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Chronic vortioxetine treatment in rodents modulates gene expression of neurodevelopmental and plasticity markers

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Abstract
The multimodal antidepressant vortioxetine displays an antidepressant profile distinct from those of conventional selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) and possesses cognitive-enhancing properties in preclinical and clinical studies. Recent studies have begun to investigate molecular mechanisms that may differentiate vortioxetine from other antidepressants. Acute studies in adult rats and chronic studies in a middle-aged mouse model reveal upregulation of several markers that play a central role in synaptic plasticity. However, the effect of chronic vortioxetine treatment on expression of neuroplasticity and neurodevelopmental biomarkers in naïve rats has not been evaluated. In the present study, we demonstrate that vortioxetine at a range of doses regulates expression of genes associated with plasticity in the frontal cortex, hippocampus, region encompassing the amygdala, as well as in blood, and displays similar effects relative to the SSRI fluoxetine in adult naïve rats. These genes encode immediate early genes (IEGs), translational regulators, and the neurodevelopmental marker *Sema4g*. Similar findings detected in brain regions and in blood provide a potential translational impact, and vortioxetine appears to consistently regulate signaling in these networks of neuroplasticity and developmental markers.

1. Introduction

Evolving preclinical evidence reveals antidepressants play a role in various neuroplasticity processes in brain regions implicated in major depressive disorder (MDD) (Castren and Hen, 2013; Pehrson et al., 2015; Russo and Nestler, 2013).

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The beneficial effects of antidepressant treatments may be partly mediated by these plasticity mechanisms and alterations in neuronal circuitry. Identification of molecular mechanisms eliciting these effects and translational studies to determine levels of plasticity-related biomarkers in response to antidepressant treatments may aid in a better understanding of their therapeutic capacity. Due to lack of accessibility of CNS tissue in patients, blood biomarkers that show similar regulation in brain regions implicated in MDD may provide a useful diagnostic in patient populations.

The multimodal-acting antidepressant vortioxetine displays a differentiated antidepressant profile relative to other conventional antidepressants in preclinical studies and possesses robust pro-cognitive properties (Sanchez et al., 2015). In addition to its activity as a serotonin (5-HT) transporter (SERT) inhibitor, it is an antagonist at 5-HT_{1D}, 5-HT_3, and 5-HT_7 receptors, agonist at 5-HT_{1A} receptors, and partial agonist at 5-HT_{1B} receptors (Bang-Andersen et al., 2011; Sanchez et al., 2015). Several clinical studies revealed vortioxetine enhances performance in tests of verbal learning and memory and processing of speed (Katona et al., 2012; Mahableshwarkar et al., 2015; McIntyre et al., 2014). At the behavioral level, numerous preclinical studies have demonstrated that vortioxetine augments working memory, including visuospatial and recognition memory (du Jardin et al., 2014; Jensen et al., 2014; Li et al., 2015a; Mork et al., 2013; Wallace et al., 2014). At the functional level, vortioxetine enhances gamma and theta power, related to memory encoding, sensory processing, and attention (Basar et al., 2000; Kaiser and Lutzenberger, 2005; Ward, 2003), pyramidal cell firing in the frontal cortex, and long-term potentiation or synaptic strengthening in hippocampal slices *in vitro* (Dale et al., 2014; Leiser et al., 2014; Riga MS et al., 2013). Vortioxetine showed differentiation from the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and escitalopram and serotonin-norepinephrine reuptake inhibitor (SNRI) duloxetine on these various measures. The localization of the various vortioxetine receptor targets on glutamatergic and GABAergic neurons may be related to its effect on enhanced glutamatergic signaling (Pehrson and Sanchez, 2014). However, the underlying molecular mechanisms by which vortioxetine can enhance memory performance, cognition, and synaptic plasticity remain incompletely understood.

Recent evidence reveals vortioxetine can promote expression of various genes that play a role in synaptic plasticity. Following acute treatment in adult rats, vortioxetine promotes expression of targets that play a role in glutamatergic signaling and dendritic spine morphology (du Jardin et al., 2013). Moreover, chronic vortioxetine treatment in middle-aged mice induces expression of a variety of plasticity targets including those involved in transcription, synaptic signaling, and maintenance of dendritic spine structure (Li et al., 2015a). A bioinformatics analysis revealed a common biology modulated by acute and chronic vortioxetine in rats and mice, respectively (Waller et al., submitted for publication). In support of its effect on expression of cytoskeletal and dendritic spine markers, vortioxetine promotes dendritic branching (Chen et al., 2015; Guilloux et al., 2013), increases spine density *in vivo* (Chen et al., 2015), and promotes a transition to a mature-like, potentially stable, spine morphology and an increase in the number of spines forming presynaptic contacts *in vitro* in hippocampal neurons (Waller et al., 2016).

A major goal has been to determine if treatment-induced alterations in gene expression in various brain regions leaves a transcriptional signature in blood, which would permit testing in patient populations and provide a potential translational link. Thus, we examined a wide array of plasticity targets in brain regions implicated in depression including the frontal cortex (Koenigs and Grafman, 2009), hippocampus (Sapolsky, 2001), region encompassing the amygdala (Hamilton et al., 2008; Yang et al., 2010), as well as in blood, following chronic vortioxetine treatment in adult rats. We also examined the effect of chronic exposure to the SSRI fluoxetine in parallel. We proposed that similar classes of targets would be regulated in response to vortioxetine in both the brain and blood.

2. Experimental procedures

2.1. Animals and dosing

All animal studies were performed in accordance with Lundbeck IACUC institutional and NIH guidelines for the care and use of laboratory animals. Adult male Sprague-Dawley rats, ages 8–12 weeks, from Charles River (Wilmington, MA, USA) were pair-housed under a 12 h light/dark cycle and given ad libitum access to food and water. Following 1 week acclimatization, vortioxetine was administered in food for 1 month, beginning at 8 weeks, at a range of clinically-relevant doses, including 0.22 g/kg of food weight, corresponding to ~50% rSERT occupancy, 0.6 g/kg, corresponding to full SERT occupancy and ~50% occupancy at the r5-HT_{1B} receptor, and a high dose of 1.8 g/kg that achieves full occupancy at SERT and ~90% occupancy at r5-HT_{1B}. Fluoxetine was administered in drinking water for 1 month at a dose of 0.16 g/L, corresponding to full SERT occupancy. Control animals received the same formulation of food without vortioxetine and same drinking water without the fluoxetine.

