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Title page

Chronic duloxetine treatment normalizes altered BDNF expression in serotonin transporter knockout rats through the modulation of specific neurotrophin isoforms.

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Running title page

Running title: Duloxetine modulation of BDNF in SERT KO

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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 recently demonstrated that SERT knockout (KO) rats, generated by ENU-induced mutagenesis, show reduced expression of the neurotrophin BDNF in 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. Conversely, the modulation of BDNF protein by DLX in frontal cortex was abolished in mutant rats.

In summary, these data suggest that animals with a genetic defect of the serotonin transporter maintain the ability to show neuroplastic changes in response to AD drugs. Since these animals show depression-like behavior, the region and isoform-specific increase of BDNF levels may be a mechanism activated by chronic antidepressant treatment to restore normal plasticity that is defective under genetic dysfunction of the serotonin transporter.

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Introduction

Major depression is thought to originate from the interaction between susceptibility genes and environmental events. Genetic susceptibility may, at least in part, be due 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 (Calabrese et al., 2009; McClung and Nestler, 2008; Pittenger and Duman, 2008; Tsankova et al., 2006). Moreover the modulation of BDNF represents a crucial step in long-term adaptive changes brought about by antidepressant drugs (Calabrese et al., 2007; Calabrese et al., 2009; Castren et al., 2007; Kozisek et al., 2008; Molteni et al., 2009a). 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; Kozisek et al., 2008; Martinowich et al., 2007; Molteni et al., 2009a). 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 pathologic 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. While 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-HT transporter is of particular relevance in the context of mood disorders because it is the target of several antidepressant drugs, but also for the presence of a

functional polymorphism within its promoter region that is associated with different neuropsychiatric disorders and mostly important with the antidepressant response (Caspi et al., 2003; Huezo-Diaz et al., 2009; Uher and McGuffin, 2008). Using target-selected mutagenesis, we have recently generated serotonin transporter (SERT) knock out rats (Smits et al., 2006), which showed an impairment of the serotonergic system (Homberg et al., 2007) associated with anxiety- and depression- like behaviors (Kalueff et al., 2009; Olivier et al., 2008).

Since SERT KO rats show a reduction of BDNF levels in selected brain regions (Molteni et al., 2009b), we decided to investigate the effect of a chronic treatment with the antidepressant drug duloxetine on BDNF expression in SERT KO rats in order to address two important points. First, we wondered if 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 chronic duloxetine treatment (Calabrese et al., 2007; Molteni et al., 2009a). To this aim we measured total (exon IX) BDNF mRNA levels as well as 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, in order to assess if 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).

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Materials and Methods

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

Animals and Pharmacological Treatment

Serotonin transporter knockout rats ($Slc6a4^{1Hubr}$) were generated by ENU-induced mutagenesis (Smits et al., 2006). All subjects were bred and reared in the Central Animal Laboratory of the University of Nijmegen. Experimental animals were derived from crossing heterozygous SERT KO rats that were out crossed for 5 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: 12 h dark–light cycle (lights on at 6:00 in the morning). Animals were treated chronically (21 days) with vehicle or duloxetine (10 mg/kg by gavage) and sacrificed 24 hours after the last injection. **Duloxetine was dissolved at a concentration of 10 mg/ml and it was administrated 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 6-10 according to the atlas of Paxinos and Watson) (Paxinos and Watson 1996) and frontal cortex (comprising regions Fr1–3, Par1 and AI from the same slice as PFC) 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.

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All experiments were carried out in accordance with the Guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC).

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

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 with the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (PCR) to assess BDNF mRNA levels.

A two 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 (Applied Biosystem). The real-time PCR reaction was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystem) with the TaqMan Gene expression Master Mix (Applied Biosystem) and the following TaqMan Gene Expression Assay (Applied Biosystem):

Total BDNF: ID Rn02531967_s1; BDNF transcript III forward primer: ATGCTTCATTGAGCCCAGTT and reverse primer: GTGGACGTTTGCTTCTTTCA; BDNF transcript IV: ID Rn01484927_m1; BDNF transcript VI: ID Rn01484928_m1, BDNF transcript IXa forward primer: TGGTGTCCCCAAGAAAGTAA and reverse primer: CACGTGCTCAAAAGTGTCAG.

