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Reversal of age-associated cognitive deficits is accompanied by increased plasticity-related gene expression after chronic antidepressant administration in middle-aged mice

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ABSTRACT

Cognitive decline occurs during healthy aging, even in middle-aged subjects, *via* mechanisms that could include reduced stem cell proliferation, changed growth factor expression and/or reduced expression of synaptic plasticity genes. Although antidepressants alter these mechanisms in young rodents, their effects in older animals are unclear. In middle-aged mice, we examined the effects of a selective serotonin reuptake inhibitor (fluoxetine) and a multimodal antidepressant (vortioxetine) on cognitive and affective behaviors, brain stem cell proliferation, growth factor and gene expression. Twelve-month-old female C57BL/6 mice exhibited impaired visuospatial memory in the novel object placement (location) task associated with reduced expression of several plasticity-related genes. Chronic treatment with vortioxetine, but not fluoxetine, improved visuospatial memory and reduced depression-like behavior in the forced swim test in middle-aged mice. Vortioxetine, but not fluoxetine, increased hippocampal expression of several neuroplasticity-related genes in middle-aged mice (*e.g.*, Nfkb1, Fos, Fmr1, Camk2a, Arc, Shank1, Nlgn2, and Rab3a). Neither drug reversed the age-associated decrease in stem cell proliferation. Hippocampal growth factor levels were not consistent with behavioral outcomes. Thus, a change in the expression of multiple genes involved in neuronal plasticity by antidepressant treatment was associated with improved cognitive function and a reduction in depression-like behavior in middle-aged mice.

1. Introduction

Although severe cognitive deficits notably occur in dementia and neurodegenerative diseases, mild cognitive deficits are also prevalent in otherwise healthy older subjects in both humans and in rodent models (Aenlle et al., 2009; Dumas et al., 2013; Ennaceur et al., 2008; Gautam et al., 2011; Gunstad et al., 2006; Salthouse, 2010). Thus, in the following we focus on the physiology of age-related cognitive changes in otherwise healthy middle-aged subjects as such studies may elucidate early changes in mechanisms of age-related cognitive deficits.

Several mechanisms have been proposed to explain age-related decline in cognitive performance, including decreased neurogenesis, reduced growth factor levels and synaptic plasticity, and morphological changes. Decreased neurogenesis in old and middle-aged subjects (Ben Abdallah et al., 2010; Kuhn et al., 1996; Spalding et al., 2013) is primarily due to decreased precursor cell (stem cell) proliferation (Olariu et al., 2007) and may be associated with deficits in hippocampal-mediated tasks (Clelland et al., 2009). Age-related changes in the levels of neurotrophic factors have also been shown in middle-aged rodents (Bimonte-Nelson et al., 2008; Hattiangady et al., 2005; Shetty et al., 2005). Decreased plasticity in middle-aged and aged rodents (Kumar, 2011; Rex et al., 2005) may also play a role in cognitive impairment (Balietti et al., 2012). In this context, it is interesting that antidepressants, which have been shown to have pro-cognitive effects in some animal models (Elizalde et al., 2008; Schilstrom et al., 2011), also alter stem cell proliferation (Ibi et al., 2008), growth factor expression (Song et al., 2006) and neuroplasticity-related gene expression (Djordjevic et al., 2012; Freitas et al., 2013), which could underlie the

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2′-deoxyuridine; ELISA, enzyme-linked immunosorbent assay; FLX, fluoxetine; IGF-1, insulin-like growth factor 1; NGF, nerve growth factor; NT-3, neurotrophin-3; PCR, polymerase chain reaction; SSRI, selective serotonin reuptake inhibitor; SERT, serotonin transporter; TBS, tris-buffered saline; VEGF, vascular endothelial growth factor; VEH, vehicle; VOR, vortioxetine.

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effects of antidepressants on cognitive function as well as mood. This is of particular importance as even sub-threshold depressive symptoms negatively impact cognitive functions in middle-aged and old subjects (Brevik et al., 2013).

Both selective serotonin reuptake inhibitors (SSRIs) and vortioxetine (1-[2-(2,4-dimethylphenylsulfanyl)-phenyl]piperazine) are antidepressants that may improve cognitive function (Biringer et al., 2009; Egashira et al., 2006; ElBeltagy et al., 2010; Mork et al., 2013; Wallace et al., 2014) and increase stem cell proliferation in young adult rodents (Guilloux et al., 2013; Ohira et al., 2013), but their effects in older animals have not been well studied. In addition, SSRIs and vortioxetine have different mechanisms of action. Vortioxetine is a multimodal antidepressant that inhibits the serotonin transporter (SERT), and is also an antagonist for 5-HT_{1D}, 5-HT_3, and 5-HT_7 receptors, a partial agonist for 5-HT_{1B} receptors and an agonist for 5-HT_{1A} receptors (Bang-Andersen et al., 2011). In clinical studies, vortioxetine improves cognitive performance in adults (McIntyre et al., 2014) as well as elderly patients with depression (Katona et al., 2012), even though this population tends to be less responsive to the effects of SSRIs (Tedeschini et al., 2011). Thus, it is of interest to examine the effects of both agents in middle-aged mice.

In the current study, we first characterized deficits in a battery of behavioral tests in healthy middle-aged (12 month) and young (3 month) mice. We then assessed the behavioral effects of vortioxetine and fluoxetine in the middle-aged mice. To elucidate the underlying mechanisms of the observed differences, we also examined age- and drug-induced effects on hippocampal cell proliferation, growth factor levels and expression of plasticity-related genes in relevant brain regions.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice of different ages (40 young — 3 months and 120 middle-aged — 11 months) were acquired from Charles River (Wilmington, MA), and group housed (2 per cage). Animals were kept under a 12:12 light:dark cycle (lights on 6 am, lights off 6 pm) with *ad libitum* access to water and food. All animal experiments were conducted in accordance to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and approved by the Institutional Animal Care and Use Committee of Lundbeck Research USA.

2.2. Experimental design

Eleven-month-old mice were randomly divided into three groups (40 mice per treatment group) and received 1 month of vortioxetine, fluoxetine or vehicle treatment. The vortioxetine group mice were fed with Purina 5001 rodent chow containing vortioxetine (synthesized by H. Lundbeck A/S, Valby, Denmark) at a concentration of 600 mg base per kg food (Research Diets Inc., New Brunswick, NJ) and drank plain tap water. The fluoxetine group mice were fed with Purina 5001 rodent chow and drank fluoxetine in tap water (synthesized by H. Lundbeck A/S, Valby, Denmark) at a concentration of 143 mg base per liter. The vehicle group was fed with Purina 5001 rodent chow and drank plain tap water. Drug concentrations of both fluoxetine and vortioxetine were chosen to reach therapeutic dose range based on brain SERT occupancy results from previous studies (Li et al., 2013) and confirmed with *ex vivo* radio-autography analysis. Body weights were also monitored to confirm no gross changes in animal's feeding behavior. At 12 months of age, 20 mice of each treatment group underwent behavioral tests. Four behaviorally tested 12 month old mice from each drug treated group were used in *ex vivo* radio-autography measurement of SERT occupancy, to confirm sufficient drug exposure. Remaining behavioral test-naive animals were used for BrdU uptake assay, qPCR and various growth factor quantifications. Similar numbers of young mice (3 months old) were included as controls. Detailed sample sizes are described in the Results section and figure legends.

2.3. Behavioral tests

Behavioral tests were conducted in the following order: open field, novel object placement, novel object recognition, social preference and forced swim test. Animals received one test per day, with at least one day between tests. Animals were brought into the laboratory for acclimatization at least 30 min prior to behavioral testing. All tests were conducted under low levels of incandescent lighting between 9 am and 5 pm.