2.2. Open array (brain regions)

Following chronic, 1-month dosing, rats were sacrificed and the frontal cortex, hippocampus, and region encompassing the amygdala were rapidly dissected in RNAlater (Ambion, Life Technologies, Carlsbad, CA, USA) on ice and stored at -20 °C prior to mRNA processing. Tissue was homogenized on ice in 1 ml of lysis buffer (Ambion RNAqueous 96 kit) using an Autogizer (Tomtec, Hamden, CT, USA). Total RNA was extracted from an aliquot of the lysate using the Ambion RNAqueous 96 automated kit according to the manufacturer's protocol. Following RNA elution from the column, a second DNase digestion was added to eliminate any residual genomic DNA in the samples. The total RNA was evaluated with an Agilent Bioanalyzer 2100 to determine RNA concentration and integrity. The average RNA integrity number (RIN) values were 6.7 for the cortex and 6.3 for the hippocampus. RNA concentration was normalized to 20 ng/μl and reverse transcription was performed using 200 ng of RNA and Superscript VILO (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The Quant-It dye intercalation assay (Life Technologies, Carlsbad, CA, USA) was used to determine cDNA yield, and the samples were normalized to a concentration of 3 ng/μl.

Pre-amplification of the samples was accomplished using 12 cycles of PCR in a reaction containing 10 ng of cDNA, 112 primer sets exactly matching targets on the OpenArray chip, and 2 ×

Taqman Preamp master mix (Life Technologies, Carlsbad, CA, USA) according to the vendor's protocol. A complete list of targets tested can be found in Supplementary Table S2. Amplified samples were analyzed on a QuantStudio 12K flex instrument (Life Technologies, Carlsbad, CA, USA). Data analysis was performed using the Expression Suite software package provided with the instrumentation. Global normalization across the entire chip was used to adjust the raw expression values for all targets. Within each of the 2 tissues, the relative expression of each target was calculated using the comparative cycle threshold values (Ct) method, with the control group set as the reference. The reported relative expression (RQ) value represents the ratio of normalized expression in each treatment group divided by the normalized expression in the control group. Values are denoted as mean $\pm$S.E.M. Statistical significance is defined as a false discovery rate (FDR) p-value of $p<0.05$.

2.3. Analysis of mRNA in blood samples

Immediately following decapitation, $\sim$0.5 ml of trunk blood was collected into EDTA-treated anti-coagulation vacutainer tubes (BD Biosciences, San Jose, CA, USA) containing 1.3 ml of RNAlater (Ambion, Life Technologies, Carlsbad, CA, USA), mixed by inversion, and stored at -20 °C until processed for mRNA levels of various targets. RNA was isolated and quantified using an Ambion RNAqueous Kit (Life Technologies, Carlsbad, CA, USA) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Quantitative PCR (qPCR) and statistical analysis was performed as described previously (Li et al., 2015a). Briefly, cDNA was generated by reverse transcription with Superscript II (Life Technologies, Carlsbad, CA, USA) and quantified using a dye intercalation assay (Quant-iT OliGreen ssDNA Assay Kit, Life Technologies, Carlsbad, CA, USA). qPCR assays were performed in duplicate using an Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). Raw qPCR data was normalized to a panel of 4 housekeeping genes (*Ppia*, *Rpl13a*, *Rplpo*, and *Tbp*) (see Li et al., 2015a). Relative expression was calculated using the comparative threshold (Ct) method, and values are represented relative to the control treatment group. Values are depicted as mean $\pm$S.E.M. The list of targets examined in blood samples is indicated in Supplemental Table S3.

2.4. *Ex vivo* receptor occupancy

A separate set of 24 adult male Sprague-Dawley rats (i.e. 3 per treatment group) were used in *ex vivo* autoradiography experiments. Animals were dosed as described above in groups corresponding to vehicle, 0.22, 0.6, or 1.8 g vortioxetine/kg of food weight or 0.16 g fluoxetine/L of water. Subsequently, all methods for estimating SERT and 5-HT_{1B} receptor occupancy were performed precisely as delineated in Leiser et al. (2014).

2.5. Plasma exposure levels

Frozen rat brains were weighed and homogenized in 3 $\times$ (weight/volume) of brain homogenization buffer (50% water, 30% 2-propanol and 20% dimethylsulfoxide). A 150 µl internal standard (Internal Compound AA34745; 50 g/mL) solution was added to 50 µl of homogenized brain sample, which was then vortexed and separated by centrifugation. The supernatant was collected for analysis of vortioxetine or fluoxetine exposure, and the internal standard solution was added to the sample as described above before thorough mixing. In addition, standard curves for vortioxetine (0-1000 ng/ml for plasma) were generated. The samples were injected directly into an Aria TLX2 coupled with a TSQ Quantum Ultra (both systems Thermo Electron, CA, USA). A Gemini column (Kinetex 2.6µ C18, 50 $\times$ 2.1 mm^2, Phenomenex, Torrance, CA, USA) was used for analytical separation. A typical 3 min gradient with the following mobile phases was used: 0.1% formic acid in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B). The mass spectrometer was equipped with a HESI probe and the source conditions were: Vaporizer temp 450 °C, Spray Voltage 3000, Sheath Gas at 40, Ion Sweep Gas and Aux Gas at 20 and Capillary temp at 300 °C. Spectra were acquired in positive SRM mode with the parent masses of: Vortioxetine 299.16 (m/z) and daughter ion (#1) 109 (m/z) at 37 collision energy (CE) and daughter ion (#2) 150 (m/z) at 27 CE, both at a tube lens of 120.

3. Results

Vortioxetine was administered to adult rats in food at a range of doses with varying SERT and 5-HT receptor occupancies including 0.22 g/kg, 0.6 g/kg, and 1.8 g/kg of food weight for 1 month, or rats were treated with fluoxetine in drinking water at a dose of 0.16 g/L for 1 month. HPLC bioanalysis confirmed appropriate exposure levels for vortioxetine and fluoxetine in plasma at each dose examined (Table S1). Major classes of targets including immediate early genes (IEGs), transcription regulation targets, and neurodevelopmental markers were modulated in response to vortioxetine treatment in all brain regions examined and in blood. Fluoxetine displayed similar effects in comparison to vortioxetine treatment.

