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Reduced function of the serotonin transporter is associated with decreased expression of BDNF in rodents as well as in humans

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ABSTRACT

In order to identify the molecular mechanisms that may contribute to the enhanced susceptibility to depression under serotonin transporter (SERT) dysfunction, we analyzed the expression of brain-derived neurotrophic factor (BDNF), a key player in neuronal plasticity, which is implicated in the etiology and treatment of depression. We found that BDNF levels were significantly reduced in the hippocampus and prefrontal cortex of SERT knockout rats, through transcriptional changes that affect different neurotrophin isoforms. The reduction of BDNF gene expression observed in prefrontal cortex is due, at least in part, to epigenetic changes that affect the promoter regions of exons IV and VI.
Moreover, BDNF gene expression is also significantly reduced in leukocytes from healthy subjects carrying the S allele of the *5-HTTLPR*, suggesting that the changes observed in SERT mutant rats may also be present in humans and may confer enhanced vulnerability to mood disorders.

Introduction

It is well known that serotonin (5-HT) modulates a variety of behavioral functions related to emotion and cognition and has an impact on neuronal circuits relevant for anxiety and depression (Lucki, 1998). Among the regulatory elements of the serotonergic system, a key role is played by the 5-HT transporter (5-HTT or SERT), which terminates the action of 5-HT by reuptaking it into the presynaptic terminals (Blakely et al., 1994).

The human serotonin transporter is encoded by a single gene, *SLC6A4* (solute carrier family 6 member 4), whose transcriptional activity is modulated by genetic variants, including a functional polymorphism in the promoter region *(5-HTTLPR*), characterized by a long (L) and a short (S) variant. Specifically, functional studies of the activity of the *SLC6A4* promoter in transfected cell lines and lymphoblasts confirmed that the L allele of the functional polymorphism *5-HTTLPR* is associated with higher levels of transcriptional activity and higher rate of serotonin uptake with respect to the S variant (Murphy et al., 2008). However, the functionality of the *5-HTTLPR* in human brains has not been consistently replicated, even though several factors like stressors and environmental adversity could mask the functional effect of the *5-HTTLPR*. Interestingly, the L allele could be a protective factor for major depression, whereas the low-functioning allele is associated with increased disease susceptibility upon exposure to adverse life events (Caspi et al., 2003; Uher and McGuffin, 2008).

On this basis, animals with SERT gene deletion represent an important tool to investigate the role of the transporter in mood disorders and to identify the molecular mechanisms contributing to the association between depression and life-long SERT dysfunction (for reviews, see Canli and Lesch, 2007; Hariri and Holmes, 2006; Murphy and Lesch, 2008). SERT knockout mice show depression- and anxiety-related behavior (Holmes et al., 2003; Lira et al., 2003) supporting the possibility that altered function of SERT may indeed be associated with increased risk for mood disorders (Gardier, 2009). We have recently developed SERT knockout rats (Homberg et al., 2007; Smits et al., 2006) that show a behavioral phenotype remarkably similar to SERT knockout mice (Kalueff et al., 2009).

In particular, SERT KO rats display negative emotionality, including anxiety and depressive behavior. This is supported by the fact that SERT KO rats spend less time in the open arm of the elevated plus maze, have increased latency to start eating in the novelty-suppressed

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feeding paradigm, have increased immobility in the forced swim test and show reduced sucrose consumption, a measure of anhedonia (Olivier et al., 2008).

During the last decade, the idea of major depression as a condition characterized by impaired neuronal plasticity has been developed, suggesting that psychopathology may be associated with reduced expression and function of proteins that are important for cellular resilience, leading to enhanced vulnerability under "challenging situations" (McClung and Nestler, 2008; Pittenger and Duman, 2008). Hence, it is feasible to hypothesize that conditions, such as those determined by a genetic impairment of SERT, that are associated with increased risk for depression, can lead to an impairment of the mechanisms that are important for neuronal plasticity and that may contribute to disease vulnerability. On this basis, we investigated if genetic abnormalities of the serotonin transporter can lead to alterations in the expression of brain-derived neurotrophic factor (BDNF), a neurotrophin that, because of the activity-dependent regulation of its expression and secretion (Bramham and Messaoudi, 2005), has emerged as crucial mediator of neuronal plasticity. BDNF is involved in the etiopathology of mood disorders as well as in the mechanism of action of antidepressant drugs (Berton and Nestler, 2006; Calabrese et al., 2007; Groves, 2007; Martinowich et al., 2007; Molteni et al., 2009), and a close link between serotonin and the neurotrophin has been suggested (Martinowich and Lu, 2008; Mattson et al., 2004).