2.3.1. Open field test (OF)

Animals were allowed to freely explore a testing arena (50 cm × 50 cm × 35 cm) for 6 min and their activity was analyzed using tracking software (Viewer, Biobserve, Bonn, Germany). General locomotor activity was assessed as total track length and anxiety-like behavior as the proportion of activity occurring in the center of the arena (% center activity = 100 × center track length / total track length).

2.3.2. Novel object placement (OP)

Visuospatial memory was examined using a novel object placement test (a.k.a. novel object location test, place recognition test, or spatial novelty test) (Ennaceur and Meliani, 1992; Yassine et al., 2013). Briefly, mice were first allowed to explore in an open field containing two identical objects (with high contrast intra-arena visual cues) for 3 min (Trial 1 — training trial). The amount of object exploration (defined as rearing on, whisking, sniffing or touching the objects with nose and/or forepaws) was scored manually using stopwatches. After a retention interval of 45 min, mice were returned to the same testing arena for another 3 min (Trial 2 — testing trial), with one object moved to a different location. Exploration of each object was again manually scored. Results of the object placement test were reported as novel object placement preference scores (100% × exploration during testing $\text{trial}_{\text{relocated object}}$ / total object exploration during testing trial). Animals with intact visuospatial memory preferentially explore the relocated (novel) object and thus would have a preference score >50%. The results of the object placement test were also reported as the proportion of animals failing and passing, with passing defined as preference score >55%. Criteria for establishing a pass/fail cutoff are detailed and justified elsewhere (Li et al., 2010). Briefly, several factors were taken into consideration: individual variability when animals repeat a task, within- and between-cohort performance stability and measurement error. In addition, typically very few subjects have preference scores between 53% and 55% and thus changing the criteria within this range does not substantively alter the results. Total novel object exploration (s) during Trial 1 is also illustrated as an internal control. Track length was measured by Viewer tracking software (Biobserve, Bonn, Germany).

2.3.3. Novel object recognition (OR)

Recognition memory was assessed using the novel object recognition test (Dere et al., 2007; Ennaceur and Delacour, 1988). Briefly, mice were allowed to explore two identical objects in the testing arena for 3 min (Trial 1 — training trial). After a retention interval of 2 h, mice were returned to the arena for another 3 min, with one of the familiar objects now replaced by a novel object (Trial 2 — testing trial). The amount of exploration was scored manually. As in the object placement test, the performance was evaluated by preference score (100% × exploration during testing $\text{trial}_{\text{novel object}}$ / total testing trial object exploration) as well as pass/fail (using preference score >55% as the criterion).

2.3.4. Social preference (SP)

Social withdrawal was measured in a social preference test (Crawley, 2004). Briefly, mice were allowed to explore a 3-chambered testing arena containing either a stimulus conspecific animal (ovariectomized female) in a wire mesh cage or a similar sized inanimate object in an identical cage for 10 min. The amount of time each animal spent exploring (defined as rearing on, whisking, sniffing or touching the mesh enclosure with nose and/or forepaws) the stimulus animal or the object was scored manually and is expressed as a social preference score (100% × $\text{exploration}_{\text{stimulus animal}}$ / total exploration).

2.3.5. Forced swim test (FS)

Depression-like behavior was assessed by a modified forced swim test (Porsolt et al., 1977). Briefly, mice were put in plexiglass cylinders (20 cm in diameter) filled with 25 °C water (30 cm deep) for 4 min. Immobility was manually scored using stopwatches, excluding the first minute of the trial. Immobility was defined as no movement other than that necessary to keep the animal's head above water. Results are presented as % immobility: 100% × immobile period (s)/180. The immobility in the forced swim test has been extensively validated as a measure of depression-like behavior. Firstly, it is sensitive to and predictive of the success of antidepressants (Cooper et al., 1980; Dulawa et al., 2004; Harkin et al., 1999; Karpa et al., 2002; Kusmider et al., 2006; Porsolt et al., 1978; Willner, 1995). Secondly, many of the circumstances thought to promote depression in humans also induce behavioral 'despair' in the forced swim test (Boccia et al., 2007; Bulduk and Canbeyli, 2004; Friedman et al., 2006; Hoshaw et al., 2006; Hwang et al., 1999; Lee et al., 2007; Pare, 1992; Ruedi-Bettschen et al., 2006; Wann et al., 2007; Willner, 1995; Willner and Mitchell, 2002). Lastly, the physiological responses, the brain regions and underlying mechanisms regulating immobility in the forced swim test behavior are similar to findings in depressed humans (Arletti and Bertolini, 1987; Flugy et al., 1992; Gil et al., 1992; Hattori et al., 2007; Hwang et al., 1999; Jesberger and Richardson, 1985; Kostowski, 1985; Kostowski et al., 1984; McKinney, 1984; Overstreet, 1986; Pare, 1989, 1992; Porsolt, 1979; Porsolt et al., 1979; White et al., 2007; Willner, 1984, 1995) and to other animal assays of depression (Gil et al., 1992; Kostowski et al., 1992; Pare, 1994; Willner, 1995).

2.4. Quantitative PCR (qPCR)

Dissected hippocampal tissues were stored in Ambion RNAlater® reagent (Life Technologies, Grand Island, NY) at −20 °C until analysis. RNA was isolated from hippocampal homogenates using an Ambion RNAqueous kit (Life Technologies) and mRNA was enriched by removing genomic DNA with a DNase digestion step. RNA concentration and quality was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription of mRNA to cDNA was accomplished using Superscript II™ (Life Technologies). Reactions were performed in duplicate using 600 ng of RNA. The cDNA from the two reactions was pooled and quantified using a dye intercalation assay (Quant-iT™ OliGreen® ssDNA Assay Kit, Life Technologies), according to the manufacturer's instructions. For qPCR assays, 384-well plates containing 2, 10 or 20 ng of each cDNA per well were used to allow the characterization of low, medium or highly expressed transcripts. All qPCR assays (10 μl) were performed in duplicate using an Applied Biosystems 7900HT Fast Real Time PCR System (Life Technologies). Primers and probes were designed using Primer Express® software (Life

Table 1
Hippocampal gene expression levels were reduced in middle-aged mice. Hippocampal gene expression levels were measured by qPCR and normalized using 3 month old mice as 100. ↓: significant reduction.