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

PCR reactions were carried out as previously reported (Molteni et al., 2009a). Briefly, after an initial step at 50 °C for 2 min and at 95 °C for 10 min, 40 cycles of PCR were

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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 and using two independent retro-transcription products. A comparative cycle threshold (*Ct*) method was used to determine the relative target gene expression. Data have been expressed as percentage calculated from the expression of the target genes normalized on rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression as control gene (ID GAPDH TaqMan probe: Rn99999916_s1).

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 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 2,000 g for 1 min at 4°C, thus obtaining a pellet corresponding to the nuclear fraction, which was resuspended in Solution A and centrifuged again (2,000 g for 2 min at 4°C). The supernatant obtained from each centrifugation-step was collected and centrifuged at 23,000 g for 5 min at 4°C to obtain a pellet (P2) corresponding to the crude synaptosomal fraction which was re-suspended in Solution A. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard.

Equal amounts of protein were run under reducing conditions on 14% SDS-polyacrylamide gels and then electrophoretically transferred onto PVDF membranes (Bio-Rad Laboratories). The blots were blocked with 10% nonfat dry milk and then incubated with the primary antibody anti-BDNF polyclonal antibody (1:1,000, 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

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were then incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:5,000) 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 ± standard error (SEM). For graphic clarity, results are presented as mean percent of WT/Vehicle-treated rats.

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Results

SERT KO rats, similarly to SERT knockout mice (Holmes et al., 2003; Lira et al., 2003), show depression- and anxiety-related behavior (Kalueff et al., 2009; Olivier et al., 2008) and have reduced expression of BDNF levels in the hippocampus and prefrontal cortex (Molteni et al., 2009b). We tested whether antidepressant treatment may restore 'normal' levels of plasticity in animals with an alteration of the serotoninergic system, which may increase the susceptibility to mood disorders (Caspi et al., 2003; Lucki, 1998; 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 (Castren et al., 2007).

Modulation of total BDNF mRNA levels by chronic duloxetine in $SERT^{+/+}$ and $SERT^{-/-}$ rats

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

Chronic 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 figure 1, duloxetine increased total BDNF mRNA levels (exon IX) in both genotypes, an effect that, in hippocampus 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%, +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). Conversely, duloxetine induced a more robust increase of the**

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neurotrophin in frontal cortex of WT (+75% vs. WT/Vehicle *p*<0.001 Fig. 1C) as compare to KO (+24% vs. KO/Vehicle *p*<0.05 Fig. 1C).

Modulation of BDNF mRNA isoforms after chronic 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 previously demonstrated that chronic duloxetine treatment specifically increased mRNA levels of isoforms III and IXa in the hippocampus of normal Sprague Dawley rats (Molteni et al., 2009a). Moreover we have shown that the mRNA levels for isoforms III, IV, IV and IXa are all significantly reduced in SERT KO rats (Molteni et al., 2009b). Thus, we decided to investigate the mRNA levels of these BDNF transcripts in the hippocampus and prefrontal cortex of WT and KO rats after chronic duloxetine treatment.

In agreement with our previous data (Molteni et al., 2009b) the mRNA levels of isoforms III, IV, VI and IXa were significantly reduced in the hippocampus and prefrontal cortex of SERT KO rats chronically treated with vehicle, an effect ranging from – 34% to -11% (Fig. 2A and Fig. 2B).

