

Long-Term Duloxetine Treatment Normalizes Altered Brain-Derived Neurotrophic Factor Expression in Serotonin Transporter Knockout Rats through the Modulation of Specific Neurotrophin Isoforms

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Received December 10, 2009; accepted February 12, 2010

ABSTRACT

Dysfunction of the serotonergic system is implicated in the etiology of many psychiatric disorders, including major depression. Major vulnerability genes for mood disorders are also related to the serotonergic system: one of these genes encodes for the serotonin transporter (SERT), which represent a major target for the action of antidepressant drugs. We have demonstrated recently that SERT knockout (KO) rats, generated by *N*-ethyl-*N*-nitrosourea-induced mutagenesis, show reduced expression of the neurotrophin brain-derived neurotrophic factor (BDNF) in the hippocampus and prefrontal cortex, suggesting that depression vulnerability can be associated with impaired neuronal plasticity. In the present study, we demonstrate that chronic treatment with the antidepressant duloxetine (DLX) was able to normalize the expression of BDNF mRNA-coding

exon (IX) in the hippocampus and prefrontal cortex of SERT KO rats through the modulation of selected neurotrophin transcripts, whose expression was up-regulated by DLX only in SERT KO rats. On the other hand, the modulation of BDNF protein by DLX in frontal cortex was abolished in mutant rats. These data suggest that animals with a genetic defect of the serotonin transporter maintain the ability to show neuroplastic changes in response to antidepressant drugs. Because these animals show depression-like behavior, the region and isoform-specific increase of BDNF levels may be a mechanism activated by long-term antidepressant treatment to restore normal plasticity that is defective under genetic dysfunction of the serotonin transporter.

Major depression is believed to originate from the interaction between susceptibility genes and environmental events. Genetic susceptibility may be due, at least in part, to deficits in neuronal resiliency and neuroprotective responses or, alternatively, to exacerbated function of systems that may lead to neuronal dysfunction and psychopathology (Duman, 2009). There is mounting evidence suggesting that heightened susceptibility in mood disorders can be due to impaired

neuronal plasticity, driven by reduced expression and function of key mediators, such as the neurotrophin brain-derived neurotrophic factor (BDNF), which are important for cellular resilience (Tsankova et al., 2006; McClung and Nestler, 2008; Pittenger and Duman, 2008; Calabrese et al., 2009). Moreover, the modulation of BDNF represents a crucial step in long-term adaptive changes brought about by antidepressant drugs (Calabrese et al., 2007, 2009; Castrén et al., 2007; Kozisek et al., 2008; Molteni et al., 2009). Although there is a general agreement that antidepressant drug treatment can modulate the expression of BDNF, controversies exist with regard to the magnitude, timing, and anatomical specificity of such changes (Berton and Nestler, 2006; Groves, 2007; Martinowich et al., 2007; Kozisek et al., 2008; Molteni et al.,

This work was supported by the Ministry of University and Research [Grant PRIN 2007STRNKH]; the Ministry of Health [Ricerca filializzata RF2007 conv/42]; and by an unrestricted grant from Eli Lilly Italia.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.109.063081.

ABBREVIATIONS: SERT, serotonin transporter; KO, knockout; WT, wild type; BDNF, brain-derived neurotrophic factor; DLX, duloxetine; PCR, polymerase chain reaction; ANOVA, analysis of variance; SCPHT, single-contrast post hoc test; UTR, untranslated region; mBDNF, mature form of the brain-derived neurotrophic factor; proBDNF, precursor of the brain-derived neurotrophic factor.

2009). A possible explanation for such discrepancies is that most of these studies have been carried out in "normal" animals, which may respond in a different manner with respect to a pathological condition.

Despite the heterogeneity of depressive symptoms in humans and the huge difference between humans and rodents, numerous attempts have been made to create animal models of depression. Although genetic approaches may be limited by the possibility to investigate one or two genes at a time, without reproducing the complex setting expected in depression, they allow the creation of a stable and unique phenotype, which may be studied with different experimental approaches.

The 5-hydroxytryptamine transporter is of particular relevance in the context of mood disorders because it is the target of several antidepressant drugs, but it is also relevant for the presence of a functional polymorphism within its promoter region that is associated with different neuropsychiatric disorders and with the antidepressant response (Caspi et al., 2003; Uher and McGuffin, 2008; Huezo-Diaz et al., 2009). Using target-selected mutagenesis, we have generated recently serotonin transporter (SERT) knockout rats (Smits et al., 2006), which showed an impairment of the serotonergic system (Homberg et al., 2007) associated with anxiety- and depression-like behaviors (Olivier et al., 2008; Kalueff et al., 2010).

Because SERT KO rats show a reduction of BDNF levels in selected brain regions (Molteni et al., 2010), we decided to investigate the effect of a long-term treatment with the antidepressant drug duloxetine on BDNF expression in SERT KO rats to address two important points. First, we wondered whether the antidepressant treatment was able to normalize the alteration of BDNF detected in this animal model. Second, we wanted to establish to what extent the serotonergic system might contribute to neuroplastic changes elicited by long-term duloxetine treatment (Calabrese et al., 2007; Molteni et al., 2009). To this aim, we measured total (exon IX) BDNF mRNA levels and the expression of selected isoforms of the neurotrophin in hippocampus and prefrontal cortex of SERT KO and wild-type rats treated for 21 days with duloxetine. Moreover, to assess whether the observed changes were specific for BDNF, we measured the mRNA levels of VGF (nonacronymic) that has only recently been proposed as a neuropeptide that may also mediate antidepressant responses (Hunsberger et al., 2007; Thakker-Varia et al., 2007).

