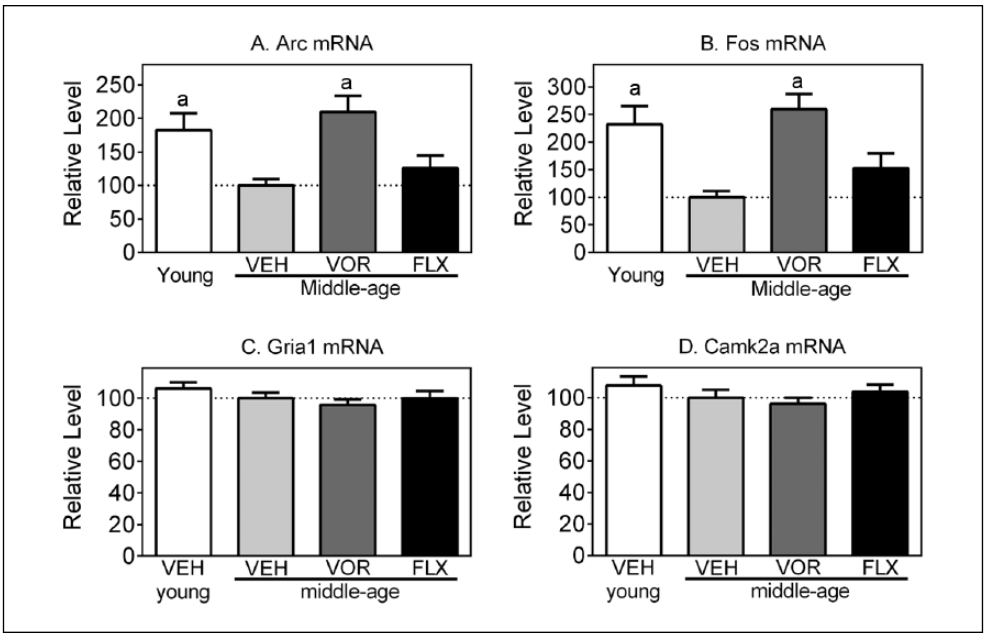

Figure 5. Effects of vortioxetine and fluoxetine on gene expression in the frontal cortex of middle-aged mice. Gene expression levels were measured using quantitative RT-PCR. Levels in young mice were included as an age control. Middle-aged mice had significantly lower mRNA levels of Arc/Arg3.1 (A) and Fos (B), compared to the young mice. These age-associated reductions were fully reversed by chronic vortioxetine, but not fluoxetine, treatment. There was no change in the transcript level of the AMPA receptor subunit GluA1 (Gria) in (C), nor the CaMKIIα (Camk2a, in D). There were $n = 10$ per group. One-way ANOVA was followed by Dunnett's comparison at $p < 0.05$, as compared to VEH-treated middle-aged mice.
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA: analysis of variance; FLX: fluoxetine; mRNA: messenger ribonucleic acid; RT-PCR: reverse transcriptase polymerase chain reaction; VEH: vehicle; VOR: vortioxetine.