3.1. Frontal cortex

Arc, the IEG involved in synaptic and structural plasticity, was downregulated in response to 0.6 g/kg (0.42 $\pm$ 0.05, VOR vs. 1.00 $\pm$ 0.17, Ctrl; *, $p=0.021$) and 1.8g/kg (0.28 $\pm$ 0.04, VOR, vs. 1.00 $\pm$ 0.17, Ctrl; **, $p=0.005$) vortioxetine in the frontal cortex in comparison to control (Figure 1A) (also see Waller et al., submitted for publication). Fluoxetine treatment at a dose of 0.16 g/L also reduced *Arc* mRNA levels (0.47 $\pm$ 0.07, FLX vs. 1.00 $\pm$ 0.17, Ctrl; *, $p=0.029$) (Figure 1A). The IEG *Egr1* was similarly downregulated following 1.8 g/kg (0.50 $\pm$ 0.03, VOR vs. 1.00 $\pm$ 0.07, Ctrl; **, $p=0.003$) vortioxetine treatment, but not in response to fluoxetine (Figure 1A). Likewise, the transcription factor *Fos* was downregulated following 0.6 g/kg (0.43 $\pm$ 0.06, VOR vs. 1.00 $\pm$ 0.15, Ctrl; *, $p=0.034$) and 1.8 g/kg (0.25 $\pm$ 0.03, VOR vs. 1.00 $\pm$ 0.15, Ctrl; **, $p=0.007$) vortioxetine treatment (also see Waller et al., submitted for publication) and fluoxetine treatment (0.42 $\pm$ 0.05, FLX vs. 1.00 $\pm$ 0.15, Ctrl; *, $p=0.028$) in the frontal cortex (Figure 1A). In addition, mRNA expression of *Esr1*, which plays a role in transcriptional regulation, was reduced in response to 0.6 g/kg (0.85 $\pm$ 0.10, VOR vs. 1.00 $\pm$ 0.04, Ctrl; **, $p=0.003$) vortioxetine as well as fluoxetine (0.73 $\pm$ 0.06, FLX vs. 1.00 $\pm$ 0.04, Ctrl; **, $p=0.002$) (Figure 1B). In contrast, the neurodevelopmental and plasticity marker *Sema4g* was upregulated by 0.22 g/kg (1.27 $\pm$ 0.13, VOR vs. 1.00 $\pm$ 0.07, Ctrl; *, $p=0.040$) vortioxetine treatment in the frontal cortex (Figure 1C) (also see Waller et al., submitted for publication). A summary of all regulated targets, related to neuroplasticity and signal transduction, in response to chronic vortioxetine and fluoxetine treatments in the frontal cortex is shown in Table 1.

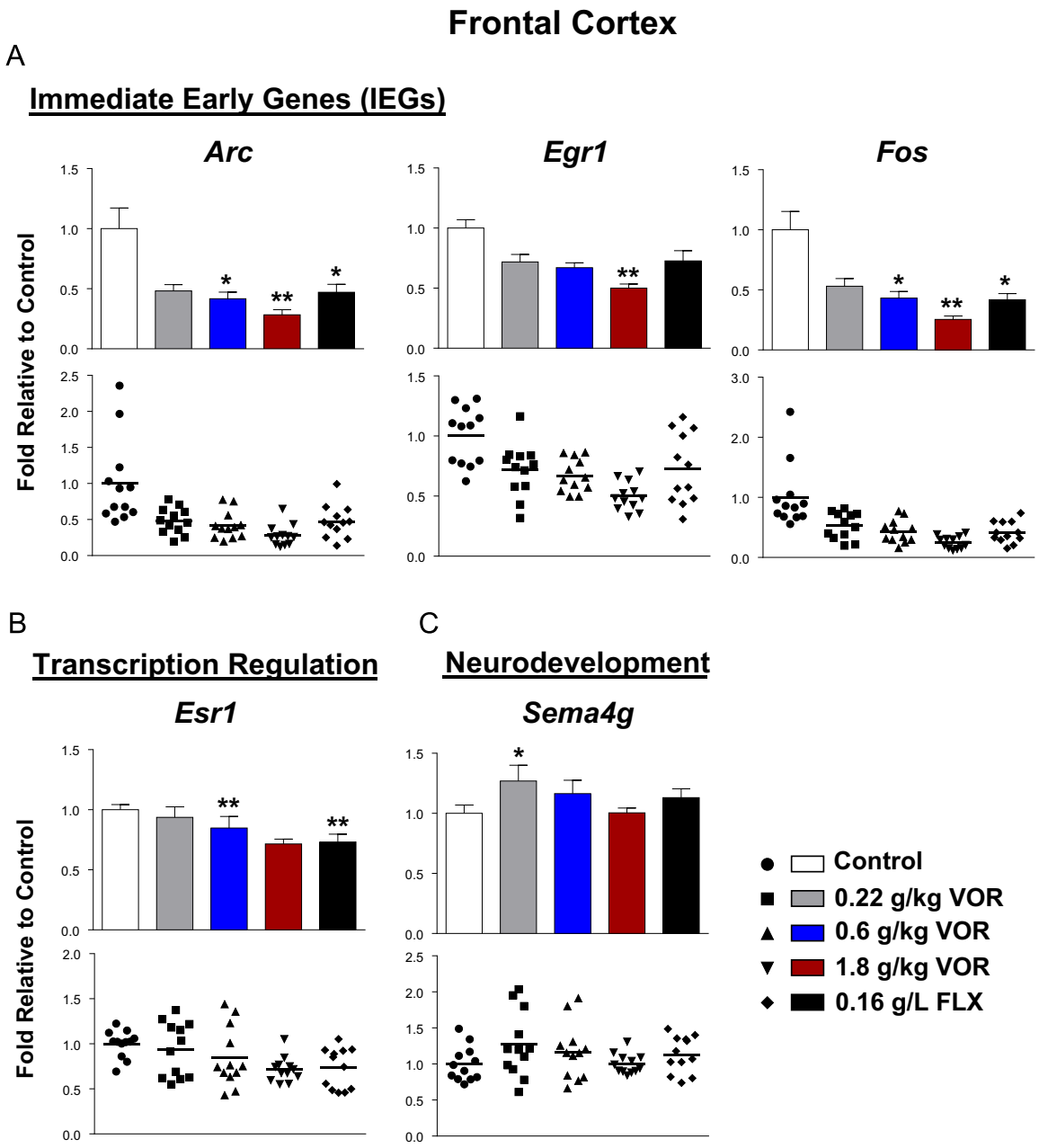

Figure 1 Genes modulated by chronic vortioxetine treatment in the frontal cortex. A panel of genes consisting of receptors, transcription factors, transporters, scaffolding, signaling, neurodevelopmental targets, and growth factors were examined by an OpenArray platform following chronic vortioxetine or fluoxetine treatment. (A) One-month chronic vortioxetine at doses of 0.6 g/kg and 1.8 g/kg of food weight led to a significant downregulation of the IEGs *Arc* (*, $p=0.021$; **, $p=0.005$) and *Fos* (*, $p=0.034$; **, $p=0.007$), and of *Egr1* (**, $p=0.003$), but only at a dose of 1.8 g/kg relative to control treatment. Chronic fluoxetine also induced a decrease in mRNA levels of *Arc* (*, $p=0.029$) and *Fos* (*, $p=0.028$) in comparison to control. (B) Chronic vortioxetine treatment promoted reduced mRNA levels of the transcriptional modulator *Esr1* at a dose of 0.6 g/kg (**, $p=0.003$) as well as chronic fluoxetine (**, $p=0.002$). (C) Chronic vortioxetine administration at a dose of 0.22 g/kg (*, $p=0.040$) led to an upregulation of the neurodevelopmental marker *Sema4g*. Significance is denoted relative to control, and scatter plots are depicted directly below the histograms. VOR = vortioxetine; FLX = fluoxetine. $n=12$ animals/group.