Materials and Methods

General reagents were purchased from Sigma-Aldrich (Milan, Italy), and molecular biology reagents were obtained from Applied Biosystem Italia (Monza, Italy), Bio-Rad Laboratories S.r.l. Italia (Segrate, Italy), GE Healthcare Europe GmbH-Italia (Milan, Italy), Roche (Monza, Italy), and Santa Cruz Biotechnology (Santa Cruz, CA).

Animals and Pharmacological Treatment. Serotonin transporter knockout rats (Slc6a4^{1Hubr}) were generated by *N*-ethyl-*N*-nitrosourea-induced mutagenesis (Smits et al., 2006). All subjects were bred and reared in the Central Animal Laboratory of the University of Nijmegen (Nijmegen, the Netherlands). Experimental animals were derived from crossing heterozygous SERT KO rats that were out crossed for five generations. After weaning at the age of 21 days, ear cuts were taken for genotyping. In all experiments, female

SERT(+/+) (WT) and SERT(−/−) (KO) were used. Animals were supplied with food and water ad libitum and were kept on a 12-h dark/light cycle (lights on at 6:00 AM). Animals were treated continuously (21 days) with vehicle or duloxetine (10 mg/kg by gavage) and sacrificed 24 h after the last injection. Duloxetine was dissolved at a concentration of 10 mg/ml, and it was administered by oral gavage once daily in the amount of 1 ml/kg according to their body weight.

Brain regions of interest (hippocampus, prefrontal cortex, frontal cortex) were rapidly dissected. Prefrontal cortex [defined as Cg1, Cg3, and IL subregions corresponding to the plates 6 to 10 according to the atlas of Paxinos and Watson (1996)] and frontal cortex (comprising regions Fr1-3, Par1, and AI from the same slice as prefrontal cortex) were dissected from 2-mm thick slices, whereas hippocampus was dissected from the whole brain. The brain specimens were frozen on dry ice and stored at −80°C for further analysis. All experiments were carried out in accordance with the guidelines set forth by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

RNA Preparation and Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction. Total RNA was isolated from tissue from different brain structures by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories) according to the manufacturer's instructions and quantified by spectrophotometric analysis. After total RNA extraction, the samples were processed for real-time polymerase chain reaction (PCR) to assess BDNF mRNA levels.

A 2-μg aliquot of each sample was treated with DNase to avoid DNA contamination and then reverse transcribed using a High-Capacity cDNA Archive commercial kit. The real-time PCR reaction was performed using the ABI Prism 7000 Sequence Detection System with the TaqMan Gene expression Master Mix and the following TaqMan Gene Expression Assay (all from Applied Biosystems, Foster City, CA): total BDNF: GenBank accession no. NM_012513.3; BDNF transcript III: forward primer, ATGCTTCATTGAGCCAGTT; reverse primer, GTGGACGTTTGCTTCTTTCA; BDNF transcript IV: GenBank accession no. EF125679.1; BDNF transcript VI: GenBank accession nos. BC087634.1/EF125680.1; and BDNF transcript IXa: forward primer, TGGTGTCCCCAAGAAAGTAA; reverse primer, CACGTGCTCAAAGTGTGTCAG.

VGF gene expression was measured in the hippocampus and in the prefrontal cortex of SERT KO rats in comparison with their wild-type counterparts by real time reverse transcriptase-PCR with the same procedure used for BDNF mRNA levels quantification and using the following primers: forward, GCTGAGGACGCAGTGT; and reverse, AAGAGCTCTCCCTGG.

PCR reactions were carried out as reported previously (Molteni et al., 2009). In brief, after an initial step at 50°C for 2 min and at 95°C for 10 min, 40 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 15 s to enable the melting process and then for 1 min at 60°C for the annealing and extension reaction. Each sample was assayed in duplicate using two independent retro-transcription products. A comparative cycle threshold (*C_t*) method was used to determine the relative target gene expression. Data have been expressed as the percentage calculated from the expression of the target genes normalized on rat glyceraldehyde 3-phosphate dehydrogenase gene expression as control gene (glyceraldehyde 3-phosphate dehydrogenase TaqMan probe for GenBank accession no. NM_017008.3).

Analysis of BDNF Protein. Western blot analysis was used to investigate BDNF protein levels in the crude synaptosomal fraction. Tissues were manually homogenized using a Dounce homogenizer in 600 μl of a pH 7.4 ice-cold buffer (solution A) containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, and a commercial cocktail of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 2000g for 1 min at 4°C, thus obtaining a pellet corresponding to the nuclear fraction, which was resuspended in solution A and centrifuged again (2000g for 2 min at

4°C). The supernatant obtained from each centrifugation step was collected and centrifuged at 23,000g for 5 min at 4°C to obtain a pellet corresponding to the crude synaptosomal fraction, which was resuspended in Solution A. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories) using bovine serum albumin as the calibration standard.