Gene	Protein	Function	Middle-aged *vs.* young	Young	Middle-aged
Transcription and translation factors					
Nfkb1[a]	Nuclear factor NF-κB p105 subunit	Part of NF-κB transcription factor	↓	100 ± 7	83 ± 3
Fos[a] (c-Fos)	FBJ murine osteosarcoma viral oncogene homolog	Part of transcription factor complex Jun/AP-1	↓	100 ± 15	44 ± 6
Fmr1[a]	Fragile X mental retardation protein	Transport mRNA	↓	100 ± 5	88 ± 4
Creb1	cAMP responsive element binding protein 1	Phosphorylation-dependent transcription factor	↓	100 ± 3	86 ± 3
Signal transduction					
Ntrk2 (TrkB)	Neurotrophic tyrosine receptor kinase	Receptor for neurotrophic factors (BDNF, neurotrophin-3 and neurotrophin-4)	↓	100 ± 5	83 ± 3
Camk2a[a]	Calcium/calmodulin-dependent protein kinase II α	Serine/threonine kinase required for hippocampal long-term potentiation (LTP) and spatial learning	↓	100 ± 7	83 ± 3
Prkca	Protein kinase C-α	Serine/threonine kinase in signal transduction pathway	↓	100 ± 5	85 ± 3
Jak2	Janus kinase 2	Tyrosine kinase in signal transduction pathway	↓	100 ± 3	84 ± 4
Gsk3b	Glycogen synthase kinase 3β	Serine/threonine kinase in signal transduction pathway	↓	100 ± 4	88 ± 3
Synaptic plasticity					
Arc[a]	Activity-regulated cytoskeleton-associated protein	Required for synaptic plasticity and memory formation	↓	100 ± 12	68 ± 5
Shank1[a]	SH3 and multiple ankyrin repeat domains 1	Adapter protein in the postsynaptic density of excitatory synapses	↓	100 ± 8	80 ± 2
Nlgn2[a]	Neuroligin 2	Cell adhesion molecule, binding partner of neurexins	↓	100 ± 6	80 ± 4
Homer1	Homer protein homolog 1	Postsynaptic density scaffolding protein	↓	100 ± 3	84 ± 3
Ncam1	Neural cell adhesion molecule 1	Cell adhesion molecule	↓	100 ± 4	88 ± 4
Dlg4 (Psd-95)	Postsynaptic density protein 95	Postsynaptic density scaffolding protein	↓	100 ± 7	85 ± 3
Neurotransmission					
Syp	Synaptophysin	Small synaptic vesicles membrane protein	↓	100 ± 4	81 ± 3
Scg3	Secretogranin-3	Protein located in secretory vesicles	↓	100 ± 4	87 ± 3
Snap25	Synaptosomal-associated protein 25	Docking of synaptic vesicles with the presynaptic plasma membrane	↓	100 ± 4	88 ± 3
Vamp1	Vesicle-associated membrane protein 1, Synaptobrevin 1	Docking of synaptic vesicles with the presynaptic plasma membrane	↓	100 ± 4	88 ± 3
Htr1d	Serotonin receptor 1D	G-protein (Gi/Go) coupled serotonin receptor	↓	100 ± 11	71 ± 6
Grin1 (Nmdar1)	Glutamate Receptor Ionotropic, NMDA 1	Ligand-gated cation channel	↓	100 ± 4	85 ± 3

[a] Indicates gene expression levels also changed by antidepressant treatment. For the full list of genes please see Table S1.

Technologies) and synthesized by Biosearch Technologies (Petaluma, CA) or were purchased premade from Applied Biosystems (Life Technologies). Enzyme for qPCR reaction (SensiFAST™ Hi-Rox) was purchased from Bioline (Taunton, MA) and used according to the manufacturer's instructions. Normalization of raw qPCR signals was performed using the geometric mean of a panel of 7 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 all 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 (when analyze age associated changes, Table 1) or vehicle-treated middle-aged mice (when analyze treatment associated changes, Fig. 4 and Table S1) defined as 100.

2.5. BrdU uptake assay

Hippocampal cell proliferation was assessed by quantifying the number of cells in the sub-granular zone of the dentate gyrus labeled by 5-bromo-2′-deoxyuridine (BrdU), based on published protocols (Wojtowicz and Kee, 2006). Briefly, mice were intraperitoneally injected 4 times with 50 mg/kg BrdU (Sigma, St. Louis, MO) with 2 h between injections and sacrificed the next day. Brains were fixed by transcardiac perfusion with phosphate-buffered saline followed by 4% paraformaldehyde (Affymetrix, Santa Clara, CA) and post fixed in 4% paraformaldehyde overnight. After equilibrating in 30% sucrose, brains were stored in −80 °C and later cryosectioned into 30 μm slices using a microtome (Thermo Scientific, Waltham, MA). Free-floating hippocampal slices underwent antigen retrieval (2 M HCl at 37 °C for 30 min followed by rinsing in 0.1 M borate buffer), membrane permeation (0.5% Triton X-100 in tris-buffered saline (TBS) at room temperature for 45 min), quenching of endogenous peroxidase activity (0.3% H_2O_2 at room temperature for 10 min), blocking of non-specific binding (5% goat serum (Vector Laboratories, Burlington, CA) in TBS at room temperature for 45 min), primary antibody labeling (1:1000 anti-BrdU antibody developed in rat (Accurate Chemical & Scientific Corporation, Westbury, NY) in 5% goat serum/TBS 4 °C overnight), secondary antibody reaction (1:200 goat anti-rat IgG, mouse adsorbed (Victor Laboratories, Burlingame, CA)) and visualized using an avidin–biotin complex labeled peroxidase system (Vectastain Elite ABC kit, Vector Laboratories) with 3,3′-diaminobenzidine (DAB) as the chromogen. Extensive wash (5 min × 5 times) were carried out between each incubation steps. Brain slices were then transferred to gelatin coated slides (Fisher Scientific, Waltham, MA), dehydrated by dipping in increasing concentrations of ethanol (50%, 70%, 95% and 100%) and washed in xylene. Coverslips were placed on slides in the presence of DPX mounting media (Electron Microscopy Sciences, Hatfield, PA). BrdU-positive cells were manually counted under a microscope (Zeiss, Oberkochen, Germany, total magnification: 100×) for every 6th hippocampal slices, throughout the rostrocaudal extent of the dentate gyrus in the subgranular zone (within a two-nucleus thick region from the granular cell layer), essentially as previously described (Walter et al., 2011). The total number of BrdU-positive cells from 10 sub-granular regions is reported.

2.6. Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Dissected hippocampi were snap frozen and stored in −80 °C. Tissue was homogenized using pellet pestles (Sigma) followed by sonication in lysis buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA) containing protease inhibitors (cOmplete® protease inhibitor cocktail, Roche, Mannheim, Germany) on ice and constant agitation at 4 °C for 1 h. After centrifugation (30 min at 13,000 rpm and 4 °C), protein concentration of the supernatant was measured using a Micro BCA kit (Thermo Scientific, Waltham, MA). Growth factor (BDNF, NT-3, IGF-1, NGF, and VEGF) levels were quantified using ELISA kits, following the instructions of the manufacturer (Genway Biotech, San Diego, CA). Results were standardized to the protein concentration of each lysate sample.

2.7. Ex vivo autoradiography to determine serotonin transporter (SERT) occupancy

To ensure that middle-aged mice in different treatment groups received antidepressants in therapeutic ranges, SERT occupancy was measured in *ex vivo* autoradiography experiments; detailed methods have been described elsewhere (Li et al., 2012).

2.8. Statistical analysis

JMP10 (SAS, Cary, NC) was used for all statistical analysis. For behavioral tests, quantification of hippocampal cell proliferation and growth factor levels, age-related differences were assessed using a *t*-test between young and middle-aged subjects. Treatment-induced changes in middle-aged mice were first compared using one-way ANOVA, followed by a protected Dunnett's *t*-test comparing the vehicle treated or fluoxetine treated group to vortioxetine treated middle-aged mice. Chi-square test was used to analyze categorical results (proportion of animals that had intact memories in object placement test-OP or in novel object recognition test-OR). Significance is defined as $p < 0.05$ for these analyses.

As most of the gene expression level results were not normally distributed, they were analyzed using non-parametric methods. Two-sample Wilcoxon/Kruskal–Wallis test (non-parametric equivalent of 2 sample *t*-test) was used to compare age-induced changes in individual genes. One-way Wilcoxon/Kruskal–Wallis test (non-parametric equivalent of one-way ANOVA) was used to compare drug-induced changes in individual genes followed by post-hoc Steel test (non-parametric equivalent of the protected Dunnett's test) comparing vehicle or fluoxetine treated groups to vortioxetine treated middle-aged mice. Significance is defined as $p < 0.05$.

3. Results

3.1. Middle-aged mice displayed deficits in visuospatial memory but had intact recognition memory, social preference, anxiety-like and depression-like behavior compared to young mice

Behaviors of otherwise healthy 12 month old female mice were compared to those of 3 month old mice in a battery of tests encompassing a variety of behavioral domains including cognitive (Fig. 1), affective, emotional and social behaviors (Fig. 2).