In this study, we investigated the expression of BDNF in different brain regions from SERT mutant rats (+/− and −/−). Moreover, given the complex genomic structure of the neurotrophin (Aid et al., 2007), we have investigated the influence of SERT deletion on its different transcripts and evaluated the involvement of epigenetic mechanisms in their modulation. Finally, we have examined if alterations on BDNF expression were also present in human leukocytes from healthy individuals with different *5-HTTLPR* genotypes.

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) and Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

Serotonin transporter knockout rats (Slc6a4^{1Hubr}) were generated by ENU-induced mutagenesis (Smits et al., 2006). All subjects have been bred and reared in the Central Animal Laboratory of the University of Nijmegen. Experimental animals were derived from crossing heterozygous SERT knockout 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, adult female SERT+/+(WT), SERT+/− and SERT−/− were used. Animals were supplied with food and water ad libitum and were kept on a 12:12-h dark–light cycle (lights on at 6:00 in the morning).

Our analyses were carried out in hippocampus and prefrontal cortex, which were rapidly dissected after rat sacrifice. Specifically, hippocampus was dissected from the whole brain whereas prefrontal cortex (defined as Cg1, Cg3 and IL subregions corresponding to the plates 6–10 according to the atlas of Paxinos and Watson, 1996) was dissected from 2-mm-thick slices. The brain specimens were frozen on dry ice and stored at −80 °C for later analysis.

To mimic the effect of SERT blockade at adulthood, separate cohorts of adult WT and SERT−/− rats were randomly assigned to receive daily injections (between 9:00 and 10:00 a.m.) of the selective-serotonin reuptake inhibitor fluoxetine (10 mg/kg i.p.) or saline for 3 weeks ($n=8$ each experimental group). Twenty-four hours after the last injection the animals were sacrificed, the prefrontal cortex was dissected and used to measure total BDNF mRNA levels.

All animals handling and experimental procedures were performed in accordance with the EC (EEC Council Directive 86/609 1987), the Italian legislation on animal experimentation (Decreto Legislativo 116/92) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

RNA preparation and quantification of BDNF mRNA expression by real-time quantitative PCR

Total RNA was isolated from tissue from different rat brain structures by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories S.r.l. Italia) 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 as previously reported (Molteni et al., 2009). Briefly, a 2-μg aliquot of each sample was treated with DNase to avoid DNA contamination, then reverse transcribed using a High-Capacity cDNA Archive commercial kit (Applera). The real-time PCR reaction was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystem Italia, Monza, MI, Italy) with the TaqMan Gene expression Master Mix (Applied Biosystem Italia, Monza, MI, Italy) and the following TaqMan Gene Expression Assay purchased from Applied Biosystem:

Total BDNF: ID Rn02531967_s1; BDNF transcript I: ID Rn01484924_m1; BDNF transcript IIa: Rn00560868_m1; BDNF transcript IIb: ID Rn01484926_m1; BDNF transcript IIc: ID Rn01484925_m1; 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.

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 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).

Preparation of protein extract and Western blot analysis

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, Monza, Italy) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 2000×*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 (2000 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 resuspended in Solution A. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories S.r.l. Italia), 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 polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories S.r.l. Italia). The blots were blocked with 10% nonfat dry milk and then incubated with the primary 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 for BDNF, Cell Signaling) and immunocomplexes were visualized by chemiluminescence utilizing the ECL Western blot kit (Amersham Life Science, Milan, Italy) according to the manufacturer's instructions. Results were standardized to β-actin as control protein, which was detected by evaluating the band density at 43 kDa after probing the membranes with a polyclonal antibody (1:10,000 dilution, Sigma) followed by a 1:10,000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma).

Epigenetic studies

Methylated DNA immunoprecipitation

Prefrontal cortex from wild-type and SERT−/− female rats was collected and used to investigate methylation status of promoters for exons IV and VI via EpiQuik Tissue Methylated DNA immunoprecipitation Kit (Epigentek, Brooklyn, NY, USA) according to the manufacturer's instruction. Briefly, tissue was lysed and genomic DNA was shearing by sonication. Samples were incubated with an antibody against 5-methylcitosine or with an equivalent amount of normal IgG (anti-mouse). A portion of the sonicated DNA was left untreated to serve as input control. Immunoprecipitated DNA was subjected to quantitative real-time PCR using primers specific for 200-bp segments corresponding to CpG island sites within rat BDNF exon IV and exon VI (Table S4). The cumulative fluorescence for each amplicon was normalized to input amplification.