Modulation of AMPA receptor subunit GluA1 phosphorylation provides a mechanism for vortioxetine's regulation of functional plasticity (LTP and LTD), which are among the cellular bases critical for memory formation. Results from the present study raise the possibility that vortioxetine may be modulating AMPA receptor trafficking. On the one hand, as vortioxetine increases glutamate neurotransmission (Dale et al., 2014), it is critical for the system to maintain sensitivity to additional stimuli, in spite of increased baseline activity via homeostatic scaling (Davis, 2006). This may be achieved by increasing Arc/Arg3.1 expression, which can upregulate the endocytosis of GluA1(Chowdhury et al., 2006); and by increasing ADF/cofilin phosphorylation, which can reduce the synaptic membrane surface expression of the AMPA receptor, independent from its effect on synaptic structure (Gu et al., 2010; Wang et al., 2013). On the other hand, S845 GluA1 phosphorylation may target the AMPA receptor to cell surface (Diering et al., 2014; Ehlers, 2000; Esteban et al., 2003; He et al., 2009) and replenish the AMPA receptor at the synaptic membrane. Therefore, vortioxetine may be facilitating AMPA receptor trafficking between synaptic and extra-synaptic spaces. In addition, vortioxetine may also modulate the channel activities of the AMPA receptor, as phosphorylation of GluA1 S845 stabilizes Ca^{2+} permeable AMPAR on the cell surface (He et al., 2009), and increases the peak open probability of AMPAR (Banke et al., 2000). The overall effect may be an increased glutamate-gated current (Roche et al., 1996) and amplified downstream signal transduction cascades. It will be interesting to directly measure the distribution of AMPA receptors at the synaptic surface, as well as the functions of Arc/Arg3.1-associated endocytosis machinery in response to vortioxetine in future studies.

The result from the present study provided some evidence that vortioxetine activates both the PKA and the CaMKII signaling cascades. Vortioxetine seems to activate PKA, as PKA-dependent phosphorylation of GluA1 S845 (Roche et al., 1996) was increased after both acute *in vitro* and chronic *in vivo* vortioxetine treatment. In addition, acute vortioxetine increases activation of CaMKII (measured as increased CaMKIIα T-286 phosphorylation), consistent with the effect of increased glutamate neurotransmission. Interestingly, CaMKII/PKC-dependent phosphorylation of GluA1 at S831 (Tan et al., 1994) was not altered after either acute *in vitro* or chronic *in vivo* vortioxetine treatment. This may be due to transient changes in S831 phosphorylation that were not detected at the time points assessed in the current study (Ai et al., 2011). Future studies to directly measure the PKA and CaMKII activities will be useful to test the hypothesis that the PKA and CaMKII signaling cascades are involved in vortioxetine's mechanism of action. Alternatively, increased phosphorylation levels of proteins after vortioxetine treatment may result from down-regulation of phosphatase activities, which also warrants further investigation.

Some age-related changes were observed in the present study. For example, middle-aged mice had lower levels of neuroplasticity-related molecules, including GluA1 protein (Figure 3), Arc/Arg3.1 and Fos mRNA (Figure 5) in the frontal cortex. Similar age-associated changes have been reported in the hippocampus (Chen et al., 2016; Li et al., 2015a; Newton et al., 2008). It is interesting that while middle-aged mice had lower GluA1 protein in synaptosomes, the total GluA1 transcript level was not