Middle-aged mice performed significantly worse than young mice in the novel object placement test (OP), as assessed by preference score (Fig. 1A, $t_{(df = 29)} = -3.71$, $p < 0.01$) and by the proportion of animals that had intact visuospatial memory (Fig. 1B: likelihood ratio $\chi^2 = 12.91$, $p < 0.01$). This visuospatial memory deficit was not due to a non-specific change in object exploration, as the exploration of the objects during the training trial (Trial 1) did not differ between middle-aged and young mice (young 18 ± 5.2 s, middle-aged: 18.3 ± 3.2 s). In contrast to the visuospatial memory deficits observed in the object placement test (OP), recognition memory was not significantly impaired in middle-aged mice tested in the novel object recognition test (OR, Fig. 1C and D).

Consistent with the fact that the middle-aged mice in this study were otherwise healthy, we did not find robust deficits in affective, emotional or social behavior compared to young mice, though some significant differences were evident (Fig. 2). In the open field test (OF), 12 month old mice explored the center more than the 3 month old mice (*i.e.*, less anxiety-like behavior) ($t_{(df = 29)} = 3.05$, $p < 0.01$, Fig. 2A), though the difference in track length was not significant

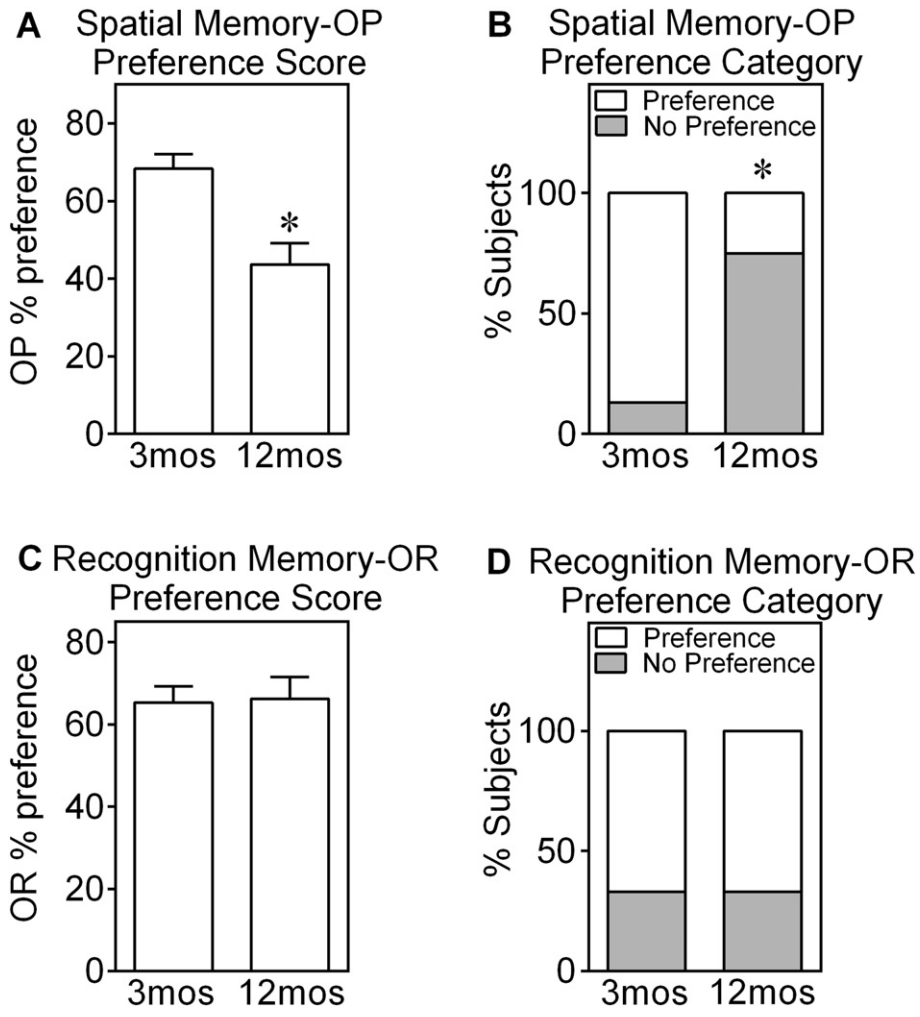

Fig. 1. Middle-aged mice display deficits in visuospatial memory. Middle-aged (12 month old) mice display deficits in visuospatial memory, assessed in the novel object placement (a.k.a. novel object location), assessed as decreased preference for the displaced (novel) object (panel A) and also as a significantly smaller proportion of 12 month old mice passing the test with a preference score >55% (panel B). Recognition memory was not impaired in 12 month old mice, assessed in the novel object recognition test (panels C and D). n = 15–16 per group. Means and standard errors are shown (panels A and C). * indicates significant difference from young mice ($p < 0.01$).

($t_{(df = 29)} = -1.45$, $p = 0.16$, Fig. 2B). In addition, 12 month old mice did not differ from 3 month old mice in terms of immobility in the forced swim test (FS), a measure for depression-like behavior (Fig. 2C), or levels of social exploration in the social preference test (SP), a measure for sociability (Fig. 2D).

3.2. Vortioxetine, but not fluoxetine, improved visuospatial memory and decreased depression-like behavior in middle-aged mice without affecting general object exploration or locomotor activity

Chronic vortioxetine treatment significantly improved cognitive performance in middle-aged mice assessed as increased preference score in the novel object placement test (OP). There was a main effect of treatment in preference scores ($F_{(2,34)} = 3.30$, $p < 0.05$, Fig. 3A) with vortioxetine-treated middle-aged mice performing better than vehicle-treated middle-aged mice. Vortioxetine treatment also significantly improved the proportion of middle-aged mice with intact visuospatial memory (likelihood ratio $\chi^2 = 4.97$, $p < 0.05$, Fig. 3B) compared to vehicle treated middle-aged mice. There was an apparent difference between vortioxetine and fluoxetine treated groups but this did not reach significance in protected post-hoc tests ($p = 0.055$ for preference scores, and $p = 0.13$ for the proportion of animals with intact visuospatial memory). The effect of drug treatments on visuospatial memory was not due to non-specific alterations of object exploration, as there was no significant change in total object exploration time during the training trial (VEH 18.3 ± 3.2 s, VOR 9.0 ± 2.8 s, FLX 11.3 ± 3.5 s, $F_{(2,28)} = 2.31$, $p = 0.11$). Chronic drug treatment *via* food did not affect body weight gains in middle-aged mice (before treatment: VEH 30.5 ± 1.0 g, VOR 28.5 ± 0.6 g, FLX 29.4 ± 0.8 g, after 1 month treatment: VEH: 31.5 ± 1.1 g, VOR 31.7 ± 0.7 g, FLX 31.1 ± 1.0 g). Consistent with previous results (Li et al., 2012, 2013), both drugs resulted in a high level of SERT occupancy after 1 month of p.o. dosing in these groups of middle-aged mice (VOR 84 ± 3%, FLX 97 ± 1%). In the novel object recognition test (OR), there was no statistically significant change in recognition memory induced by any treatment (Fig. 3C).