Chromatin immunoprecipitation assay

Prefrontal cortex from wild-type and SERT−/− female rats was collected and used for chromatin immunoprecipitation assay by EpiQuik Tissue Acetyl-Histone H3 ChiP Kit (Epigentek) according to the manufacturer's instruction. Briefly, tissue was disaggregated and incubated in 1% formaldeide (cross-link). Chromatin was sheared by sonication. Samples were incubated with primary antibody (anti-acetyl H3) or with an equivalent amount of normal IgG (anti-mouse). A portion of the sonicated DNA was left untreated to serve as input control. Immunoprecipitated DNA was subjected to quantitative real-time PCR using primers specific to the rat BDNF exon IV and exon VI (Table S4). The cumulative fluorescence for each amplicon was normalized to input amplification.

Quantification of Gadd45b mRNA expression by real-time quantitative PCR

Gadd45b gene expression was measured 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 the following TaqMan Gene Expression Assay purchased from Applied Biosystem: forward primer: GCTGCGACAATGACATTGACATC and reverse primer: CTCGTTTGTGCCTAGAGTCTCT.

Analyses of BDNF mRNA levels in human leukocytes

Human subjects

A group of 50 healthy volunteers (34 females, 16 males; mean age = 48.09 ± 16.77 years, mean body max index = 24.29 ± 2.45 kg/m^2) with a negative anamnesis for any Axis I disorder (confirmed by the MINI interview), a negative family history for psychoses and mood disorders and without any regular treatment for a medical condition was enrolled for the study. The study was approved by the local ethic committee and all subjects enrolled gave their informed consent to the participation. Venous blood samples for DNA and RNA isolation were collected in the morning after an overnight fast (between 8:00 and 9:00 a.m.) in anticoagulant tubes (Chimica Omnia, Brescia, Italy) for DNA isolation and in PaxGene tubes (Qiagen S.p.A., Milan, Italy) for RNA isolation.

5-HTTLPR and rs25531 genotyping analyses

Genomic DNA was isolated from peripheral white blood cell samples by Puregene kit (Gentra System, Minneapolis, MN. USA) according to the manufacturer's protocol. Genotyping assay of the *5-HTTLPR* polymorphism was performed according to Gelernter et al. (1997). Moreover, a restriction digestion with *Msp*I enzyme (Fermentas International Inc., Burlington, Canada) was performed in samples where at least one L allele was identified in order to evaluate also the *rs25531* polymorphism (Nakamura et al., 2000; Wendland et al., 2006). Subjects were genotyped with biallelic (*5-HTTLPR* S and L alleles) and triallelic (S, L_A, L_G) classification.

BDNF mRNA analysis

After blood samples were withdrawn, PaxGene Tubes were stores at −80 °C until their processing. RNA isolation was performed using the PAXGene Blood RNA Kit (Qiagen S.p.A., Milan, Italy) according to the manufacturer's protocols. The RNA quantity was assessed by evaluation of the $A_{260/280}$ and $A_{260/230}$ ratios using a Nanodrop spectrometer (NanoDrop Technologies, Wilmington, DE, USA) and the RNA quality was determined using an Agilent Bioanalyzer (Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Italy). Two micrograms of total RNA was used for cDNA synthesis using random hexamer primers (Invitrogen, Carlsbad, CA, USA) and the Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocols. The expression levels of the total BDNF mRNA and of the housekeeping genes GAPDH, β-actin, β2 microglobulin (B2 M), cytochrome c-1 (Cyc1) and ATP synthase, H^+ transporting mitochondrial F1 complex subunit (Atp5b) have been analyzed by real-time PCR using a StepOne Real-Time System (Applied Biosystem) with TaqMan Gene Expression Master Mix (Applera) and TaqMan Gene Expression Assays (BDNF forward primer: GCCCAAGGCAGGTTCAAGA, BDNF reverse primer: CCTCATCCAACAGCTCTTCTATCAC, BDNF probe sequence: TTGGCTGACACTTTCG) as previously described. BDNF mRNA levels have been normalized on the geometric mean of the five housekeeping genes.