3.2. Hippocampus

Similar to the frontal cortex, mRNA expression of the IEGs *Arc*, *Egr1*, and *Fos* was downregulated in response to vortioxetine treatment. *Arc* levels were reduced following 1.8 g/kg (0.64 ± 0.05, VOR vs. 1.00 ± 0.13, Ctrl); *, $p=0.033$) vortioxetine treatment in the hippocampus relative to control (also see Waller et al., submitted for publication), and fluoxetine had no effect on *Arc* mRNA expression in the hippocampus (Figure 2A). Likewise, mRNA levels of the IEG *Egr1* were decreased following 0.22 g/kg vortioxetine administration (0.85 ± 0.04, VOR vs. 1.00 ± 0.08, Ctrl; *,

Table 1 Summary of regulated genes – frontal cortex. Major classes of targets modulated by chronic vortioxetine and fluoxetine include IEGs, transcriptional regulators, receptors, transporters, neurodevelopmental targets, and other markers involved in vesicle recycling/endocytosis.

Gene Name	Function	0.22 g/kg VOR	0.6 g/kg VOR	1.8 g/kg VOR	0.16 g/L FLX
Arc	AMPAR trafficking; Spine density/morphology; Plasticity	n.s.	↓	↓	↓
Egr1	IEG; Stimulates transcription; Induced by neuronal activity	n.s.	n.s.	↓	n.s.
Epn1	Clathrin-mediated endocytosis	n.s.	↑	n.s.	↑
Esr1	Activates transcription; Cell proliferation/ differentiation	n.s.	↓	↓	↓
Fos	IEG/Cell activation marker	n.s.	↓	↓	↓
Gabrd	Memory; Epilepsy susceptibility	n.s.	↑	n.s.	n.s.
Grin2a	Binds glutamate; Fast kinetics; Modulates LTP	↓	↓	↓	↓
Grm1	Metabotropic glutamate receptor; Coupled to G_q, PKC	n.s.	↓	n.s.	n.s.
Prkca	Kinase activated by mGluR1/5	n.s.	↓	↓	↓
Sema4g	Neuronal development; Axon guidance	↑	n.s.	n.s.	n.s.
Slc1a6	Glutamate transporter	n.s.	↑	n.s.	n.s.
Vamp1	Vesicle targeting/fusion	n.s.	n.s.	↓	n.s.

n.s. = not significant.

$p=0.038$), whereas fluoxetine had no effect (Figure 2A). Moreover, the IEG *Fos* was downregulated in response to 0.6 g/kg (0.53 ± 0.06, VOR vs. 1.00 ± 0.12, Ctrl; **, $p=0.009$) and 1.8 g/kg (0.43 ± 0.05, VOR vs. 1.00 ± 0.12, Ctrl; **, $p=0.004$) vortioxetine (also see Waller et al., submitted for publication) and fluoxetine (0.47 ± 0.04, FLX vs. 1.00 ± 0.12, Ctrl; **, $p=0.004$) treatments (Figure 2A). Expression of the transcriptional regulator *Mapk1* was reduced to nearly significant levels ($p=0.054$) in response to 1.8 g/kg (0.90 ± 0.06, VOR vs. 1.00 ± 0.06, Ctrl) vortioxetine, but not fluoxetine treatment (Figure 2B). The list of all neuroplasticity targets modulated by chronic vortioxetine and fluoxetine in the hippocampus is indicated in Table 2.

3.3. Amygdala region

In the region encompassing the amydgala structures, mRNA expression of similar classes of targets was also modulated by vortioxetine. The IEG *Fos* was downregulated, as seen in the frontal cortex and hippocampus, following 0.6 g/kg (0.50 ± 0.08, VOR vs. 1.00 ± 0.17, Ctrl; *, $p=0.045$) and 1.8 g/kg (0.37 ± 0.04, VOR vs. 1.00 ± 0.17, Ctrl; *, $p=0.025$) vortioxetine, as well as following fluoxetine (0.41 ± 0.06, FLX vs. 1.00 ± 0.17, Ctrl; *, $p=0.019$) administration (Figure 3A). Vortioxetine treatment also led to an increase in mRNA levels of the transcriptional modulators *Mapk1* at a dose of 1.8 g/kg (1.42 ± 0.12, VOR vs. 1.00 ± 0.07, Ctrl; *, $p=0.044$), *Mapk3* at doses of 0.6 g/kg (1.40 ± 0.14, VOR vs. 1.00 ± 0.06, Ctrl; *, $p=0.049$) and 1.8 g/kg (1.58 ± 0.14, VOR vs. 1.00 ± 0.06, Ctrl; *, $p=0.014$) as well as fluoxetine (1.36 ± 0.11, FLX vs. 1.00 ± 0.06, Ctrl; *, $p=0.028$), and *Mapk9* at a dose of 1.8 g/kg (1.13 ± 0.04, VOR vs. 1.00 ± 0.08, Ctrl; *, $p=0.036$) (Figure 3B). Moreover, the neurodevelopmental marker *Sema4g* was upregulated in response to 1.8 g/kg (1.53 ± 0.14, VOR vs. 1.00 ± 0.06, Ctrl; *, $p=0.022$) vortioxetine and fluoxetine (1.49 ± 0.09, FLX vs. 1.00 ± 0.06, Ctrl; *, $p=0.019$) treatments (Figure 3C). A summary of all neuroplasticity-related targets regulated by chronic vortioxetine and fluoxetine in the amygdala region is indicated in Table 3.

3.4. Blood samples

Similar to expression changes detected in brain regions, vortioxetine treatment modulated the same classes of targets in blood samples. Vortioxetine treatment at doses of 0.6 g/kg (0.69 ± 0.04, VOR vs. 1.00 ± 0.08, Ctrl; **, $p=0.003$) and 1.8 g/kg (0.71 ± 0.04, VOR vs. 1.00 ± 0.08, Ctrl; **, $p=0.004$) led to a downregulation of the IEG *Fos* (Figure 4A). Also, similar to that seen in the frontal cortex and amygdala regions, the neurodevelopmental target *Sema4g* was upregulated in response to 0.22 g/kg (1.28 ± 0.07, VOR vs. 1.00 ± 0.07, Ctrl; **, $p=0.008$) vortioxetine administration (Figure 4B). In contrast, fluoxetine treatment had no effect on these markers in the blood. The list of all targets modulated by chronic vortioxetine and fluoxetine in blood is shown in Table 4.