Depression-like behavior was assessed in the forced swim test (FS). Vortioxetine, but not fluoxetine, significantly decreased immobility in middle-aged mice. There was a significant treatment effect ($F_{(2,54)} = 3.87$, $p < 0.05$, Fig. 3D and E), primarily due to vortioxetine ($p < 0.05$, vortioxetine compared to vehicle). There was an apparent difference between vortioxetine and fluoxetine treatment but this did not reach statistical significance ($p = 0.07$). The vortioxetine-induced reduction of immobility was not due to a global or non-specific change in locomotor activity, as there was no significant change in total track length in the open field test (OF): VEH 2467 ± 158 cm; VOR 2402 ± 96 cm; and FLX 2161 ± 100 cm. Neither

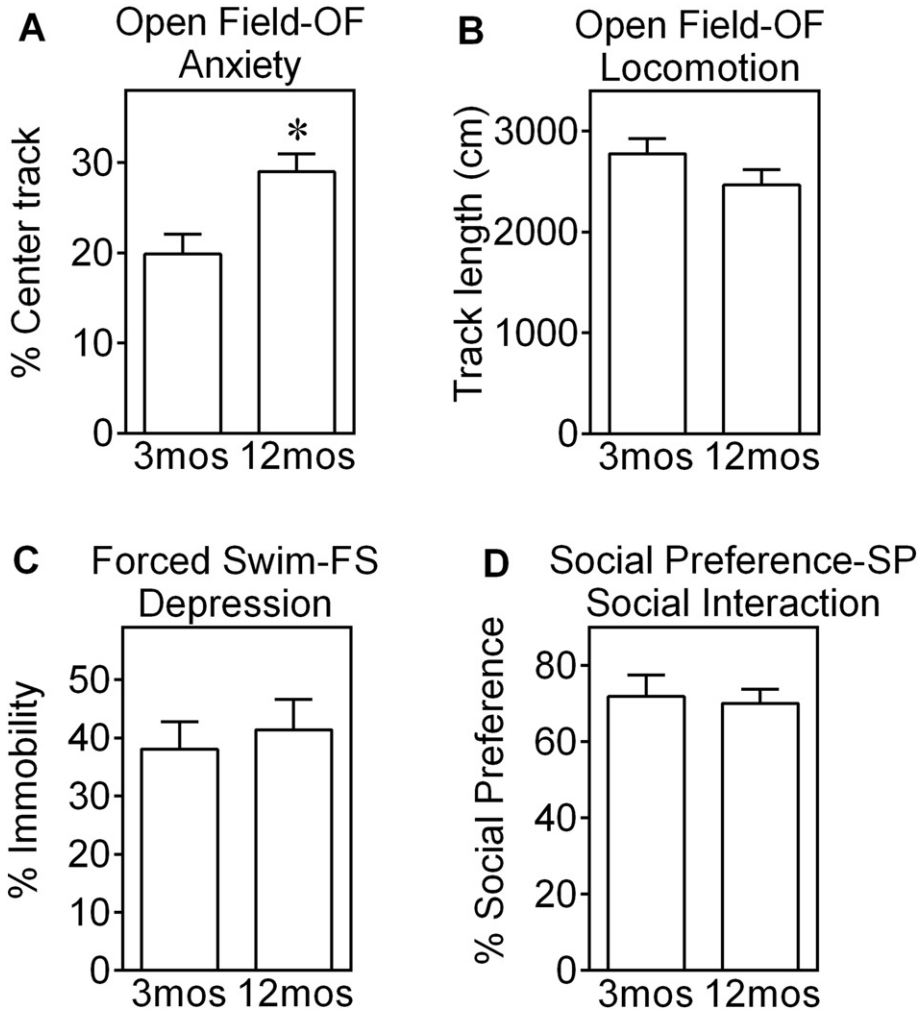

Fig. 2. Characterization of young and middle-aged mice in affective, emotional and social domains. In the open field test, 12 month old mice had less anxiety-like behavior, assessed as higher levels of center exploration (panel A) and did not differ in voluntary ambulation (track length, panel B) compared to 3 month old mice. There was no significant alteration of immobility in the forced swim test (panel C) or social exploration in the social preference test (panel D) in 12 month old mice. n = 15–19 per group for panels A–C, n = 10 per group for panel D. Means and standard errors are shown. * indicates significant difference from young mice ($p < 0.05$).

vortioxetine nor fluoxetine had a significant effect on anxiety-like behavior in middle-aged mice assessed by center exploration (Fig. 3F) in the open field test (OF).

3.3. Middle-aged mice had wide-spread reduction in gene expression levels compared to young mice, vortioxetine treatment increased expression of a subset of these genes

The most robust age-related deficit was detected in visuospatial memory. As the hippocampus is critical in spatial memory (Mumby et al., 2002), we focused our subsequent studies on this brain region. The hippocampal gene expression levels were measured using quantitative RT-PCR.

Compared to 3 month old mice, there was a widespread reduction in gene expression in 12 month old mice (Table 1). These genes include transcription and translation factors (Nfkb1, Fos, Creb1, and Fmr1), genes related to signal transduction (Ntrk2, Camk2a, Jak2, Prkca, and Gsk3b), neuroplasticity (Arc, Shank1, Nlgn2, Homer1, Dlg4, and Ncam1) and neurotransmission (Scg3, Syp, Snap25, Vamp1, Htr1d, and Grin1).

Chronic vortioxetine treatment of middle-aged mice increased expression of specific genes in several functional categories compared to vehicle treated mice (Fig. 4), including: transcription and translation factors (Nfkb1, Fos, and Fmr1), neuroplasticity (Arc, Shank1, and Nlgn2), signal transduction (Camk2a) and neurotransmission (Rab3a and Dat). The effects of vortioxetine on the expression of some of these genes were also significantly different from that of fluoxetine, which was largely ineffective in modifying gene expression. In addition, almost all of these genes were reduced in 12 month old mice compared to 3 month old mice. Therefore, changes in expression of these genes were consistent with vortioxetine-induced improvements in the visuospatial memory test. Please refer to Table S1 for common names, brief descriptions of functions and statistical analysis for all genes measured in this study.

3.4. Neither vortioxetine nor fluoxetine altered hippocampal cell proliferation in middle-aged mice

Middle-aged mice had reduced levels of hippocampal cell proliferation compared to young mice. There was a significant reduction in the number of cells in the subgranular zone of the dentate gyrus that incorporated the thymidine analog BrdU in 12 month old mice *vs.* 3 month old mice (total number of BrdU positive cells in 10 dentate gyrus subgranular zone regions in each animal: 3 month old 182 ± 9, 12 month old 36 ± 7, $t_{(df = 14)} = -12.5$, $p < 0.01$). Chronic treatment with vortioxetine did not significantly change the number of BrdU positive cells in the sub-granular zone of the dentate gyrus of middle-aged mice ($F_{(2,20)} = 0.54$, $p = 0.59$, Fig. 5) compared to vehicle or fluoxetine treated mice. We found essentially the same pattern of results