Within the hippocampus (Fig. 2A) chronic duloxetine treatment significantly increased the expression of isoform 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) as well as in SERT KO (+30% for isoform III and +37% for isoform IXa) rats. Conversely the modulation of isoform 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 chronic duloxetine treatment in SERT knockout

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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 chronic duloxetine treatment produced a qualitatively similar up-regulation of isoforms III mRNA levels in the prefrontal cortex of both genotypes (Fig. 2B), we observed a significant genotype X drug ($F_{1,37}$=7.35313, p<0.05), possibly due to the fact that 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 isoforms IXa in prefrontal cortex seems to be somewhat different from the hippocampus, since duloxetine was completely ineffective in regulating its mRNA levels in wild type as well as in SERT KO rats (Fig. 2B).

Modulation of BDNF 3' UTR mRNA levels by chronic 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. Since 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% vs. WT/Vehicle, p<0.05; Fig. 3A), as well as in prefrontal cortex (-25% vs. WT/Vehicle, p<0.05; Fig. 3B). Chronic treatment with duloxetine increased the mRNA levels for the long 3'UTR in SERT KO rats both in hippocampus (+30% vs. KO/Vehicle, p<0.0001; Fig. 3A) and in prefrontal cortex (+33% vs. KO/Vehicle, p<0.05; Fig. 3B), but not in WT animals (hippocampus, +8% vs. WT/Vehicle, p>0.05; prefrontal cortex, -2% vs.

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WT/Vehicle, $p>0.05$).

Modulation of BDNF protein levels in the frontal cortex of SERT$^{+/+}$ but not of SERT$^{-/-}$ rats following chronic duloxetine treatment

We have previously demonstrated that the mature from of BDNF (mBDNF) was markedly increased in the crude synaptosomal fraction of frontal cortex from normal Sprague Dawley rats chronically 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 (the neurotrophin precursor) were not modulated by the genotype as well as by the pharmacological treatment (Fig. 4). Consistent with our previous results (Calabrese et al., 2007), chronic treatment with duloxetine increased mBDNF levels in the crude synaptosomal fraction of WT animals (+46% vs. WT/Vehicle, $p<0.01$; Fig. 4). This effect appears to depend upon the integrity of the serotonin transporter, since mBDNF levels in SERT KO rats were not significantly modulated by chronic duloxetine treatment (-4% vs. KO/Vehicle, $p>0.05$).

Regulation of VGF mRNA levels in SERT$^{+/+}$ and SERT$^{-/-}$ rats after chronic duloxetine treatment

It has been recently shown that other neurotrophic factors may be involved in etiology 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, in press). We found that, similarly to BDNF, VGF gene expression was significantly decreased in SERT KO rats (hippocampus: -11%, vs. WT/Vehicle, $p<0.01$; Fig. 5A; prefrontal cortex: -24%; vs. WT/Vehicle, $p<0.01$; Fig. 5B). Chronic duloxetine treatment increased VGF mRNA levels in both regions of WT rats (hippocampus: +14%, $p<0.001$; prefrontal cortex: +20%; vs. WT/Vehicle, $p<0.01$; Fig. 5A, 5B), an

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effect also found in the hippocampus (+10% vs. KO/Vehicle, $p<0.05$; Fig. 5A) and in the prefrontal cortex (+10% vs. KO/Vehicle, $p<0.05$; Fig. 5B) of SERT KO animals.

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Discussion

In the present study we show that chronic 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 appears to be less effective in promoting neuroplastic changes in the frontal cortex of SERT knockout rats.

As previously demonstrated (Calabrese et al., 2007; Molteni et al., 2009a), chronic duloxetine treatment increased total BDNF mRNA levels in hippocampus and cortical regions (prefrontal and frontal cortex) of wild type animals. Since in the present study we used female Wistar rats, these data confirm the involvement of BDNF in the action of this antidepressant drug, independently from 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 5' untranslated exons, each linked to individual promoter regions, and a 3' coding exon (IX), which codes for the BDNF pre-protein amino acid sequence (Aid et al., 2007). The analysis of specific neurotrophin transcripts has proven quite informative in order 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 following chronic duloxetine may be a qualitative rather than a quantitative trait. We show that, within the hippocampus, duloxetine increases the expression of isoform III and IXa in wild type animals as well in SERT knockout rats. The changes in wild type rats resemble the effects we have previously reported in normal male Sprague-Dawley rats (Molteni et al., 2009a) and

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suggest that these changes may not be strictly dependent upon a 'pathologic' phenotype. Interestingly in prefrontal cortex isoform III is also up-regulated by duloxetine in both genotype, whereas isoform IXa was not regulated by the antidepressant independently from 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.