Equal amounts of protein were run under reducing conditions on 14% SDS-polyacrylamide gels and then electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). The blots were blocked with 10% nonfat dry milk and then incubated with the primary antibody anti-BDNF polyclonal antibody (1:1000, 4°C, overnight; Santa Cruz Biotechnology) able to recognize both the mature form of the neurotrophin (mBDNF; 14 kDa) and its precursor (proBDNF; 32 kDa). Membranes were then incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:5000), and immunocomplexes were visualized by chemiluminescence using the ECL Western Blotting kit (GE Healthcare Europe GmbH-Italia). Results were standardized using β -actin as the control protein, which was detected by evaluating the band density at 43 kDa after probing the membranes with a polyclonal antibody (1:10,000; Sigma-Aldrich) followed by a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich). Protein levels were calculated by measuring the optical density of the autoradiographic bands using Quantity One software (Bio-Rad Laboratories). To ensure that autoradiographic bands were in the linear range of intensity, different exposure times were used.

Statistical Analyses. The effect of the duloxetine on BDNF mRNA and protein levels was analyzed with a two-way analysis of variance (ANOVA) followed by single-contrast post hoc test (SCPHT). Significance for all tests was assumed for $p < 0.05$. Data are presented as means \pm S.E.M. For graphic clarity, results are presented as the mean percentage of WT/vehicle-treated rats.

Results

SERT KO rats, similarly to SERT knockout mice (Holmes et al., 2003; Lira et al., 2003), show depression- and anxiety-related behavior (Olivier et al., 2008; Kalueff et al., 2010) and have reduced expression of BDNF levels in the hippocampus and prefrontal cortex (Molteni et al., 2010). We tested whether antidepressant treatment may restore “normal” levels of plasticity in animals with an alteration of the serotonergic system, which may increase the susceptibility to mood disorders (Lucki, 1998; Caspi et al., 2003; Uher and McGuffin, 2008). To this aim, we treated SERT KO rats with duloxetine, a noradrenaline and serotonin transporter blocker, and measured the expression of BDNF and VGF, two neurotrophic molecules that play a role in neuroplastic defects associated with mood disorders (Castrén et al., 2007).

Modulation of Total BDNF mRNA Levels by Long-Term Duloxetine Treatment in SERT(+/+) and SERT(-/-) Rats. In agreement with our recent work, the mRNA levels of BDNF are significantly lower in the hippocampus (-12% versus WT, $p < 0.05$; Fig. 1A) and in the prefrontal cortex (-28% versus WT, $p < 0.001$; Fig. 1B), whereas an increase was observed in the frontal cortex (+17% versus WT, $p < 0.05$; Fig. 1C) of vehicle-injected SERT KO rats.

Long-term administration of duloxetine had a significant effect on BDNF mRNA levels in the hippocampus ($F_{1,25} = 57.323$, $p < 0.001$; Fig. 1A), in the prefrontal cortex ($F_{1,26} = 54.564$, $p < 0.001$; Fig. 1B), and in the frontal cortex ($F_{1,37} = 47.504$, $p < 0.001$; Fig. 1C). Indeed, as shown in Fig. 1, duloxetine increased total BDNF mRNA levels (exon IX) in both genotypes, an effect that, in hip-