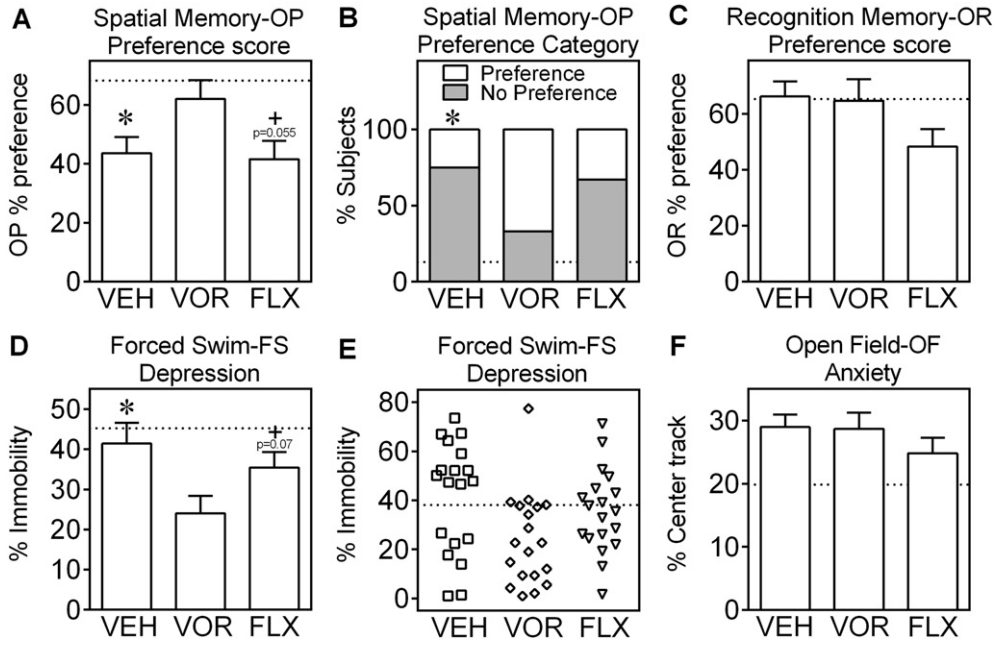

Fig. 3. Behavioral effects of chronic antidepressant treatments in middle-aged mice. Vortioxetine (VOR) but not fluoxetine (FLX) significantly improved visuospatial memory in the novel object placement test in middle-aged mice, assessed as preference score (panel A) or as the proportion of mice passing (panel B). There was no significant treatment effect in the novel object recognition test (panel C). In the forced swim test, vortioxetine, but not fluoxetine, significantly reduced depression-like behavior in middle-aged mice, assessed as increased immobility (panel D, group summary and panel E, scatterplot). Neither vortioxetine nor fluoxetine treatment altered anxiety-like behavior (center exploration in open field, panel F). n = 7–19 per group. Means and standard errors are shown (panels A, C, D and F). * indicates significant difference between vortioxetine and vehicle groups ($p < 0.05$) and $^{+}$ indicates the comparison between vortioxetine and fluoxetine (p value shown in graph) groups following overall significant one-way ANOVA ($p < 0.05$). Dotted lines indicate the performance of young mice for comparison only.

using the Ki-67 antibody (number of Ki-67 positive cells in 10 subgranular zone regions of dentate gyrus: young 78 ± 23, middle-aged + VEH 20 ± 7, middle-aged + VOR 21 ± 4, middle-aged + FLX 18 ± 5, n = 3–5 per group).

3.5. The effects of vortioxetine on visuospatial memory were independent of hippocampal levels of growth factors

Several growth factors were quantified in hippocampal homogenates using commercial ELISA kits. In addition, TrkB (Ntrk2, the receptor for BDNF and NT-3) was quantified by qPCR. There was no consistent decrease or increase of growth factors in middle-aged mice compared to young mice. Middle-aged mice had a higher level of NGF, lower level of TrkB, and non-significant changes in the levels of BDNF, IGF-1, NT-3 and VEGF, compared to young mice (dotted line in Fig. 6, and Table S2). There was also no age-related change in BDNF mRNA levels measured by qPCR (data not shown). The effects of antidepressants on growth factors levels were variable and patterns of change were not consistent with the results of the object placement test (Fig. 6). Thus, the observed changes in growth factors did not reflect the functional differences observed in behavioral tests (impaired in aged mice and altered by vortioxetine but not fluoxetine treatment), nor did they vary consistently by age. Please refer to Table S2 for detailed statistical analysis results.

4. Discussion

Mild cognitive deficits occur as early as middle-age and are predictive of progressive deficits in humans and in rodents (Larrabee and Crook, 1994; Stone et al., 1997). Visuospatial ability shows a strong association with age, in both humans and in rodent models (Aenlle et al., 2009; Ennaceur et al., 2008; Hoogendam et al., 2014). This study demonstrated an age-related cognitive deficit in visuospatial memory in healthy middle-aged mice. This was accompanied by global decreases in gene expression related to diverse physiological functions and by extensive reduction in hippocampal cell proliferation, but not by a consistent pattern of changes in growth factor levels. Chronic treatment with the multimodal antidepressant, vortioxetine, but not fluoxetine, improved visuospatial memory and decreased depression-like behavior in middle-aged mice, consistent with clinical findings that vortioxetine improves both cognitive function and depression in old patients (Katona et al., 2012). The improved cognitive function in middle-aged mice treated with vortioxetine was accompanied by increased mRNA levels of transcription factors, members of signal transduction pathways and neuroplasticity markers. Most of these genes had lower transcript levels in the hippocampus of middle-aged *vs.* young mice. In contrast, neither hippocampal cell proliferation nor growth factor levels were related to improved performance in the behavioral tasks.

The cognitive behavioral tests used in the study are not confounded by stressors or aversive stimuli, do not require food or water deprivation (Ennaceur and Meliani, 1992; Gulinello et al., 2009) and can be repeated in the same subjects. The object placement task is dependent on intact hippocampal function and is analogous to the visuospatial memory tests used in humans (Haladjian and Mathy, 2015; Raber, 2015), which are likely associated with mental rotation ability (Kelly et al., 2013; Vandenberg and Kuse, 1978). This type of cognitive test has been used in assessing cognitive impairments in patients (Caterini et al., 2002; Lawrence et al., 2000).

In otherwise healthy middle-aged mice, age-associated impairments in cognitive performance were evident in visuospatial memory but not in object recognition memory, consistent with other reports (Ron-Harel et al., 2008; Wimmer et al., 2012). However, some behavioral signs of aging could still be detected. There was a modest reduction in total track length in open field. In the forced swim test, even though there was no significant overall increase of immobility in aged *vs.* young mice, there appeared to be a subpopulation of middle-aged mice displaying higher depression-like behavior (more than 60% of the middle-aged mice displayed high levels of immobility compared to less than 45% of young mice showing high levels of immobility). The reduction in depression-like behavior seen after vortioxetine treatment