At a difference from the modulation of isoforms III and IXa, the mRNA levels of transcripts IV and VI were significantly up regulated by chronic duloxetine 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 in SERT knock out rats, as compared to 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., 2009b), 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 since Dwivedi et al.** (Dwivedi et al., 2006) have **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 corticosterone-mediated decrease in BDNF.** Since specific transcriptional regulatory sites are present in the promoter regions of BDNF exons, it may be postulated that duloxetine treatment affects signalling 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' untranslated region (UTR) (Ghosh et al., 1994; Timmusk et al., 1993). 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

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in SERT knock out 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's interesting to note that in the frontal cortex, differently from what 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, at a difference from PFC and HIP, the expression of BDNF is not reduced in the frontal cortex of SERT knockout rats. We have previously shown that chronic pharmacological blockade of SERT (fluoxetine) is not sufficient to increase mBDNF levels in frontal cortex. **Based on the present data, we may hypothesize that duloxetine can effectively up-regulate mBDNF protein through a synergic modulation of serotonin and noradrenaline systems. Conversely 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 pathologic 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 chronic duloxetine in wild type and SERT KO rats.

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We have previously shown that the expression of BDNF and VGF is also significantly reduced in another animal model of depression (prenatal stress), which relies on an environmental manipulation during development (Fumagalli et al., 2004; Cattaneo et al., in press). **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., in press).

These data suggest that reduced neuronal plasticity associated with major depression may be supported by intermingled deficits of different neurotrophic factors, including BDNF (Calabrese et al., 2009; Pittenger et al., 2007), VGF (Hunsberger et al., 2007) as well as basic Fibroblast Growth Factor (FGF-2) 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; Calabrese et al., 2009; Castren et al., 2007).

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 AD drugs, possibly via the blockade of the noradrenaline transporter. Since these animals show depression-like behaviour (Homberg et al., 2007; Olivier et al., 2008), the region and isoform-specific increase of BDNF levels may be a mechanism activated by chronic antidepressant treatment to normalize defective plasticity under genetic dysfunction of the serotonin transporter.

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Footnotes

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Legends for figures

Figure 1

Chronic duloxetine treatment increases total BDNF mRNA levels in WT as well as SERT KO rats.

Total BDNF mRNA levels were measured in hippocampus (A), prefrontal cortex (B) and **frontal cortex** (C) of wild-type (WT) and SERT-/- (KO) rats treated for 21 days with vehicle or duloxetine and killed 24 hours after the last injection. The data, expressed as a percentage of WT/Vehicle (set at 100%), are the mean ± SEM from at least 6 independent determinations. *p<0.05 and *** p<0.001 vs. WT/Vehicle; $^{\$}$***p*<0.05** and $^{\$\$\$}$ p<0.001 vs. KO/Vehicle (two-way ANOVA with SCPHT).

Figure 2

The modulation of BDNF isoforms following chronic duloxetine treatment is influenced by SERT genotype.

The mRNA levels for different BDNF mRNA transcripts (III, IV, VI, IXa) were measured in the hippocampus (A) and in the prefrontal cortex (B) of wild-type (WT) and of SERT-/- (KO) rats treated for 21 days with vehicle or duloxetine and killed 24 hours after the last injection. The data are expressed as a percentage of WT/Vehicle (set at 100%), are the mean ± SEM from at least 6 independent determinations. *p<0.05, **p<0.01, ***p<0.001 vs WT/Vehicle; $^{\$}p$<0.05, $^{\$\$\$}$ p<0.001 vs. KO/Vehicle (two-way ANOVA with SCPHT).