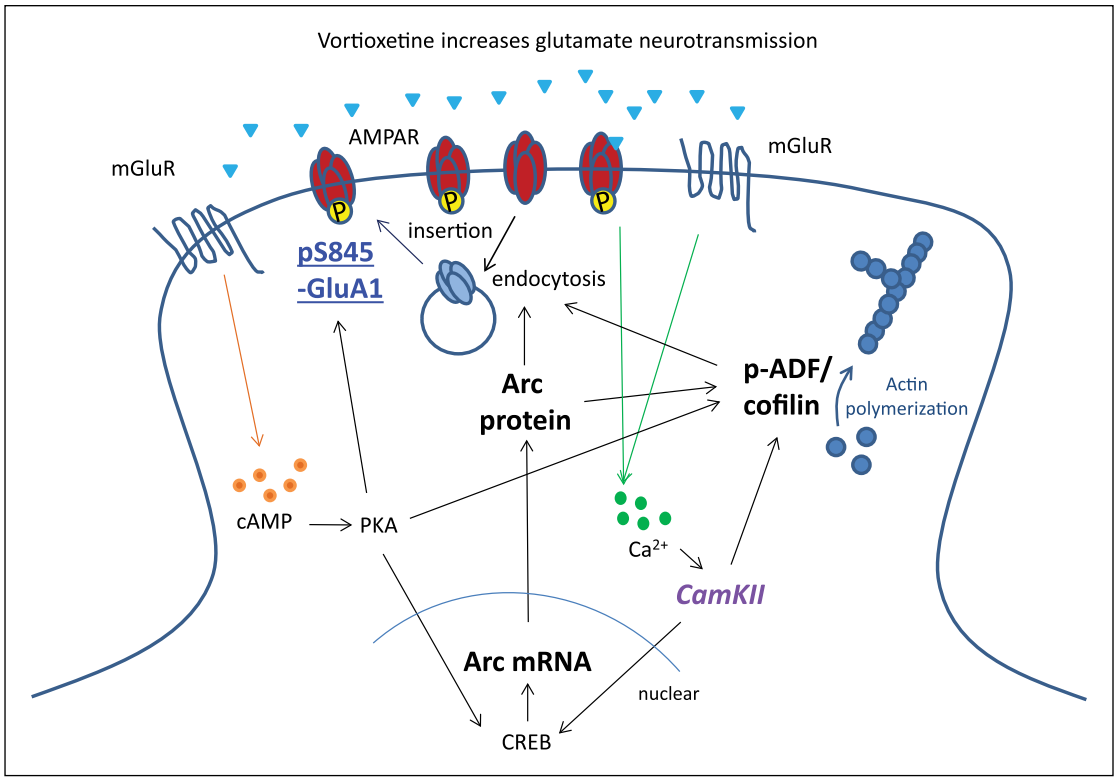

Figure 6. Hypothesized mechanisms for vortioxetine's upregulation of neuroplasticity. The current study demonstrated increases in Arc/Arg3.1 expression, CamKII activation, GluA1 phosphorylation at S845, and phosphorylation of ADF/cofilin (highlighted with bold black font for *in vivo* observations, bold italic purple font for *in vitro* observations, and bold underlined blue font for both *in vivo* and *in vitro* observations). Combined with prior observed electrophysiological effects, we hypothesize some molecular underlying mechanisms for vortioxetine. Vortioxetine-induced increase of glutamate neurotransmission leads to upregulations of the Ca^{2+} and cAMP signaling cascades. These second messengers activate PKA and CaMKII, and increase the expression of immediate-early genes such as Arc/Arg3.1 via upregulating transcription factors such as CREB. Consequences of activation of Arc/Arg3.1 expression and second messenger signaling cascades include an increase in growth and maturation of dendritic spines via inactivation of ADF/cofilin by phosphorylation; an upregulation of AMPA receptor trafficking, including both the removal (via Arc/Arg3.1 and ADF/cofilin-related mechanisms) and insertion (via PKA phosphorylation) of AMPA receptors; and altered AMPA channel activities. The overall effect is increased structural and functional neuroplasticity.

ADF: actin-depolymerizing factor; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; cAMP: cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; PKA: protein kinase A.

different. This may result from a difference in GluA1 subcellular localization (synaptic versus cytosolic localization), a reduction in GluA1 protein synthesis, or an increase in GluA1 protein degradation in middle-aged mice. In addition, middle-aged mice responded to vortioxetine treatment differently compared to young mice. Even though vortioxetine increased Arc/Arg3.1 expression at the transcript and protein levels, vortioxetine did not change the phosphorylation levels of cofilin and GluA1 in middle-aged mice. It will therefore be interesting to investigate if alternative pathways downstream from Arc/Arg3.1, such as PSD95 (Soule et al., 2008) and components of the NMDA receptor complex (Husi et al., 2000), may mediate behavioral outcomes following vortioxetine treatment of middle-aged mice (Li et al., 2015a). These differences may reflect the age-related changes in synaptic plasticity (Oberman and Pascual-Leone, 2013) that are critical for cognitive and affective functions.