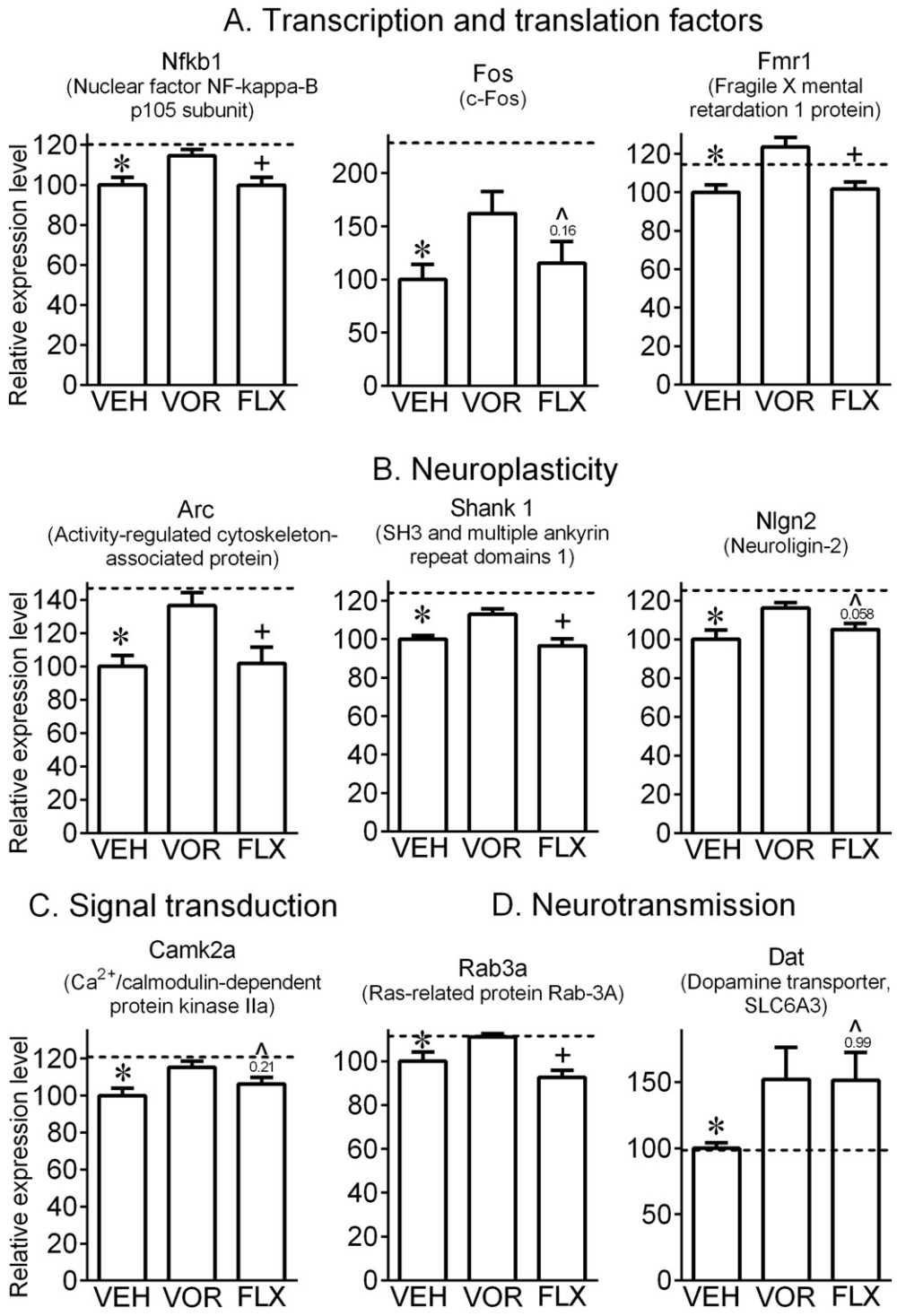

Fig. 4. Chronic vortioxetine, but not fluoxetine, increased plasticity-related gene expression in middle-aged mice. Data from qPCR are expressed as relative expression levels to that of vehicle-treated 12 month old mice. These genes include transcription and translation factors (panel A), synaptic plasticity markers (panel B), signal transduction (panel C) and proteins involved in neurotransmission (panel D). VEH: vehicle; VOR: vortioxetine; FLX: fluoxetine. Expression levels in 3 month old mice are indicated with a dotted line for comparison only. n = 10 per group. Means and standard errors are shown. * indicates significant difference between vortioxetine and vehicle groups ($p < 0.05$), $^{+}$ indicates significant ($p < 0.05$) difference between vortioxetine and fluoxetine groups following a significant ($p < 0.05$) Wilcoxon/Kruskal–Wallis test (non-parametric equivalent of one-way ANOVA). $^{\wedge}$ indicates non-significant difference between vortioxetine and fluoxetine (p values shown in graph).

may be due to changes in the population of aged mice susceptible to depression-like behavior. This is of particular importance as even mild depressive symptoms negatively impact cognitive function in middle-aged and old subjects (Brevik et al., 2013).

Modulations of specific subtypes of 5-HT receptors are thought to be critically involved in cognitive functions (for a review, see Meneses, 2013). For example, the 5-HT_3 receptor antagonist ondansetron improved spatial memory in aged rats (Fontana et al., 1995; Pitsikas et al., 1993) and increased c-Fos expression (Urzedo-Rodrigues et al., 2014). In addition, 5-HT_{1A} receptor (du Jardin et al., 2014; Haider et al., 2012) and 5-HT_7 receptor modulation (Roberts and Hedlund, 2012) affects hippocampal dependent cognitive functions in rodents. Results from the current study support the hypothesis that direct receptor activities may contribute to the effects of vortioxetine (du Jardin

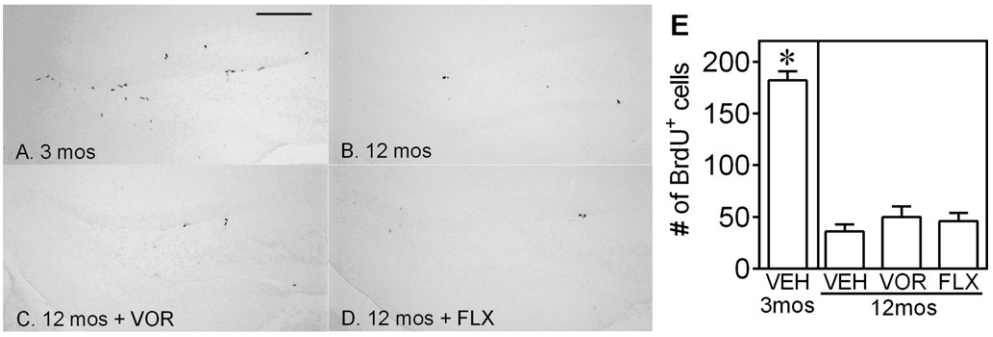

Fig. 5. Neither vortioxetine nor fluoxetine reversed the age-related reduction in hippocampal stem cell proliferation. Panels A–D: representative microscopic images of dentate gyrus stained for BrdU. Panel A: young mice. Panel B: middle-aged mice treated with vehicle (VEH). Panel C: middle-aged mice treated with vortioxetine (VOR). Panel D: middle-aged mice treated with fluoxetine (FLX). Scale bar: 400 μm. Panel E: total numbers of BrdU-positive cells from 10 dentate gyrus regions. Sample size = 7–8 per group. Means and standard errors are shown. * indicates young mice significantly different from vehicle-treated middle-aged mice in pair-wise *t*-test ($p < 0.01$).

et al., 2014; Jensen et al., 2014; Li et al., 2013) in these middle-aged mice. First, vortioxetine improved visuospatial memory while fluoxetine was not effective. Second, whereas vortioxetine selectively increased transcription of multiple genes in the hippocampus, fluoxetine had no effect on the majority of genes assessed. Furthermore, vortioxetine significantly decreased depression-like behavior in 12 month old mice while fluoxetine did not, which is consistent with the clinical observation that elderly patients have a lower response to SSRIs (Tedeschini et al., 2011) and that cognitive deficits in depressed and/or elderly patients are also relatively insensitive to SSRI treatment (Herzallah et al., 2013). Therefore, our results support that vortioxetine is working *via* a different mechanism than the SSRI fluoxetine in this model of age-related cognitive deficits.