Figure 3

Chronic 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 wild-type (WT) and of SERT-/- (KO) rats treated for 21 days with vehicle or duloxetine and killed 24 hours after the last injection. The data,

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expressed as a percentage of WT/Vehicle rats (set at 100%), are the mean ± SEM from at least 6 independent determinations. *p<0.05 vs WT/Vehicle; $^{\$}p$<0.05, $^{\$\$\$}p$<0.001 vs. KO/Vehicle (two-way ANOVA with SCPHT).

Figure 4

The modulation of BDNF protein levels in the frontal cortex of rats chronically 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 wild-type (WT) and SERT-/- (KO) rats treated for 21 days with vehicle or duloxetine and killed 24 hours after the last injection. The data, expressed as a percentage difference of WT/Vehicle (set at 0), are the mean ± SEM from at least 5 independent determinations. **p<0.01 vs. WT/Vehicle (two-way ANOVA with SCPHT).

Figure 5

Chronic duloxetine treatment increases VGF mRNA levels in WT rats.

VGF mRNA levels were measured in hippocampus (A) and prefrontal cortex (B) of wild-type (WT) and SERT-/- (KO) rats treated for 21 days with vehicle or duloxetine and killed 24 hours after the last injection. The data, expressed as a percentage of WT/Vehicle (set at 100%), are the mean ± SEM from at least 6 independent determinations. **p<0.01 and *** p<0.001 vs. WT/Vehicle; $^{\$}p$<0.05 vs. KO/Vehicle (two-way ANOVA with SCPHT).