Statistical analyses

The effect of the genotype on BDNF mRNA and protein levels was analyzed with one-way analysis of variance (ANOVA), whereas the effect of fluoxetine chronic treatment on WT and SERT−/− rats was evaluated by two-way ANOVA with genotype (WT vs. SERT−/− rats) and treatment (saline vs. fluoxetine) as independent factors and BDNF levels as dependent variable. When appropriate, further differences were analyzed by single contrast post hoc test (SCPHT). Conversely the results of epigenetic studies were evaluated by Student *t* test. Significance for all tests was assumed for $p<0.05$. Data are presented as means ± standard error (SEM). For graphic clarity, results of animal studies were presented as mean percent of wild-type rats whereas the relative mRNA levels of BDNF in human leukocytes were expressed in arbitrary units (AU) as a relative expression ratio of the target gene in comparison to a reference gene.

Results

Rodent studies

Since the SERT mutant rat is a model of depression (Olivier et al., 2008), we investigated possible changes in the expression of the

neurotrophin BDNF, a key marker of neuronal plasticity (Berton and Nestler, 2006; Groves, 2007; Martinowich et al., 2007).

As a first step, we measured total BDNF (exon IX) mRNA levels in different brain regions obtained from SERT+/− and SERT−/− mutant rats as compared to wild-type (WT) animals. A significant genotype effect was found in both hippocampus ($F_{2,41}=7.690$, $p<0.05$) and prefrontal cortex ($F_{2,38}=45.081$, $p<0.001$). Specifically, as shown in Fig. 1, total BDNF mRNA levels were significantly reduced in the hippocampus of SERT KO rats (−14%, $p<0.05$; Fig. 1A) as well as in the prefrontal cortex of SERT+/− (−16%, $p<0.001$; Fig. 1B) and SERT−/− (−34%, $p<0.001$; Fig. 1B) rats. It is known that the transcription of the rat BDNF gene is controlled by separate promoters, each driving the expression of different 5^{I} exons spliced onto a common 3^{I} coding exon (IX) (Aid et al., 2007). On this basis, to establish which BDNF exon may contribute to the reduction of total BDNF, we have analyzed the expression of transcripts containing exons IV and VI since, up to date, their promoters have been well characterized (Tabuchi et al., 2000; Takeuchi et al., 2002) and, as shown in Fig. S1, they are prominently expressed in the brain regions considered.

We found a significant genotype effect on BDNF exon IV and exon VI mRNAs in hippocampus ($F_{2,41}=3.513$, $p<0.05$ and $F_{2,41}=3.282$, $p<0.05$, respectively) as well as in prefrontal cortex ($F_{2,38}=10.655$, $p<0.001$ and $F_{2,38}=13.161$, $p<0.001$, respectively). Specifically, similarly to what observed for total BDNF, the mRNA levels for isoform IV were significantly reduced within the hippocampus of SERT KO rats (−16%, $p<0.05$, Fig. 2A) and in the prefrontal cortex of SERT+/− (−18%, $p<0.05$, Fig. 2B) and SERT−/− (−33%, $p<0.001$, Fig. 2B). Conversely, the expression of transcript VI was unchanged in heterozygous animals but significantly down-regulated in the hippocampus (−11%, $p<0.05$, Fig. 2A) and in the prefrontal cortex (−30%, $p<0.001$, Fig. 2B) of SERT KO rats.

We have also investigated the influence of SERT deletion on the other BDNF transcripts expressed in the brain regions examined and, as shown in table S2, a significant reduction of BDNF exon IIb, exon III and exon IXa mRNAs ranging from −8% to −16% was present in the hippocampus of SERT KO rats. Conversely, the expression of all transcripts was significantly reduced in the prefrontal cortex of SERT KO rats (from −19% to −34%, Table S3), whereas the mRNA levels for some isoforms (I, IIa and IXa) were also down-regulated in SERT+/− rats (from −15% to −30%, Table S3).