Altering gene expression and the consequent changes in protein levels may be one mechanism of the improved cognitive function in middle-aged mice after chronic antidepressant administration. First, as synaptic plasticity is an active process, it is plausible that manipulating this process will affect cognitive function, including visuospatial memory. Second, results from this study showed that changes in gene expression were consistent with the observed behavioral changes. In middle-aged mice, the profound reduction in gene expression in the hippocampus was accompanied with visuospatial memory impairment in untreated and fluoxetine treated animals. Increased hippocampal transcription of a subset of these genes (including Nfkb1, Fos, Fmr1, Camk2a, Arc, Shank1, Nlgn2, Rab3a, and Ndor1) was accompanied with improvement of performance in this hippocampal-dependent task in animals treated with vortioxetine. Third, the products of those genes affected by vortioxetine can be considered as related to neuroplasticity, which plays a key role in learning and memory (Mayford et al., 2012). For instance, Arc is an immediate early gene critical to neuroplasticity, learning and memory (for a review, see Korb and Finkbeiner, 2011). Its post-synaptic expression is induced by exposure to novelty and the resultant increase in synaptic activity, the dysfunction of which has been indicated as a fundamental mechanism of memory impairment. Another example is Fmr1. The protein coded by Fmr1 (the Fragile X mental retardation protein, FMRP) regulates translation of a variety of mRNAs (Sethna et al., 2014). Impaired expression of Fmr1 has been related to cognitive dysfunction in patients with

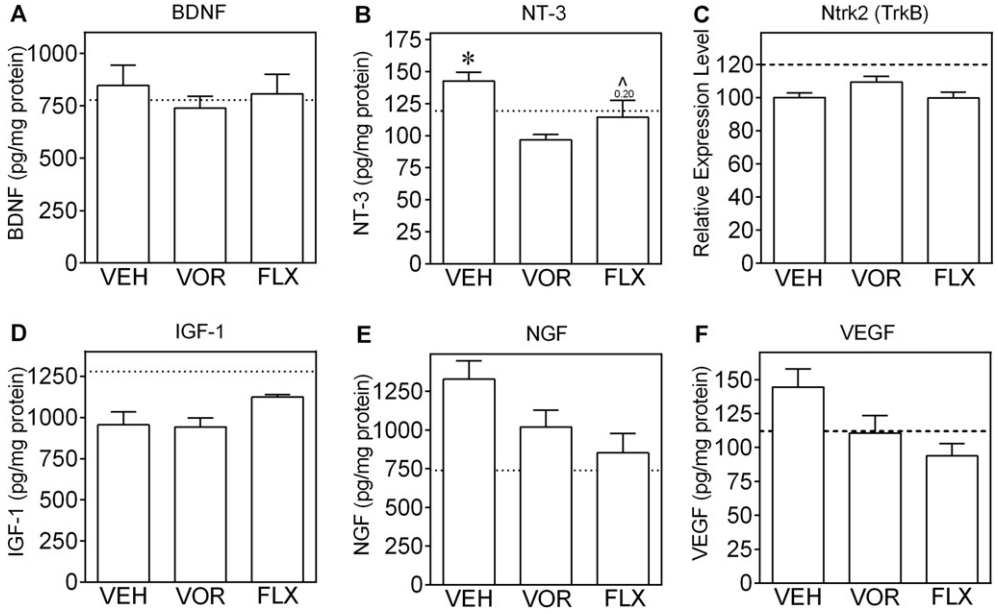

Fig. 6. Hippocampal growth factors and growth factor receptor levels after antidepressant treatment in middle-aged mice. Growth factor levels were quantified by ELISA (n = 4–9 per group) and standardized to the protein concentration of the tissue homogenate (pg/mg protein). The growth factor receptor TrkB (Ntrk2) was quantified by qPCR (n = 10 per group) and expressed as relative expression level using vehicle-treated middle-aged mice as 100. Dotted line denotes the levels in young mice. Means and standard errors are shown. Only differences in NT-3 level reached overall significance in one-way ANOVA ($p < 0.05$). * indicates significant difference between the vortioxetine and vehicle groups ($p < 0.05$) and ^ indicates the comparison between vortioxetine and fluoxetine (*p* value shown in graph). VEH: vehicle; VOR: vortioxetine; FLX: fluoxetine.

Fragile X syndrome and in carriers (Verkerk et al., 1991). Our data suggest that altered expression of Fmr1 may also play a role in cognitive deficits in middle-aged subjects as well as in developmental disorders. Therefore, results from the current study support the hypothesis that long-term changes in gene expression may contribute to the age-related decline in cognition. We hypothesize that up-regulation of the transcription factors that are reduced in middle-aged animals may be a necessary prerequisite for the adequate expression of the specific genes needed for maintaining synaptic plasticity and cognitive functions.

Neurogenesis which has been shown to be involved in cognitive impairment and depression in humans and in a variety of rodent models, is a function of serotonergic regulation and may affect the response to antidepressants in both cognitive and affective behavioral domains (Adeosun et al., 2014; Alenina and Klempin, 2015; Anacker, 2014; Biscaro et al., 2012; Braun and Jessberger, 2014; Chadwick et al., 2011; Cho et al., 2015; Dimitrov et al., 2014; Gundersen et al., 2013; Hill et al., 2015; Jiang et al., 2015; Lin and Wang, 2014; Mendez-David et al., 2013; Morais et al., 2014; O'Leary and Cryan, 2014; Parihar et al., 2013; Pereira Dias et al., 2014; Ransome et al., 2012; Rotheneichner et al., 2014; Schoenfeld and Cameron, 2015; Seib et al., 2013; Serafini et al., 2014; Shetty, 2014; Suarez-Pereira et al., 2015; Yau et al., 2014). In young adult mice, antidepressants increase stem cell proliferation (Hodes et al., 2010; Santarelli et al., 2003; Tanti et al., 2013). However, in the current study, amelioration of the deficits in the hippocampal-dependent object placement task (OP) was not related to levels of stem cell proliferation, nor was there evidence of increased levels of apoptosis markers or gliosis (Gfap, Table S1), consistent with the relatively specific behavioral deficits and otherwise normal behavior of healthy middle-aged mice. These data are also consistent with previous studies dissociating performance in hippocampal tasks from levels of stem cell proliferation in aged mice (Walter et al., 2011). The lack of treatment effect on hippocampal stem cell proliferation in the middle-aged animals is also consistent with previous reports indicating that fluoxetine does not increase stem cell proliferation in older (>8 months) rodents (Couillard-Despres et al., 2009; Cowen et al., 2008; Guirado et al., 2012). While the reasons that antidepressants do not increase stem cell proliferation in middle-aged subjects are unclear, it appears that alternative mechanisms (*i.e.*, increased neuroplasticity and gene expression) are sufficient to improve the cognitive functions in middle-aged mice.

The relationship between hippocampal growth factor levels and cognitive function seems to be complex and insufficient to explain either the existence or the reversal of cognitive impairment. Growth factor levels have a variable relationship with aging, with either increased, decreased or unchanged levels reported compared to young animals (Croll et al., 1998; Hattiangady et al., 2005; Katoh-Semba et al., 1998; Muller et al., 2012; Shetty et al., 2005; Silhol et al., 2005, 2007). In young adult rodents, the effects of fluoxetine on growth factors in hippocampus are just as variable (Altar et al., 2003; Coppell et al., 2003; Dias et al., 2003; Nibuya et al., 1996). Furthermore, although we found some changes in growth factor levels after chronic antidepressant treatment in middle-aged mice, none of these are consistent with the observed changes in visuospatial memory. It has been reported elsewhere that improvement in spatial memory in aged rodents were not accompanied by a change in growth factor levels (Kumar et al., 2012). It is important to note that this does not preclude an important role for drug-induced changes in hippocampal growth factors in other behavioral domains not addressed here. Regulation of growth factor levels in other brain regions could also have important functional consequences.

5. Conclusions

In conclusion, aging in healthy middle-aged mice is associated with specific, mild deficits in cognitive function concomitant with widespread reduction of gene expression, including reduced expression of several transcription factors that may herald the inability to respond to stimuli with critical regulation of downstream protein synthesis. Cognitive function in middle-aged mice was improved by the multimodal antidepressant vortioxetine. The results from current study support the hypothesis that the underlying mechanisms by which vortioxetine affect cognition involve increased expression of genes related to neuroplasticity.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pbb.2015.05.013.

Conflict of interest

Drs. Yan Li, Joseph A Tamm, Alan Pehrson, Connie Sánchez and Ms. Aicha Abdourahman are full-time employees at Lundbeck Research USA. Dr. Maria Gulinello received compensation as a consultant for H. Lundbeck A/S.

Role of funding source

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