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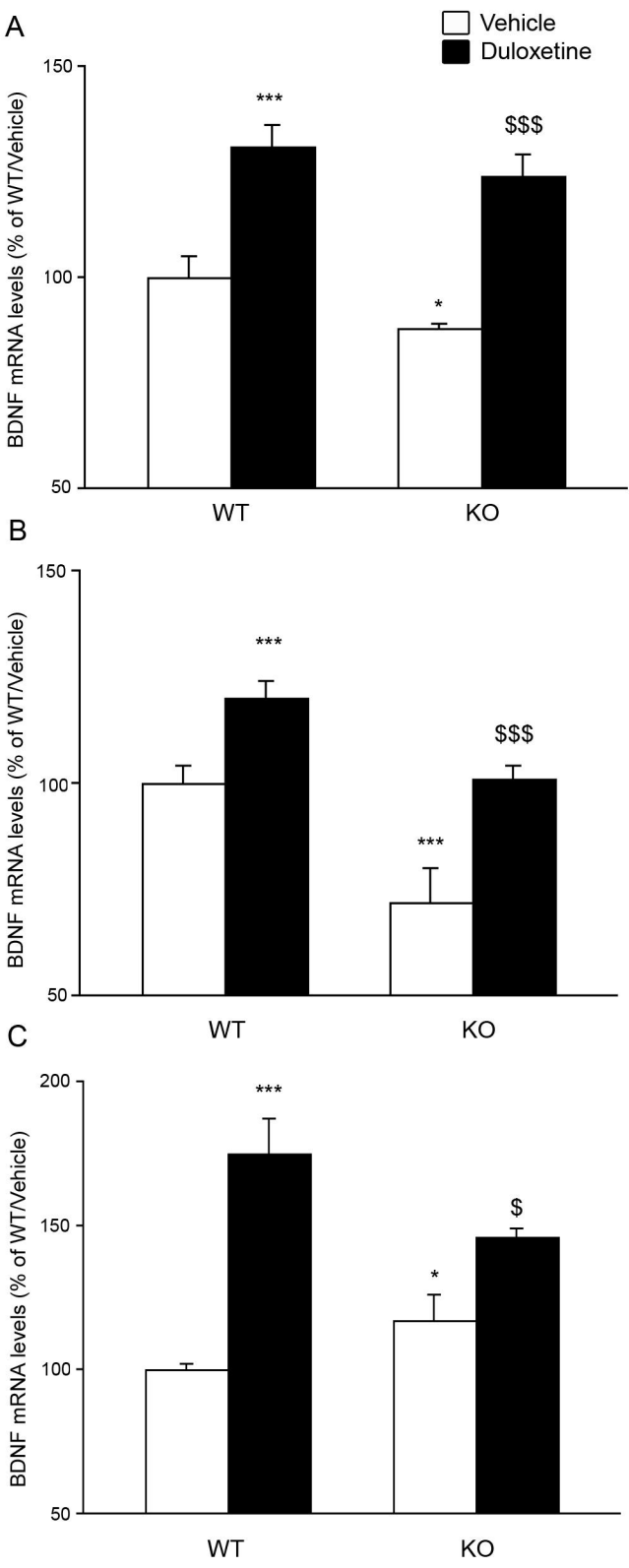
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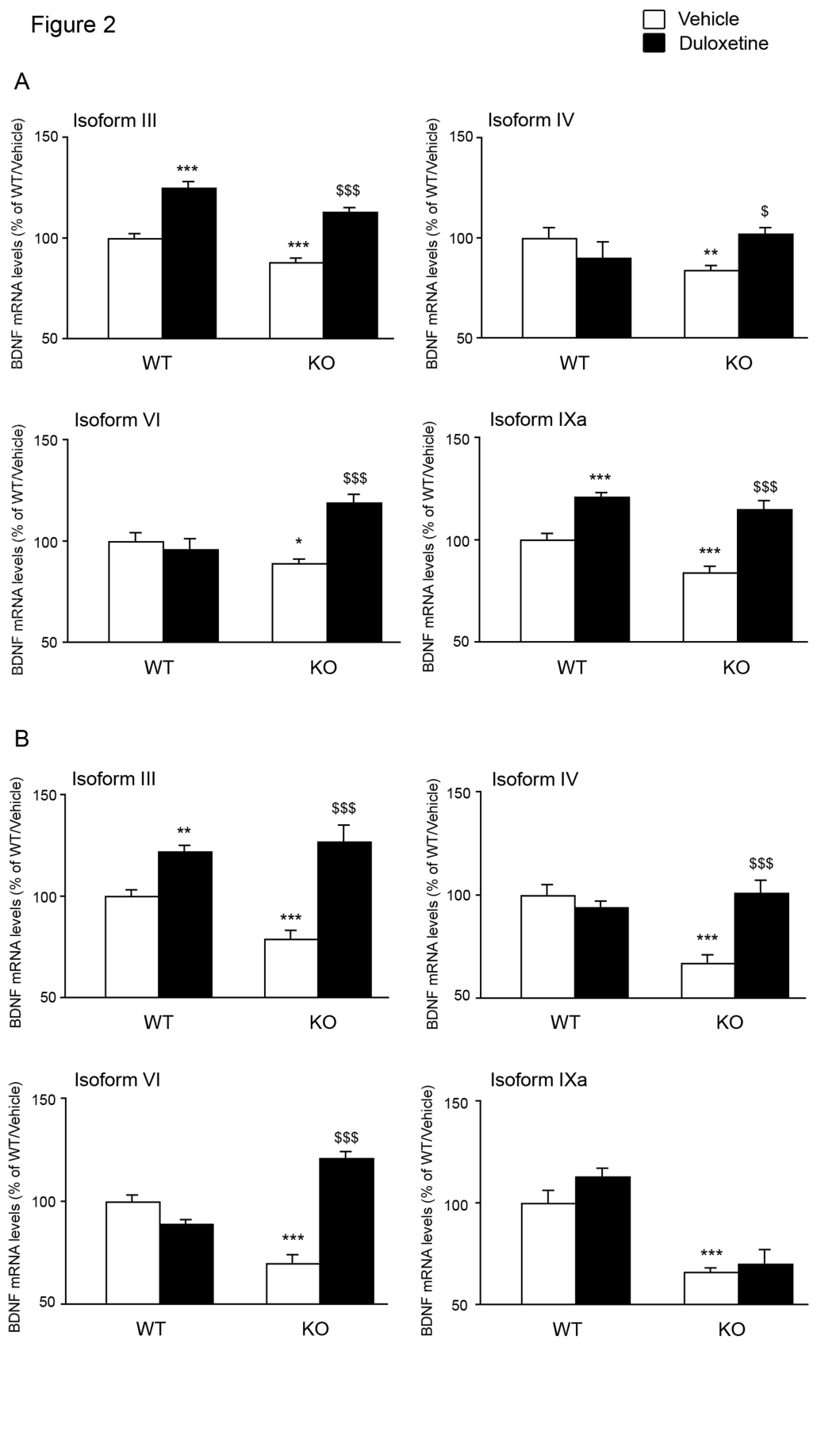
Figure 2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BDNF mRNA levels (% of WT/Vehicle)
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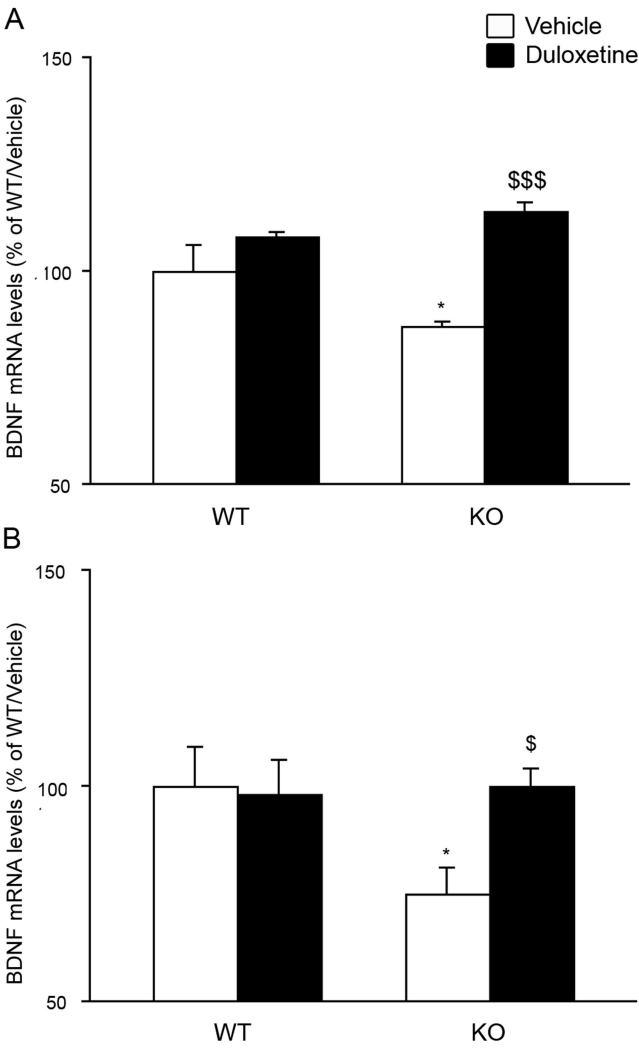
Figure 3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Duloxetine
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BDNF mRNA levels (% of WT/Vehicle)
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BDNF mRNA levels (% of WT/Vehicle)
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*
WT
KO