In order to evaluate if the reduction of BDNF gene expression observed in the hippocampus and prefrontal cortex of SERT mutant rats was paralleled by similar effects at translational level, we performed Western blot analysis of the crude synaptosomal fraction from these animals. We found a significant genotype effect on BDNF protein in hippocampus ($F_{2,33}=8.114$, $p<0.001$) and prefrontal

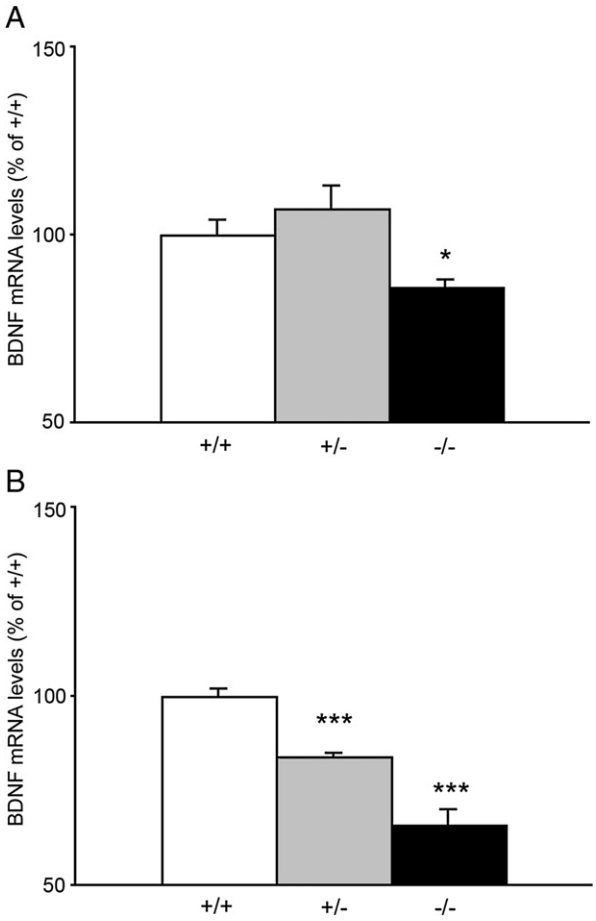

Fig. 1. Deletion of *SERT* gene reduces BDNF gene expression in hippocampus and prefrontal cortex. Total BDNF (exon IX) mRNA levels were measured in the hippocampus (A) and prefrontal cortex (B) of SERT heterozygous (+/−) and homozygous (−/−) rats, as compared to their wild-type (+/+) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean ± SEM from 7 to 14 independent determinations. *$p<0.05$, ***$p<0.001$ vs. +/+ rats (one-way ANOVA with Fischer's PLSD).

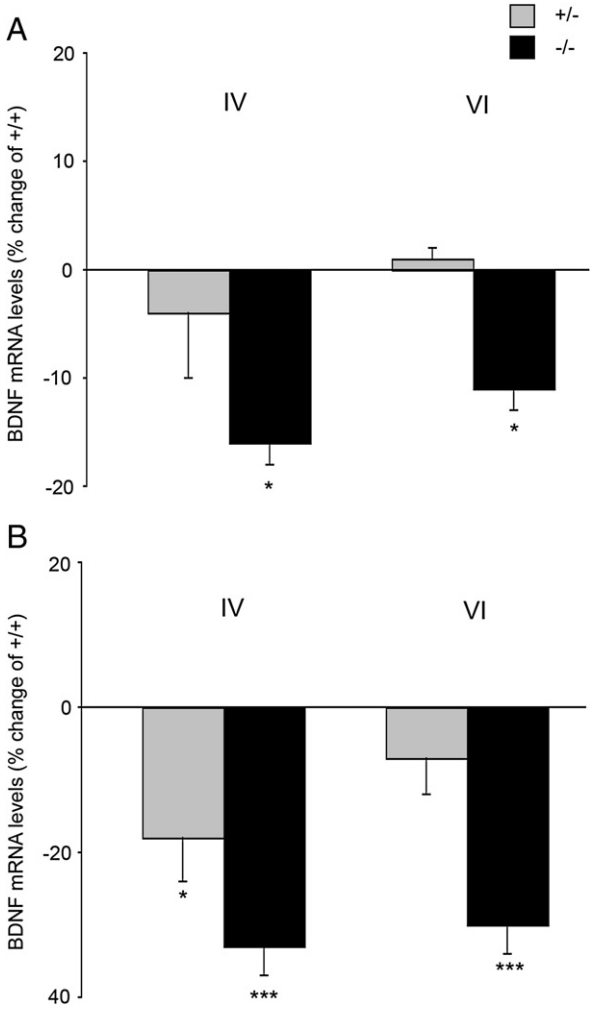

Fig. 2. Deletion of *SERT* gene reduces the mRNA levels of BDNF IV and VI transcripts in rat hippocampus and prefrontal cortex. The mRNA levels for BDNF exons IV and VI were measured in the hippocampus (A) and prefrontal cortex (B) of SERT heterozygous (+/−) and homozygous (−/−) rats, as compared to their wild-type (+/+) counterparts. The data, expressed as a percentage difference of +/+ animals (set at 0), are the mean ± SEM from 7 to 14 independent determinations. *$p<0.05$, ***$p<0.001$ vs. +/+ rats (one-way ANOVA with Fischer's PLSD).