4. Discussion

We examined the effect of chronic vortioxetine treatment and the SSRI fluoxetine in parallel in naïve animals on expression of a wide array of neuroplasticity markers in brain regions implicated in depression and investigated whether similar effects were detected in the blood. Similar patterns of gene expression of plasticity markers in brain

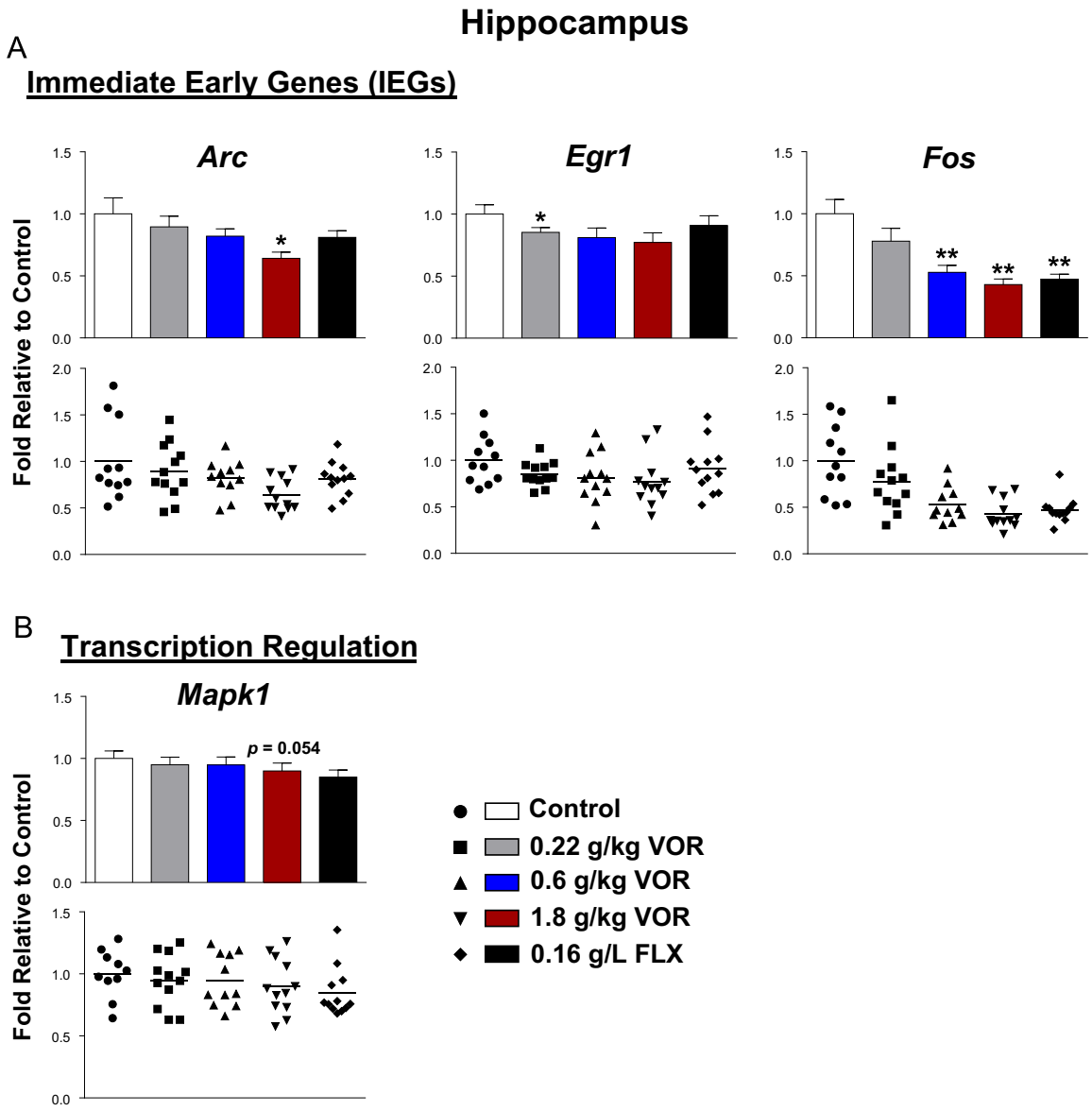

Figure 2 Genes modulated by chronic vortioxetine treatment in the hippocampus. Chronic vortioxetine and fluoxetine treatments promoted similar patterns in expression of genes related to neuronal activity (IEGs) and transcriptional regulation. (A) Chronic 1.8 g/kg (*, $p=0.033$) vortioxetine treatment led to a decrease in *Arc* gene expression, 0.22 g/kg (*, $p=0.038$) vortioxetine promoted a decrease in *Egr1* mRNA expression, and *Fos* gene expression was significantly reduced in response to 0.6 g/kg (**, $p=0.009$) and 1.8 g/kg (**, $p=0.004$) vortioxetine as well as fluoxetine (**, $p=0.004$) to similar levels. (B) *Mapk1* gene expression was reduced to nearly significant levels at a dose of 1.8 g/kg ($p=0.054$) vortioxetine relative to control. Significance is denoted relative to control, and scatter plots are depicted directly below the histograms. VOR = vortioxetine; FLX = fluoxetine. n =12 animals/group.

Table 2 Summary of regulated genes – hippocampus. Major classes of targets regulated by chronic vortioxetine and fluoxetine include IEGs, transcriptional regulators, and signaling molecules.

Gene name	Function	0.22 g/kg VOR	0.6 g/kg VOR	1.8 g/kg VOR	0.16 g/L FLX
Arc	AMPAR trafficking; Spine density/morphology; Plasticity	n.s.	n.s.	↓	n.s.
Egr1	IEG; Stimulates transcription; Induced by neuronal activity	↓	n.s.	n.s.	n.s.
Fos	IEG/Cell activation marker	n.s.	↓	↓	↓
Jak2	Cytokine/STAT signaling	n.s.	n.s.	↓	n.s.
Mapk1	Transcription regulation	n.s.	n.s.	↓	n.s.

n.s. = not significant.