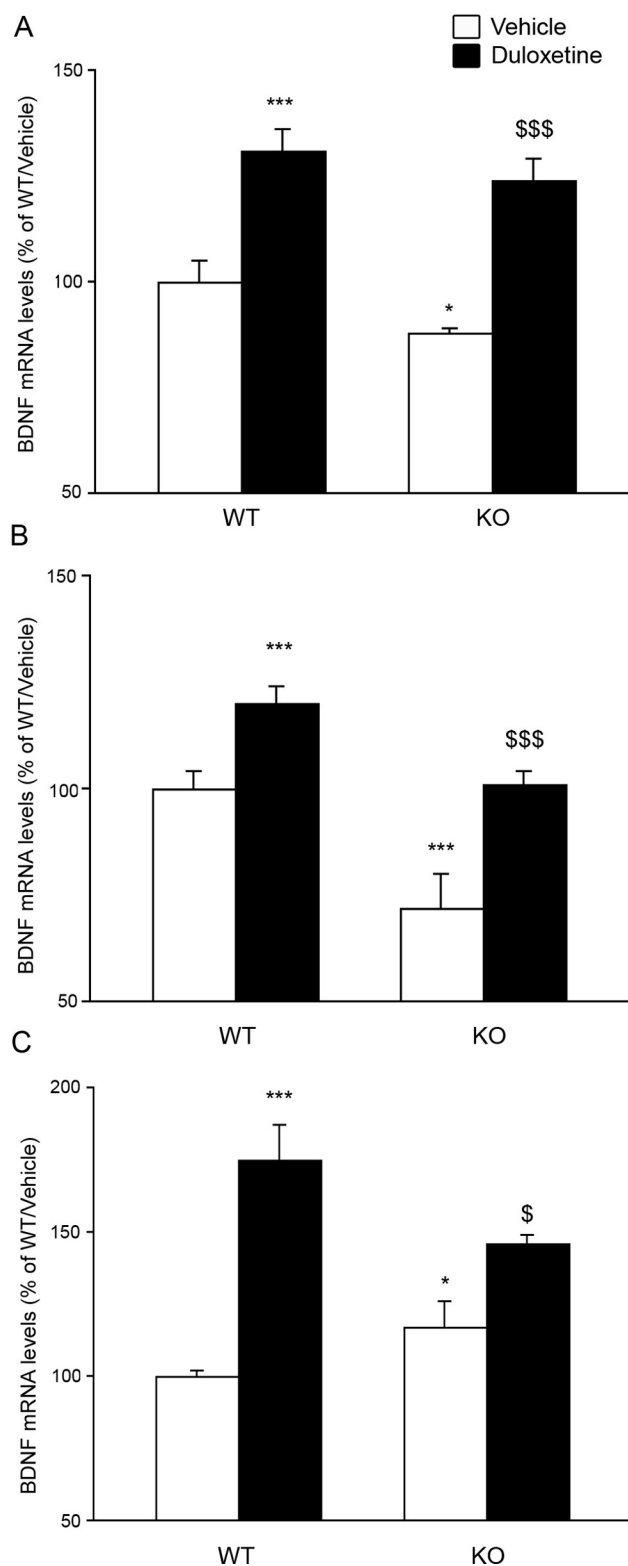


Fig. 1. Long-term duloxetine treatment increases total BDNF mRNA levels in WT and SERT KO rats. Total BDNF mRNA levels were measured in hippocampus (A), prefrontal cortex (B) and frontal cortex (C) of WT and KO rats treated for 21 days with vehicle or duloxetine and killed 24 h after the last injection. The data, expressed as a percentage of WT/vehicle (set at 100%), are the mean \pm S.E.M. from at least six independent determinations. *, $p < 0.05$; ***, $p < 0.001$ versus WT/vehicle; \$, $p < 0.05$; \$\$\$, $p < 0.001$ versus KO/vehicle (two-way ANOVA with SCPHT).

pocampus and prefrontal cortex, is more pronounced in SERT KO than in wild-type rats. Specifically, BDNF levels were increased by duloxetine treatment in the hippocampus of WT and SERT KO rats (respectively, +31 and +42% of their vehicle-injected counterpart, $p < 0.001$; Fig. 1A) and in the prefrontal cortex (+20% in WT and +41% in KO rats, $p < 0.001$ Fig. 1B). On the other hand, duloxetine induced a more robust increase of the neurotrophin in frontal cortex of WT (+75% versus WT/vehicle, $p < 0.001$; Fig. 1C) compared with KO (+24% versus KO/vehicle, $p < 0.05$; Fig. 1C).

Modulation of BDNF mRNA Isoforms after Long-Term Duloxetine Treatment in SERT(+/+) and SERT(-/-) Rats. It is known that the *BDNF* gene has a complex organization, and it is transcribed from at least eight promoters, each of which drives the transcription of a 5' exon alternatively spliced onto a common 3' exon-encoding BDNF protein (Aid et al., 2007). We have demonstrated previously that long-term duloxetine treatment specifically increased mRNA levels of isoforms III and IXa in the hippocampus of normal Sprague-Dawley rats (Molteni et al., 2009). Moreover, we have shown that the mRNA levels for isoforms III, IV, VI, and IXa are all significantly reduced in SERT KO rats (Molteni et al., 2010). Thus, we decided to investigate the mRNA levels of these BDNF transcripts in the hippocampus and prefrontal cortex of WT and KO rats after long-term duloxetine treatment.

In agreement with our previous data (Molteni et al., 2010), the mRNA levels of isoforms III, IV, VI, and IXa were significantly reduced in the hippocampus and prefrontal cortex of SERT KO rats continuously treated with vehicle, an effect ranging from -34 to -11% (Fig. 2, A and B).

Within the hippocampus (Fig. 2A), long-term duloxetine treatment significantly increased the expression of isoforms III and IXa in both genotypes without a significant genotype X drug interaction ($F_{1,39} = 0.06202$, $p > 0.05$; $F_{1,39} = 2.16343$, $p > 0.05$, respectively). In fact, the mRNA levels for isoforms III and IXa were significantly up-regulated in SERT WT (+25% for isoform III and +21% for isoform IXa) and in SERT KO (+30% for isoform III and +37% for isoform IXa) rats. On the other hand, the modulation of isoforms IV and VI mRNA levels showed a significant genotype X drug interaction ($F_{1,39} = 8.33993$, $p < 0.05$; $F_{1,39} = 16.20351$, $p < 0.05$, respectively), being that the expression of the two isoforms was significantly increased by long-term duloxetine treatment in SERT knockout rats (+22% for isoform IV and +33% for isoform VI) but not in wild-type animals (-10% for isoform IV and -4% for isoform VI).

Although long-term duloxetine treatment produced a qualitatively similar up-regulation of isoform III mRNA levels in the prefrontal cortex of both genotypes (Fig. 2B), we observed a significant genotype X drug interaction ($F_{1,37} = 7.35313$, $p < 0.05$), possibly because the magnitude of the observed changes was larger in SERT KO rats (+61%) with respect to WT animals (+22%). Likewise to the hippocampus, the expression of BDNF isoforms IV and VI was significantly increased by duloxetine treatment only in SERT KO rats (+51 and +73%, respectively), as confirmed by the significant genotype X drug interaction ($F_{1,37} = 17.090265$, $p < 0.05$ for isoform IV; $F_{1,37} = 68.334212$, $p < 0.05$ for isoform VI). Finally, the transcriptional modulation of isoform IXa in the prefrontal cortex seems to be somewhat different from the hippocampus, because duloxetine was completely ineffective in regulating its mRNA levels in wild-type and SERT KO rats (Fig. 2B).