Figure 4

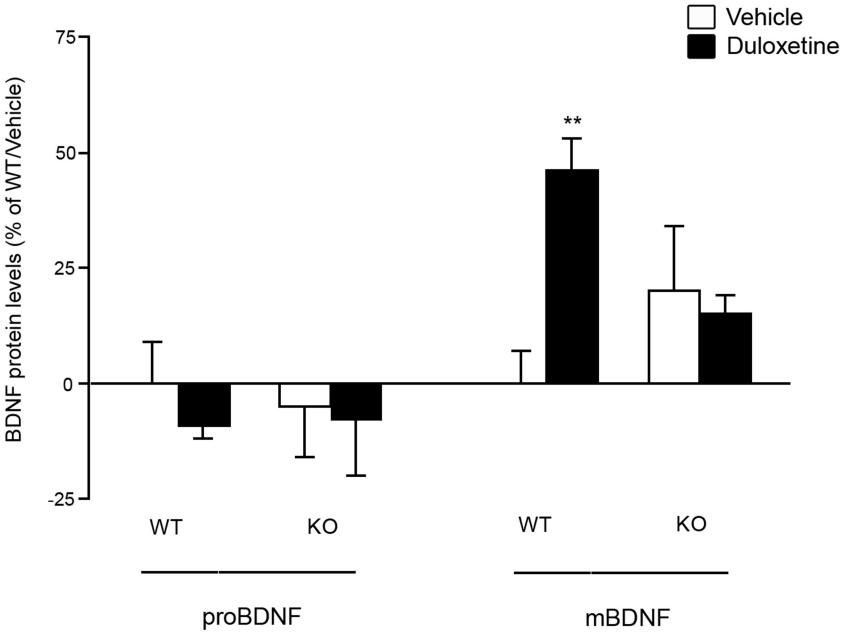
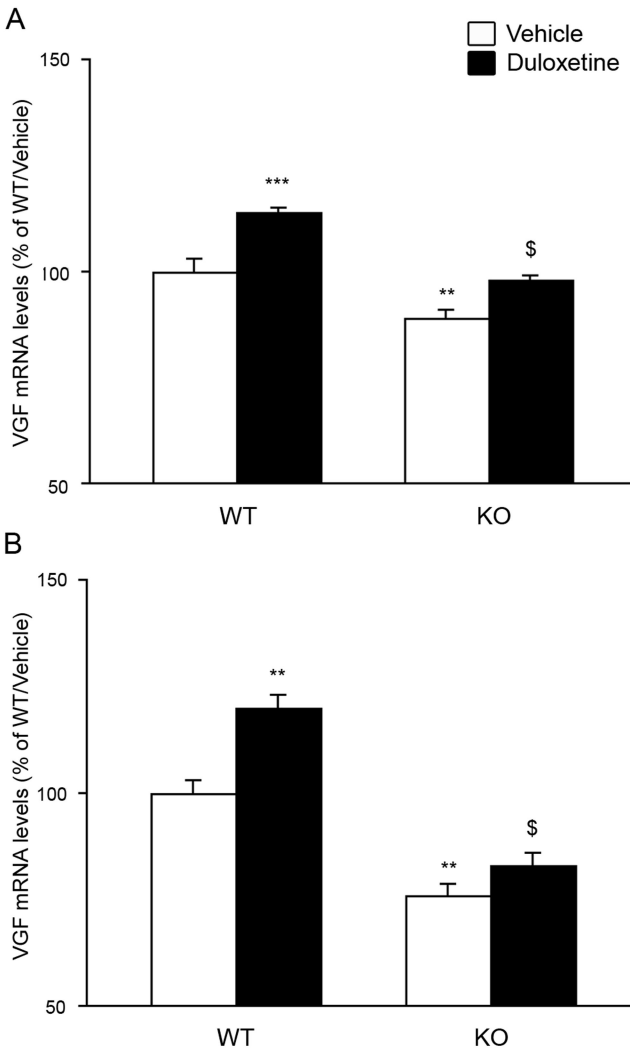

Vehicle
Duloxetine
BDNF protein levels (% of WT/Vehicle)
75
50
25
0
-25
**
WT
KO
WT
KO
proBDNF
mBDNF

Figure 5


A
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VGF mRNA levels (% of WT/Vehicle)
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VGF mRNA levels (% of WT/Vehicle)
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