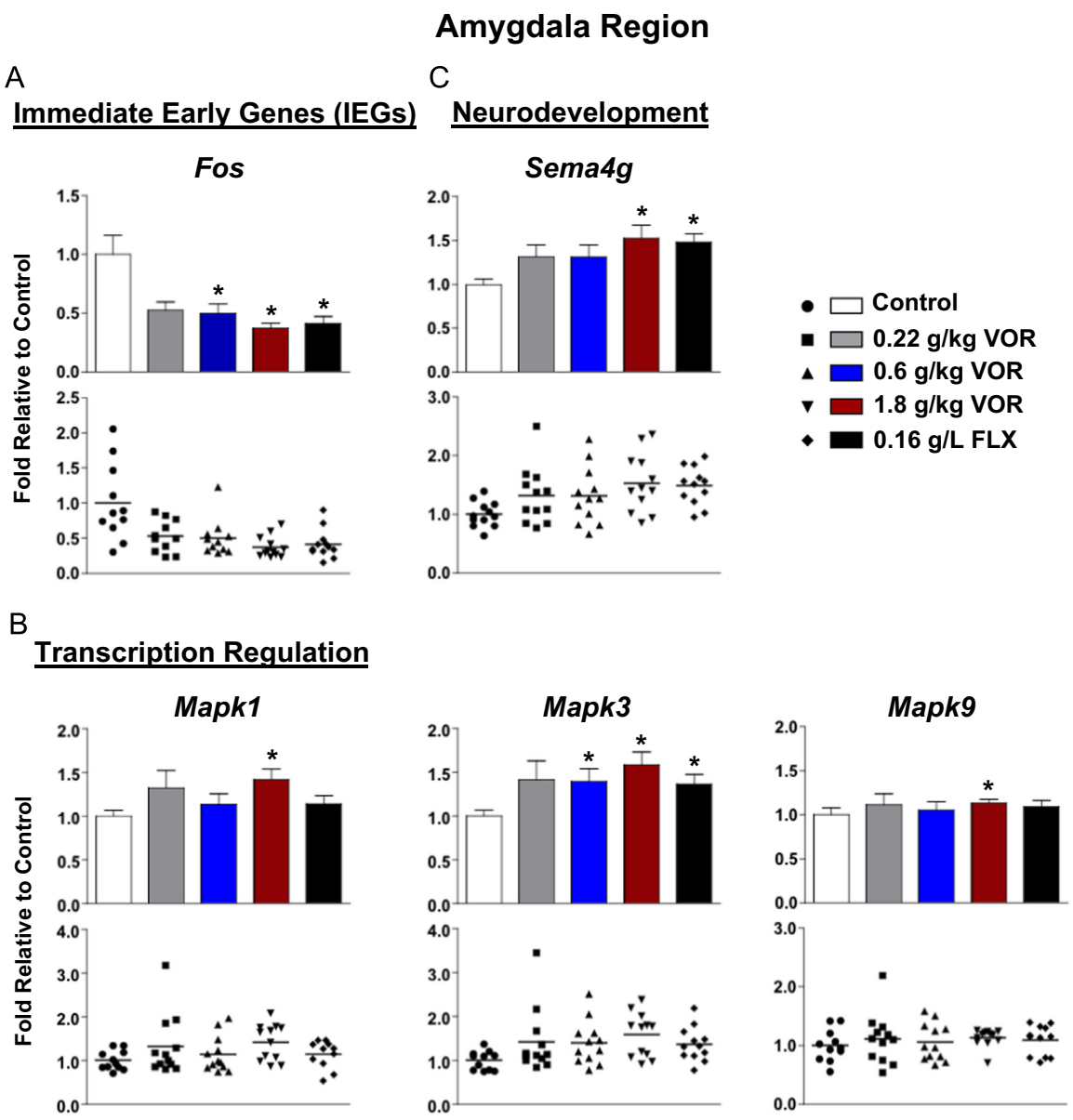

Figure 3 Genes modulated by chronic vortioxetine treatment in the amygdala region. Chronic vortioxetine and fluoxetine produced some similar changes in gene expression in the same class of targets as seen in the frontal cortex and hippocampus. (A) Relative to control treatment, chronic 0.6 g/kg (*, $p=0.045$) and 1.8 g/kg (*, $p=0.025$) vortioxetine led to a downregulation of *Fos* mRNA levels. Chronic fluoxetine (*, $p=0.019$) treatment promoted a similar decrease in *Fos* mRNA. (B) In contrast to the frontal cortex and hippocampus, chronic vortioxetine induced an increase in the *Mapk* class of genes involved in transcriptional regulation at 1.8 g/kg (*, $p=0.044$) for *Mapk1*, at doses of 0.6 g/kg (*, $p=0.049$) and 1.8 g/kg (*, $p=0.014$) for *Mapk3*, and at a dose of 1.8 g/kg (*, $p=0.036$) for *Mapk9*. Chronic fluoxetine (*, $p=0.028$) promoted an increase in *Mapk3* levels too. (C) Chronic vortioxetine at a dose of 1.8 g/kg (*, $p=0.022$), as well as chronic fluoxetine (*, $p=0.019$), also promoted an analogous increase in mRNA levels of the neurodevelopmental and plasticity marker *Sema4g*. Significance is denoted relative to control, and scatter plots are depicted directly below the histograms. VOR = vortioxetine; FLX = fluoxetine. n =12 animals/group.

and blood would provide possible translational potential. We reveal that similar targets, including IEGs (*Arc*, *Fos*, *Egr1*), and targets involved in transcriptional regulation (*Esr1*, *Mapks*), neurodevelopment, and LTP (*Sema4g*), are consistently modulated in response to chronic vortioxetine treatment in both the brain and blood. Furthermore, chronic fluoxetine treatment altered gene expression of similar classes of markers.

Gene expression of IEGs including *Arc*, *Fos*, and *Egr1*, were consistently downregulated by vortioxetine, and in some cases fluoxetine, across all brain regions tested and in the blood. *Arc* encodes the major cytoskeletal protein and plasticity target Arc and regulates all forms of plasticity including LTP, long-term depression (LTD), a form of synaptic weakening, and homeostatic plasticity (Bramham et al., 2010; Li et al., 2015b). *Fos*, which encodes c-fos, and *Egr1*, which encodes zif268, are markers for neuronal activity. Fos

Table 3 Summary of regulated genes – amygdala region. Major classes of targets modulated in response to chronic vortioxetine and fluoxetine include IEGs, growth factors, scaffold molecules, transcriptional/translational regulators, receptors, transporters, and signaling and neurodevelopmental markers.

Gene name	Function	0.22 g/kg VOR	0.6 g/kg VOR	1.8 g/kg VOR	0.16 g/L FLX
Akap9	Links PKA and PP1 to NMDAR complex	n.s.	↓	↓	↓
B2m	Component of Class I MHC; Immune function	n.s.	n.s.	n.s.	↑
Bdnf	Transmission; Dendritic growth and morphology	n.s.	n.s.	↓	n.s.
Cacng4	AMPAR trafficking, gating	n.s.	n.s.	↑	↑
Casp1	Apoptosis, necrosis	n.s.	↑	↑	n.s.
Dlg1	PSD/NMDAR scaffold	n.s.	n.s.	↑	n.s.
Dlg4	PSD/NMDAR scaffold; Plasticity	n.s.	n.s.	↑	↑
Epn2	Clathrin-mediated endocytosis	n.s.	n.s.	n.s.	↑
Esr1	Activates transcription; Cell proliferation, differentiation	n.s.	n.s.	n.s.	↑
Esr2	Activates transcription; Cell proliferation, differentiation	n.s.	n.s.	n.s.	↑
Fmr1	RNA transport; Translation repressor	n.s.	n.s.	↑	↑
Fos	IEG/Cell activation marker	n.s.	↓	n.s.	n.s.
Gabra2	Anxiolytic; Alcoholism susceptibility	n.s.	n.s.	n.s.	↑
Gabra5	Tonic inhibition; Stroke; Ethanol reinforcement	n.s.	n.s.	↓	n.s.
Gria1	Ca^{2+}-permeable; Activity-dept transport to synapse; Synaptic transmission	n.s.	n.s.	n.s.	↑
Gria3	Ca^{2+}-permeable ; Constitutive transport to synapse	n.s.	n.s.	n.s.	↑
Grik2	Ionotropic glutamate receptor; Plasticity	n.s.	n.s.	n.s.	↑
Grik5	Ionotropic glutamate receptor; Plasticity	n.s.	n.s.	n.s.	↑
Htr1a	GPCR-coupled to G_i/G_o	n.s.	n.s.	↓	n.s.
Htr4	GPCR-coupled to G_s	n.s.	n.s.	n.s.	↑
Jak2	Cytokine/STAT signaling	n.s.	n.s.	↑	n.s.
Map2	Microtubule-associated protein 2	n.s.	n.s.	↑	↑
Mapk1	Transcription regulation	n.s.	n.s.	↑	n.s.
Mapk3	Transcription regulation	n.s.	↑	↑	↑
Mapk9	Transcription regulation	n.s.	n.s.	↑	n.s.
Mtor	Protein synthesis	n.s.	n.s.	↑	n.s.
Nlgn3	Cell adhesion; Synapse function, formation	n.s.	n.s.	n.s.	↑
Ntrk2	Neurotrophic Tyrosine Kinase Receptor Type 2/TrkB	n.s.	n.s.	n.s.	↑
Rplp0	Component of ribosomes	n.s.	n.s.	n.s.	↑
S100b	Marker of astroglial activation	n.s.	n.s.	n.s.	↑
Sema4g	Neural development; Axon guidance	n.s.	n.s.	↑	↑
Slc1a3	Glia-specific glutamate transporter	n.s.	n.s.	n.s.	↑