Modulation of BDNF 3'-Untranslated Region (UTR) mRNA Levels by Long-Term Duloxetine Treatment in SERT(+/+) and SERT(-/-) Rats. A further degree of complexity for the BDNF gene is due to the presence of two alternative polyadenylation sites in its mRNAs, thus leading to a short and long 3'-UTR. Because the long 3'-UTR may be associated with dendritic targeting of specific neurotrophin transcripts (An et al., 2008), we measured its mRNA levels in SERT KO rats under basal condition or after duloxetine treatment. SERT KO rats show a significant decrease of long 3'-UTR BDNF mRNA levels in the hippocampus (-13% versus WT/vehicle, $p < 0.05$; Fig. 3A) and in prefrontal cortex (-25% versus WT/vehicle, $p < 0.05$; Fig. 3B). Long-term treatment with duloxetine increased the mRNA levels for the long 3'-UTR in SERT KO rats both in hippocampus (+30% versus KO/vehicle, $p < 0.0001$; Fig. 3A) and in prefrontal cortex (+33% versus KO/vehicle, $p < 0.05$; Fig. 3B) but not in WT animals (hippocampus, +8% versus WT/vehicle, $p > 0.05$; prefrontal cortex, -2% versus WT/vehicle, $p > 0.05$).

Modulation of BDNF Protein Levels in the Frontal Cortex of SERT(+/+) but Not of SERT(-/-) Rats after Long-Term Duloxetine Treatment. We have demonstrated previously that mBDNF was markedly increased in the crude synaptosomal fraction of frontal cortex from normal Sprague-Dawley rats continuously treated with duloxetine (Calabrese et al., 2007).

Hence, we decided to investigate the relative contribution of SERT in the modulation of BDNF protein levels by duloxetine. The levels of proBDNF were not modulated by the genotype or the pharmacological treatment (Fig. 4). Consistent with our previous results (Calabrese et al., 2007), long-term treatment with duloxetine increased mBDNF levels in the crude synaptosomal fraction of WT animals (+46% versus WT/vehicle, $p < 0.01$; Fig. 4). This effect seems to depend on the integrity of the serotonin transporter, because mBDNF levels in SERT KO rats were not significantly modulated by long-term duloxetine treatment (-4% versus KO/vehicle, $p > 0.05$).

Regulation of VGF mRNA Levels in SERT(+/+) and SERT(-/-) Rats after Long-Term Duloxetine Treatment. It has been shown recently that other neurotrophic factors may be involved in cause of depression and in the action of antidepressant drugs. For example, it has been demonstrated that VGF (nonacronymic) has antidepressant effects in animal models of depression (Hunsberger et al., 2007; Thakker-Varia et al., 2007) and may be modulated by antidepressant treatments (Altar et al., 2004; Thakker-Varia et al., 2007; Cattaneo et al., 2010). We found that, similarly to BDNF, VGF gene expression was significantly decreased in SERT KO rats (hippocampus, -11% versus WT/vehicle, $p < 0.01$; Fig. 5A; prefrontal cortex, -24% versus WT/vehicle, $p < 0.01$; Fig. 5B). Long-term duloxetine treatment increased VGF mRNA levels in both regions of WT rats (hippocampus, +14%, $p < 0.001$; prefrontal cortex, +20% versus WT/vehicle, $p < 0.01$; Fig. 5, A and B), an effect also found in the hippocampus (+10% versus KO/vehicle, $p < 0.05$; Fig. 5A) and in the prefrontal cortex (+10% versus KO/vehicle, $p < 0.05$; Fig. 5B) of SERT KO animals.

Discussion

In the present study, we show that long-term treatment with the antidepressant duloxetine is able to normalize BDNF mRNA levels, which are reduced in the hippocampus

and prefrontal cortex of SERT KO rats. This effect occurs through the modulation of specific BDNF isoforms, whose expression is up-regulated by duloxetine in mutant but not in normal rats. In contrast, duloxetine seems to be less effective in promoting neuroplastic changes in the frontal cortex of SERT knockout rats.

As demonstrated previously (Calabrese et al., 2007; Moltani et al., 2009), long-term duloxetine treatment increased total BDNF mRNA levels in hippocampus and cortical regions (prefrontal and frontal cortex) of wild-type animals.

Because we used female Wistar rats in this study, these data confirm the involvement of BDNF in the action of this antidepressant drug, independent of gender and animal strains used in the study. Although the effects produced by duloxetine on total BDNF mRNA levels are qualitatively similar between WT and SERT KO rats, the magnitude of these changes is larger in SERT KO rats. This may occur because the basal levels are lower in SERT KO rats but, as discussed later, also because different regulatory mechanisms can take place in SERT KO rats. The rat BDNF gene consists of nine