cortex ($F_{2,29}=6.661$, $p<0.01$). Specifically, the levels for mature BDNF were reduced in the hippocampus of SERT KO (Fig. 3A: −19%, $p<0.01$) and SERT+/− (−21%, $p<0.01$) rats, whereas they were unchanged at cortical level (Fig. 3B). Conversely, the levels of the precursor form of the neurotrophin (proBDNF) were significantly reduced in the prefrontal cortex of SERT KO (Fig. 3B: −31%, $p<0.01$) and, to less extent, in SERT+/− rats (−19%, $p<0.05$) without significant changes in the hippocampus (Fig. 3A).

The reduction of BDNF expression observed in SERT mutant rats might be due to the lack of the transporter during the entire life, encompassing development as well as adulthood. To discriminate between these possibilities, we have used a pharmacological strategy and we investigated the neurotrophin expression in the prefrontal cortex after subchronic (21 days) treatment of adult +/+ and −/− rats with fluoxetine, a selective-serotonin reuptake inhibitor. Chronic administration of fluoxetine produced a significant effect on BDNF mRNA levels ($F_{1,23}=11.964$, $p<0.01$; Fig. 1A) with a significant genotype x treatment interaction ($F_{3,23}=4.029$, $p<0.05$). Indeed, as shown in Fig. 4, the antidepressant significantly increased total BDNF mRNA levels in WT rats (+32%, $p<0.01$). Conversely, the reduced neurotrophin expression in vehicle-injected SERT KO rats (−40% vs. vehicle-injected WT rats, $p<0.001$) was not significantly affected by fluoxetine administration ($p>0.05$). These results suggest that prolonged blockade of SERT at adulthood is not sufficient to determine the reduction of BDNF expression observed in SERT KO rats. Moreover, the lack of fluoxetine effect in SERT mutant rats confirms that these animals do not express a functional serotonin transporter.