The present study demonstrates that vortioxetine is different from fluoxetine in terms of effects on neuroplasticity. In contrast to increased Arc/Arg3.1 and Fos expression in the frontal cortex by vortioxetine treatment, an equivalent dose of fluoxetine was ineffective in middle-aged mice. This is consistent with the findings in the hippocampus (Li et al., 2015a). While the brain-derived neurotrophic factor (BDNF) signaling pathway has been shown to mediate regional effects of fluoxetine on Arc/Arg3.1 expression in young male rodents (Alme et al., 2007), vortioxetine does not increase BDNF at the mRNA level in hippocampal (Li et al., 2015a) or in frontal cortical (results not shown) homogenate in middle-aged female mice. It will be interesting to examine the effects of vortioxetine on BDNF protein levels in different brain regions. In addition, vortioxetine modulates GluA1 phosphorylation at the PKA site without affecting the total GluA1 levels, which is different from the effects of fluoxetine (Ampuero et al., 2010). Differences between vortioxetine and SSRIs (fluoxetine or escitalopram) in other measures of neuroplasticity have been reported, such as structural plasticity (Chen et al., 2016; Waller et al., 2015), functional plasticity (Dale et al., 2014) and neurogenesis (Betry et al., 2015; Guilloux et al., 2013). Direct modulations of 5-HT receptors contribute to the effects of vortioxetine (Betry et al., 2013; Dale et al., 2014). Therefore, the multimodal mechanism of action may be essential for vortioxetine's

modulation of neuroplasticity. This notion is further supported by preclinical studies demonstrating that vortioxetine, but not SSRIs, improved performance of rodents in both cortical- and hippocampal-dependent behavioral tests (Sanchez et al., 2015). We hypothesize that 5-HT_3 antagonism may be the key that differentiates vortioxetine from SSRIs such as fluoxetine.

The current study utilized different experimental systems to examine the effects of vortioxetine on neuroplasticity related molecules. For *in vivo* studies, cortical changes were measured in young adult and middle aged mice, after chronic dosing (1 month). For the *in vitro* study, effects of acute drug treatment (1 h) were measured in isolated embryonic rat hippocampal neurons. Some effects were observed in both young adult mice and in cultured neurons: Vortioxetine increased phosphorylation of GluA1 protein at the S845 residue, without changing the phosphorylation of the S831 residue, nor the total GluA1 protein levels. A hypothetical model of the molecular mechanism of vortioxetine is proposed, combining results from different experimental systems; however, it should be kept in mind that links between *in vivo* and *in vitro* effects are tentative, and some parts of the model are experimental system specific.

We hypothesize that vortioxetine increases neuroplasticity via modulation of multiple molecular targets (Figure 6). Vortioxetine increases glutamate neurotransmission, likely through a 5-HT_3 receptor antagonism-mediated attenuation of GABAergic inhibitory control of glutamate signaling (Dale et al., 2014, 2016; Riga et al., 2014). This may lead to increased activities of Ca^{2+} and cAMP signaling cascades via stimulation of ionotropic and metabotropic glutamate receptors (Menard et al., 2015), which in turn increases the expression of immediate-early genes such as Arc/Arg3.1 and Fos. Consequences of Arc/Arg3.1 activation and signaling cascades include:

1. Increased growth and maturation of dendritic spines via inactivation of ADF/cofilin by phosphorylation; and
2. Upregulation of AMPA receptor trafficking, including both the removal (via Arc/Arg3.1- and ADF/cofilin-related mechanisms) and insertion (via PKA phosphorylation) of AMPA receptors.

The net effect of vortioxetine treatment is increased structural and functional neuronal plasticity, which may be the underlying mechanisms for the positive effects of vortioxetine on cognitive function observed in both pre-clinical and clinical studies (Sanchez et al., 2015).

Declaration of conflicting interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Pirathiv Kugathasan, Jessica Waller, Ligia Westrich, Aicha Abdourahman, Joseph A Tamm, Elena Dale, Connie Sanchez and Yan Li were all employees of Lundbeck Research USA at the time of this study. Maria Gulinello was a consultant for Lundbeck Research USA at the time of this study.

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