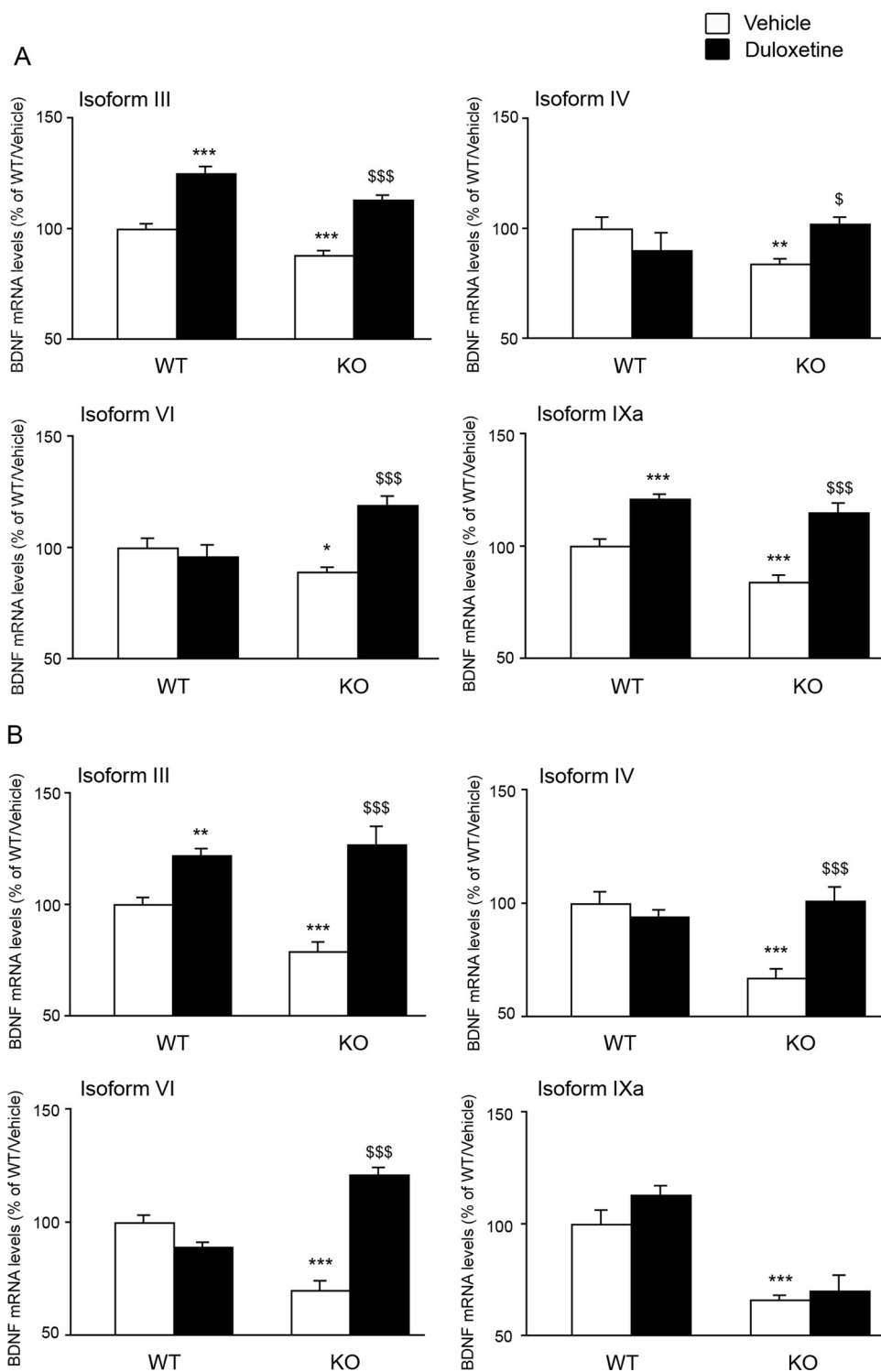


Fig. 2. The modulation of BDNF isoforms after long-term duloxetine treatment is influenced by SERT genotype. The mRNA levels for different BDNF mRNA transcripts (III, IV, VI, and IXa) were measured in the hippocampus (A) and the prefrontal cortex (B) of WT and KO rats treated for 21 days with vehicle or duloxetine and killed 24 h after the last injection. The data are expressed as a percentage of WT/vehicle (set at 100%) and are the mean \pm S.E.M. from at least six independent determinations. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus WT/vehicle; \$, $p < 0.05$, \$\$\$, $p < 0.001$ versus KO/vehicle (two-way ANOVA with SCPHT).

5'-untranslated exons, each linked to individual promoter regions, and a 3'-coding exon (IX), which codes for the BDNF preprotein amino acid sequence (Aid et al., 2007). The analysis of specific neurotrophin transcripts has proven quite informative to establish potential differences in neuroadaptive mechanisms set in motion by the pharmacological treatment in a vulnerable genotype, with respect to normal animals. Indeed, our results suggest that differences in the modulation of BDNF transcripts after long-term duloxetine treatment may be a qualitative rather than a quantitative trait. We show that, within the hippocampus, duloxetine increases the expression of isoforms III and IXa in wild-type animals and in SERT knockout rats. The changes in wild-type rats resemble the effects we have reported previously in normal male Sprague-Dawley rats (Molteni et al., 2009) and suggest that these changes may not be strictly dependent on a "pathological" phenotype. It is interesting that the prefrontal cortex isoform III is also up-regulated by duloxetine treatment in both genotype, whereas isoform IXa was not regulated by

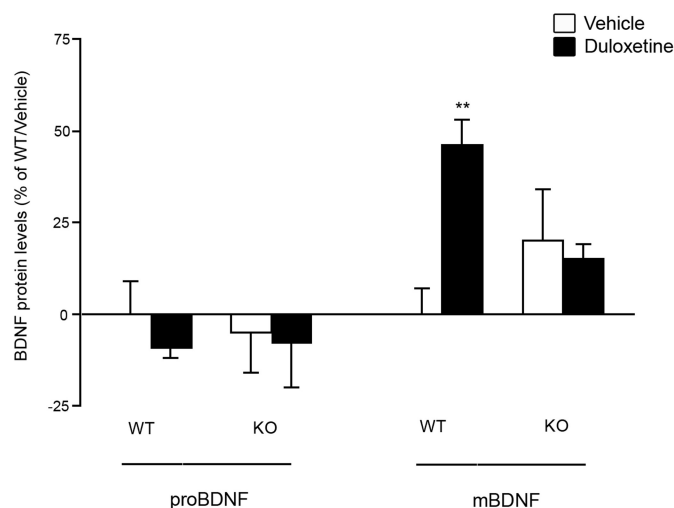


Fig. 4. The modulation of BDNF protein levels in the frontal cortex of rats continuously treated with duloxetine is influenced by the SERT genotype. The levels of the precursor (proBDNF) and mature form (mBDNF) of the neurotrophin were measured in the crude synaptosomal fraction of the frontal cortex of WT and KO rats treated for 21 days with vehicle or duloxetine and killed 24 h after the last injection. The data, expressed as a percentage difference of WT/vehicle (set at 0), are the mean \pm S.E.M. from at least five independent determinations. **, $p < 0.01$ versus WT/vehicle (two-way ANOVA with SCPHT).