n.s. = not significant.

plays a role in spatial learning and NMDA receptor-dependent LTP (Fleischmann et al., 2003), and zif268 is also tightly associated with synaptic plasticity (Knapska and Kaczmarek, 2004). Consistent with these results, chronic vortioxetine can promote a cell-type specific decrease in c-fos expression in certain subregions of the hippocampus by immunohistochemistry (unpublished observations). However, previous studies have revealed that vortioxetine promotes an upregulation of *Arc* and *Fos* mRNA in the hippocampus of middle-aged mice (Li et al., 2015a). These discrepancies may be attributed to both species- and age-specific differences. In addition, there may be cell-type specific variations in expression that cannot be detected in whole brain regions tested here. At the age examined in this study, the differential gene expression patterns induced by chronic vortioxetine dosing may be maintaining a certain state of plasticity and level of neurotransmission (Kollack-Walker et al., 1999). Moreover, IEGs are typically rapidly or transiently induced and may undergo posttranslational modifications. Further studies are needed to determine protein levels of Arc, Fos, and Egr1, in these samples. Nevertheless, regulation of *Arc*, *Fos*, and *Egr1* expression may be associated with vortioxetine's role in morphological plasticity and LTP (Chen et al., 2015; Dale et al., 2014; Waller et al., 2016).

Transcriptional regulation is very critical to synaptic gene and protein expression and can strongly influence synaptic signaling. Chronic vortioxetine promoted downregulation of mRNA levels of the *Mapk* family of genes, which encode the MAP kinases (MAPKs) or extracellular signal-related kinases (ERKs) in the frontal cortex and hippocampus, while it induced an upregulation in the region encompassing the

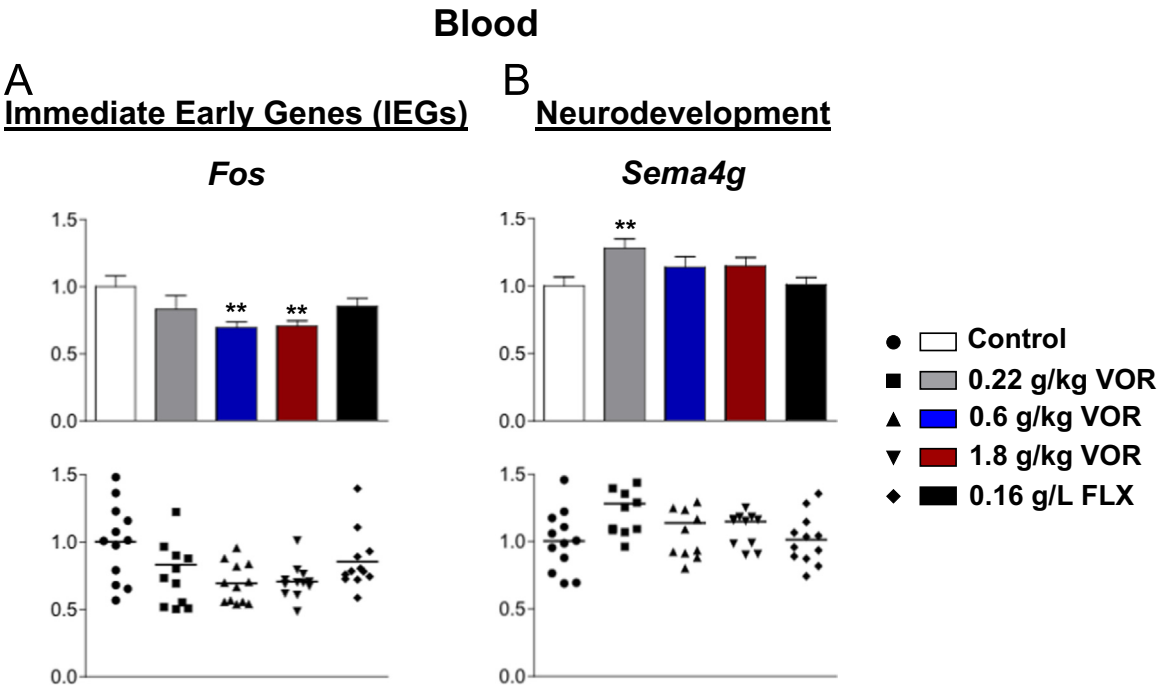

Figure 4 Genes modulated by chronic vortioxetine treatment in blood. A parallel pattern of gene expression was detected for the IEG *Fos* and neurodevelopmental marker *Sema4g* in blood samples. (A) Chronic vortioxetine treatment, but not fluoxetine, at doses of 0.6 g/kg (**, $p=0.003$) and 1.8 g/kg (**, $p=0.004$) promoted decreases in *Fos* mRNA levels. (B) Chronic vortioxetine at a dose of 0.22 g/kg (**, $p=0.008$), but not fluoxetine, led to an upregulation of *Sema4g* mRNA. Significance is denoted relative to control (Unpaired *t*-test), and scatter plots are depicted directly below the histograms. VOR = vortioxetine; FLX = fluoxetine. $n=12$ animals/group.

Table 4 Summary of regulated genes – blood samples. Major classes of targets regulated by vortioxetine consist of IEGs and mitotic, signaling, and neurodevelopmental markers.