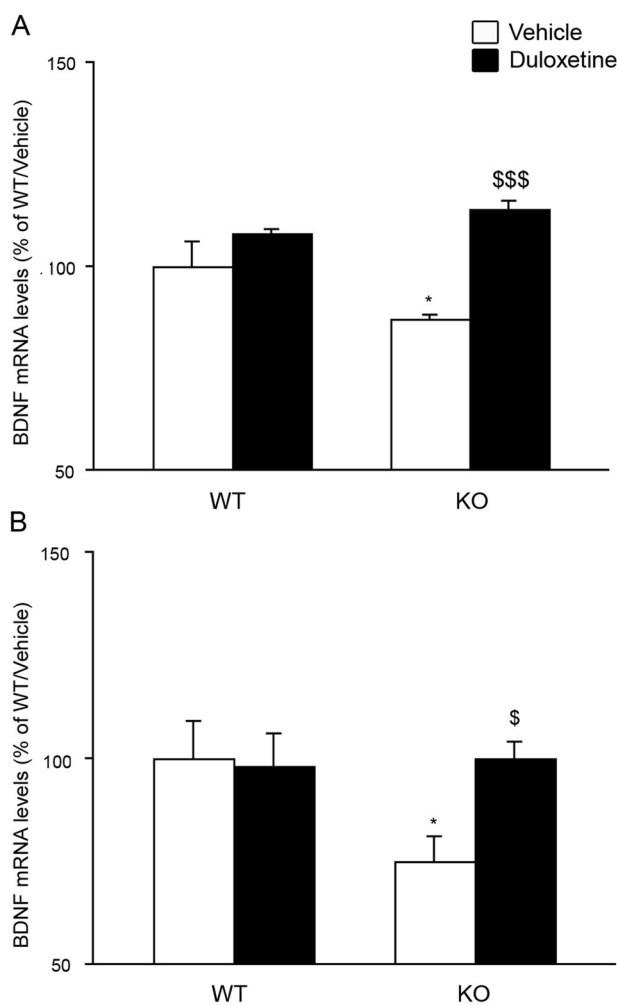


Fig. 3. Long-term duloxetine treatment increases 3'-UTR long BDNF mRNA levels in SERT KO rats. 3'-UTR long BDNF mRNA levels measured in the hippocampus (A) and in the prefrontal cortex (B) of WT and KO rats treated for 21 days with vehicle or duloxetine and killed 24 h after the last injection. The data, expressed as a percentage of WT/vehicle (set at 100%), are the mean \pm S.E.M. from at least six independent determinations. *, $p < 0.05$ versus WT/vehicle; \$, $p < 0.05$; \$\$\$, $p < 0.001$ versus KO/vehicle (two-way ANOVA with SCPHT).

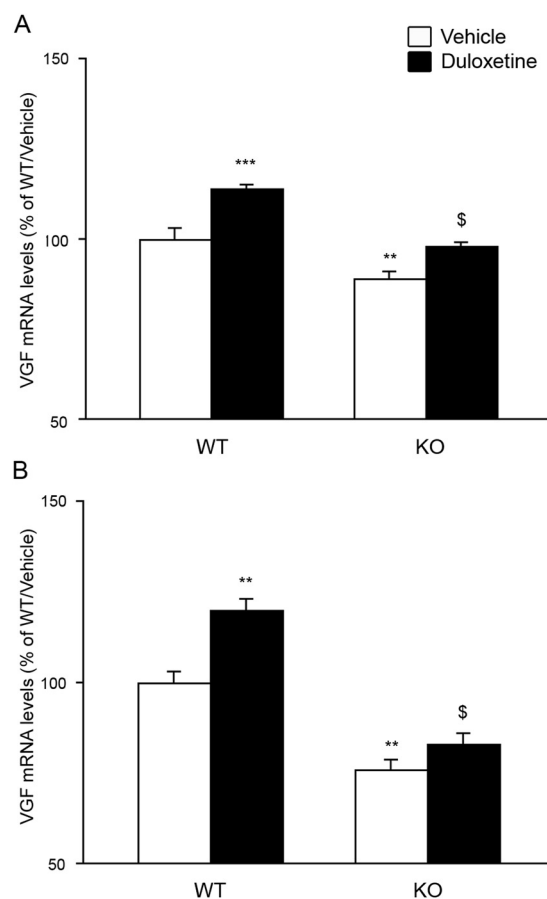


Fig. 5. Long-term duloxetine treatment increases VGF mRNA levels in WT rats. VGF mRNA levels were measured in hippocampus (A) and prefrontal cortex (B) of WT and KO rats treated for 21 days with vehicle or duloxetine and killed 24 h after the last injection. The data, expressed as a percentage of WT/vehicle (set at 100%), are the mean \pm S.E.M. from at least six independent determinations. **, $p < 0.01$ and ***, $p < 0.001$ versus WT/vehicle; \$, $p < 0.05$ versus KO/vehicle (two-way ANOVA with SCPHT).

the antidepressant independent of the fact that its mRNA levels were significantly diminished in SERT knockout rats. This observation provides further support to the complexity and anatomical specificity in the regulation of BDNF transcripts.