Gene name	Function	0.22 g/kg VOR	0.6 g/kg VOR	1.8 g/kg VOR	0.16 g/L FLX
Fos	IEG/Cell activation marker	n.s.	↓	↓	n.s.
Jun	Mitosis; Anti-apoptotic	n.s.	n.s.	↑	n.s.
Psen1	Catalytic component of γ-secretase	n.s.	↑	n.s.	n.s.
S100a10	S100 Calcium-Binding Protein A10/P11	n.s.	n.s.	↑	n.s.
Sema4g	Neural development; Axon guidance	n.s.	n.s.	↑	n.s.

n.s. = not significant.

amygdala. MAPKs are localized to the postsynaptic density (PSD) of dendritic spines, where they can phosphorylate a number of substrates, including cytoskeletal/structural proteins, signaling molecules, and receptors, to modulate synaptic transmission, LTP, and LTD (Mao and Wang, 2015). MAPKs can also function as transcriptional repressors, independent of their kinase activity (Jain et al., 1998). *Esr1*, which encodes the nuclear hormone receptor estrogen receptor α (ERα), was similarly downregulated by vortioxetine in the frontal cortex. Estrogen signaling via ERα promotes a decrease in neuroinflammatory genes in the frontal cortex of middle-aged female rats (Sarvari et al., 2011). The region-specific differences in expression are consistent with opposing effects reported in depression models in cortical and hippocampal *vs.* amygdala regions. Depression is typically characterized by impaired cortical and hippocampal activity and concurrent elevated activity in the amygdala (Andrade and Rao, 2010; Duman, 2004; Krishnan and Nestler, 2010). Protein levels and epigenetic signatures of the MAPKs and ERα and effects downstream of MAPK and estrogen signaling in response to vortioxetine, which may be linked to vortioxetine's role in plasticity and neurotransmission (Dale et al., 2014; Riga et al., 2013), in this model remain to be determined.

The neurodevelopmental marker *Sema4g*, which encodes Semaphorin 4 g, was consistently upregulated by vortioxetine in the frontal cortex and region encompassing the amygdala, and also by fluoxetine in the amygdala region. *Sema4g* was identified as a potential marker modulated by acute and chronic vortioxetine treatment in a large-scale

Table 5 SERT and 5-HT_{1B} receptor occupancy following subchronic vortioxetine and fluoxetine treatments. *Ex vivo* receptor occupancy of SERT and 5-HT_{1B} in subcortical regions of adult Sprague-Dawley rats following 14-day vortioxetine administration at doses of 0.22 g/kg, 0.6 g/kg, and 1.8 g/kg and 0.16 g/L fluoxetine treatment.

Drug/Dose	SERT	5-HT_{1B}
Vehicle	0±3.9	0±2.2
0.22 g/kg VOR	53±2.2	N.D.
0.6 g/kg VOR	88±1.0	52±3.6
1.8 g/kg VOR	98±0.2	86±1.9
0.16 g/L FLX	96±1.9	N.D.

Values denote % occupancy±S.E.M. N.D.=not determined.

network analysis of interacting proteins (Waller et al., submitted for publication). We provide further biological validation that chronic vortioxetine regulates *Sema4g* expression levels in the brain. Notably, *Sema4g* was upregulated by vortioxetine in the periphery as well. Semaphorin 4g is an axonal guidance molecule, but its expression persists in adulthood where it plays a role in neurotransmission and synaptic plasticity (Bouzioukh et al., 2006; Burkhardt et al., 2005). More specifically, Semaphorin 4g localizes to the postsynaptic density, where it interacts with the major NMDAR scaffold postsynaptic density-95 (PSD-95) (Burkhardt et al., 2005). With its role in modulating NMDAR-mediated signaling pathways, regulation of Semaphorin 4g expression by vortioxetine may be related to its enhancement of LTP and cortical activity (Dale et al., 2014; Leiser et al., 2014; Riga MS et al., 2013).

Changes in gene expression were observed across all vortioxetine doses, and there was some level of differentiation following treatment with the SSRI fluoxetine in all brain regions examined. Fluoxetine had no effect on gene expression levels of *Sema4g* and *Egr1* in the frontal cortex and hippocampus, *Arc* in the hippocampus, *Fos*, *Mapk1*, and *Mapk9* in the amygdala region, and no effect on gene expression in blood on all markers examined. This indicates some level of 5-HT receptor target engagement by vortioxetine must be critical for these differential effects (Table 5). In the case of *Sema4g* in the frontal cortex and blood and *Egr1* in the hippocampus, vortioxetine led to alterations in gene expression only at the low dose of 0.22 g/kg. At this dose of vortioxetine, it occupies ~53% of the SERT (Table 5), in contrast to full SERT occupancy for fluoxetine (Table 5) at the dose used in this study, and likely full 5-HT_3 receptor occupancy. This indicates that the 5-HT_3 antagonism of vortioxetine, and not its effect on SERT inhibition, may play a role in expression of *Sema4g* and *Egr1*. The effect of 5-HT_3 antagonism alone on *Sema4g* and *Egr1* expression has not been determined.

There were also many similarities in genes altered by vortioxetine and fluoxetine treatments as well as in the magnitude and direction of changes. The commonalities between vortioxetine and fluoxetine may be attributed to the longer-term chronic dosing regimen performed in this study. Differential effects of vortioxetine with SSRIs are typically evident acutely or following subchronic dosing. Following 1-week vortioxetine administration in adult rats, vortioxetine displayed differential effects on spine density and dendritic branching relative to 1-week fluoxetine treatment (Chen et al., 2015). Vortioxetine promoted an increase in dendritic spine density, dendritic length, and dendritic branching after 1 week, whereas fluoxetine only showed these effects at the 4-week timepoint (Chen et al., 2015). Moreover 2-week vortioxetine treatment p.o., but not fluoxetine, significantly increased dendritic length and branching in the dentate gyrus in mice (Guilloux et al., 2013). Similar effects of vortioxetine and fluoxetine on cell maturation were not detected until 3 weeks following administration (Guilloux et al., 2013). Thus, consequences of vortioxetine relative to fluoxetine treatment would be expected to converge at longer dosing timepoints, with vortioxetine having more rapid-acting effects due to its 5-HT receptor mechanisms and modulation of 5-HT feedback inhibition (Sanchez et al., 2015).

A recent bioinformatics analysis (Waller et al., submitted for publication) revealed that vortioxetine modulates a common biology of neuroplasticity networks in different species and in response to different treatment regimens in the frontal cortex and hippocampus. Vortioxetine may be enhancing neurotransmission by modulating expression of these neuroplasticity-related genes. We present further evidence that chronic vortioxetine treatment can regulate similar biological pathways in the region encompassing the amygdala and in blood. Thus, there is a consistent theme of classes of genes regulated by vortioxetine among brain regions involved in the pathogenesis of depression and in the periphery, providing potential translational potential.

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Conflicts of interest

Jessica A. Waller, Joseph A. Tamm, Aicha Abdourahman, Alan L. Pehrson, Yan Li, Manuel Cajina, and Connie Sanchez, were full-time employees of Lundbeck Research U.S.A. at the time the research was performed.

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Appendix A. Supporting information

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