As opposed to the modulation of isoforms III and IXa, the mRNA levels of transcripts IV and VI were significantly up-regulated by long-term duloxetine treatment only in the hippocampus and prefrontal cortex of SERT KO rats, an effect that normalizes the reduced BDNF expression found in mutant rats. This suggests that the larger increase of total BDNF mRNA levels brought about by duloxetine treatment in SERT knockout rats compared with wild-type animals, may be ascribed to the modulation of these isoforms. Although the expression of several transcripts is reduced in SERT KO rats (Molteni et al., 2010), it may be inferred that the modulation of isoforms IV and VI might represent a specific mechanism through which antidepressant drugs, such as duloxetine, can restore defective plasticity in a vulnerable genotype. A role of noradrenaline in the modulation of exon III can be postulated because Dwivedi et al. (2006) demonstrated that desipramine, a selective noradrenaline reuptake inhibitor, produces a robust up-regulation of exon III in rat hippocampus and frontal cortex, which may reverse a corticosterone-mediated decrease in BDNF. Because specific transcriptional regulatory sites are present in the promoter regions of BDNF exons, it may be postulated that duloxetine treatment affects signaling pathways that are different between WT and SERT KO rats.

It has been shown that two distinct populations of BDNF transcripts exist, respectively, with a short or long 3'-UTR (Timmusk et al., 1993; Ghosh et al., 1994). Similar to the changes of total BDNF mRNA levels, the expression of the long 3'-UTR was also significantly reduced in SERT KO rats. However, we demonstrate that the levels of the long 3'-UTR BDNF mRNA are increased after duloxetine treatment only in SERT knockout rats, suggesting that this post-transcriptional mechanism can be pharmacologically regulated. Recent studies have suggested that the long 3'-UTR has a role in targeting BDNF mRNA to dendrites (An et al., 2008). The differential modulation of the long 3'-UTR in SERT knockout rats treated with duloxetine may be related to the increased expression of exon VI, which is one of the BDNF isoforms found in dendrites (Chiaruttini et al., 2008). Although the functional implications of these changes remain to be established, it may be inferred that higher levels of long 3'-UTR BDNF mRNA may enhance the synaptic pool of BDNF transcripts that, upon stimuli, may undergo local translation (An et al., 2008).

It is interesting to note that in the frontal cortex, different from what was observed in the other two brain regions, the up-regulation of BDNF mRNA levels observed in wild-type animals treated with duloxetine is greatly diminished in mutant rats, whereas the increase of mBDNF protein levels was completely abolished in SERT knockout rats. The reason for this differential modulation is unknown. However, it must be pointed out that, different from prefrontal cortex and hippocampus, the expression of BDNF is not reduced in the frontal cortex of SERT knockout rats. We have shown previously that long-term pharmacological blockade of SERT (fluoxetine) is not sufficient to increase mBDNF levels in frontal cortex. Based on the present data,

we hypothesize that duloxetine can effectively up-regulate mBDNF protein through a synergic modulation of serotonin and noradrenaline systems. On the other hand, within the hippocampus and the prefrontal cortex, the ability of duloxetine to block the noradrenaline transporter may be sufficient to promote neuroadaptive changes leading to the up-regulation of BDNF expression.

Deletion of the SERT gene is also associated with a significant reduction of VGF expression, suggesting that the pathological phenotype may be associated with a diminished neurotrophic support in key brain regions. In line with BDNF changes, VGF mRNA levels are similarly up-regulated by long-term duloxetine treatment in wild-type and SERT KO rats.

We have shown previously that the expression of BDNF and VGF is also reduced significantly in another animal model of depression (prenatal stress), which relies on an environmental manipulation during development (Fumagalli et al., 2004; Cattaneo et al., 2010). Hence, the observation that these two neurotrophic molecules are defective in the genetic model of SERT KO rats provides further support to the role of neurotrophic factors in depression, and it is confirmed by the fact that similar alterations of BDNF and VGF levels have been described in depressed subjects (Sen et al., 2008; Cattaneo et al., 2010).

These data suggest that reduced neuronal plasticity associated with major depression may be supported by intermingled deficits of different neurotrophic factors, including BDNF (Pittenger et al., 2007; Calabrese et al., 2009), VGF (Hunsberger et al., 2007), and basic fibroblast growth factor, whose expression is also affected under similar conditions (Molteni et al., 2001; Turner et al., 2006). The pharmacological modulation of these systems may therefore represent a relevant and valuable strategy for patient recovery (Berton and Nestler, 2006; Castrén et al., 2007; Calabrese et al., 2009).

In summary, our data suggest that animals with a genetic defect of the serotonin transporter maintain the ability to show neuroplastic changes in response to antidepressant drugs, possibly via the blockade of the noradrenaline transporter. Because these animals show depression-like behavior (Homborg et al., 2007; Olivier et al., 2008), the region and isoform-specific increase of BDNF levels may be a mechanism activated by long-term antidepressant treatment to normalize defective plasticity under genetic dysfunction of the serotonin transporter.

Acknowledgments

We thank Dr. Juliet Richetto, Dr. Paola F. Maj, and Dr. Jocelien D. A. Olivier for contributing to part of this research.

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