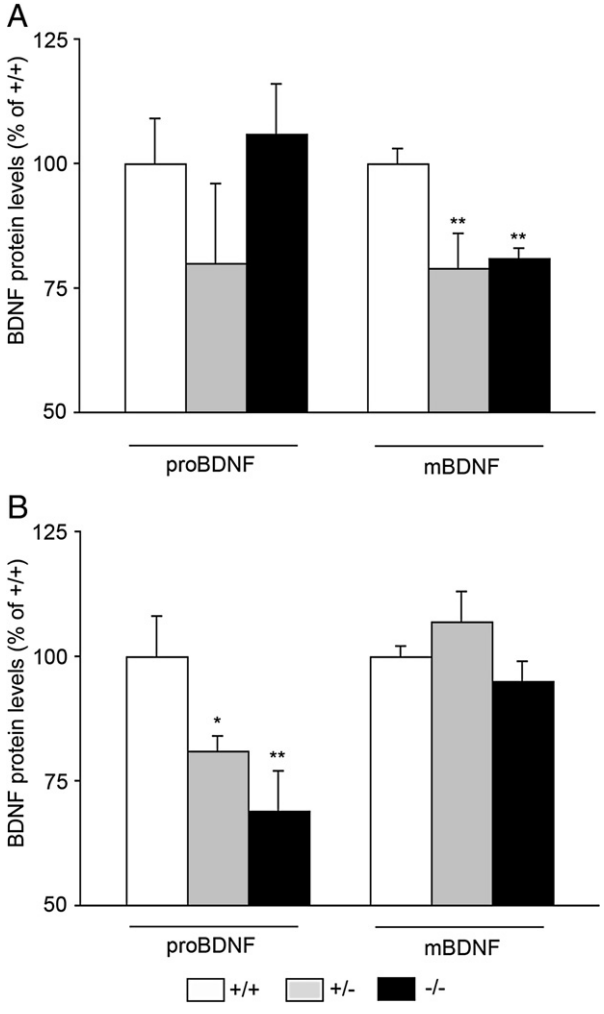

Fig. 3. Altered levels of BDNF protein in the hippocampus and in the prefrontal cortex of SERT mutant rats. The precursor of the neurotrophin (proBDNF) and its mature form (mBDNF) were measured in the crude synaptosomal fraction of hippocampus (A) and prefrontal cortex (B) from SERT heterozygous (+/−) and homozygous (−/−) rats, as compared to their wild-type (+/+) counterparts. Results were standardized to β-actin as control protein. Data, expressed as a percentage of +/+ animals (set at 100%), are the mean ± SEM from 9 to 14 independent determinations. *$p<0.05$, **$p<0.01$ vs. +/+ rats (one-way ANOVA with Fischer's PLSD).

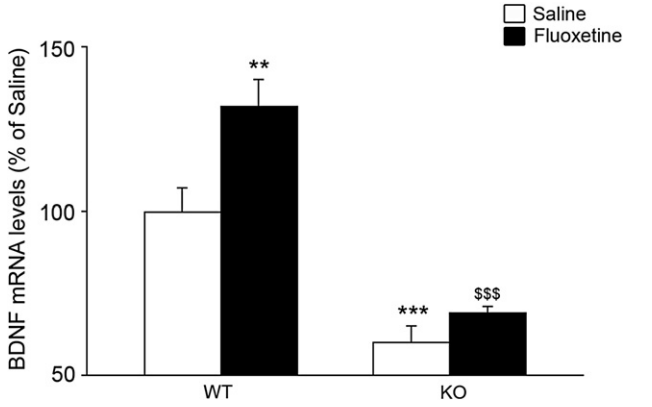

Fig. 4. Chronic fluoxetine treatment increases total BDNF mRNA levels in prefrontal cortex of adult wild-type rats but not in SERT KO rats. Adult wild-type and SERT−/− female Wistar rats received daily injection of the selective-serotonin reuptake inhibitor fluoxetine (10 mg/kg i.p.) or saline for 3 weeks ($n=8$ each experimental group). Twenty-four hours after the last injection, the animal were sacrificed, the prefrontal cortex was dissected and used to measure total BDNF gene expression. The data, expressed as a percentage of WT/Saline (set at 100%), are the mean ± SEM from at least 6 independent determinations. **$p<0.01$ and ***$p<0.001$ vs. WT/Saline; *$$p<0.001$ vs. WT/Fluoxetine (two-way ANOVA with SCPHT).

Since impairment of SERT in adulthood does not appear to be responsible for BDNF down-regulation in the prefrontal cortex, it is feasible to hypothesize that the reduction of the neurotrophin may represent a long-lasting consequence of transporter dysfunction during development, in agreement with what has been shown for the behavioral phenotype of SERT KO mice (Ansorge et al., 2004). Hence, we hypothesized that such persistent changes in neurotrophin expression may occur through epigenetic mechanisms that affect the function of specific BDNF promoters.

On this basis, we investigated if the reduced levels of BDNF exon IV and exon VI mRNAs in the prefrontal cortex of SERT KO rats were associated with altered DNA methylation or histone acetylation of their corresponding promoters. We choose these specific transcripts since they are among the most affected in SERT mutant rats and because their expression can be regulated through epigenetic mechanisms (Roth et al., 2009; Tsankova et al., 2006). As shown in Fig. 5A, we found that the down-regulation of BDNF exon VI mRNA in prefrontal cortex of SERT KO rats correlated with a significant threefold increase of the DNA methylation at CpG sites of its promoter (+2,7 fold change vs. WT rats, $p<0.001$), whereas no changes were observed on the DNA methylation state of the promoter for exon IV. Consistent with the increased methylation at *Bdnf* P6, the mRNA levels of Growth arrest and DNA-damage-inducible, beta (Gadd45b), which is involved in DNA demethylation of several gene promoters including BDNF (Ma et al., 2009), were significantly reduced in SERT KO rats (−20%, $p<0.001$; Fig. 6).

On the other hand, the significant reduction of BDNF exon IV mRNA in the prefrontal cortex of SERT KO rats correlated with a significant decrease of H3 acetylation at its promoter ($p<0.001$, Fig. 5B). Conversely, no significant change in H3 acetylation at the promoter for exon VI was found in the prefrontal cortex of SERT KO rats (Fig. 5B).

Human studies

It is known that the human serotonin transporter gene contains a functional polymorphism (*5-HTTLPR*) resulting in a long (L)/short(S)

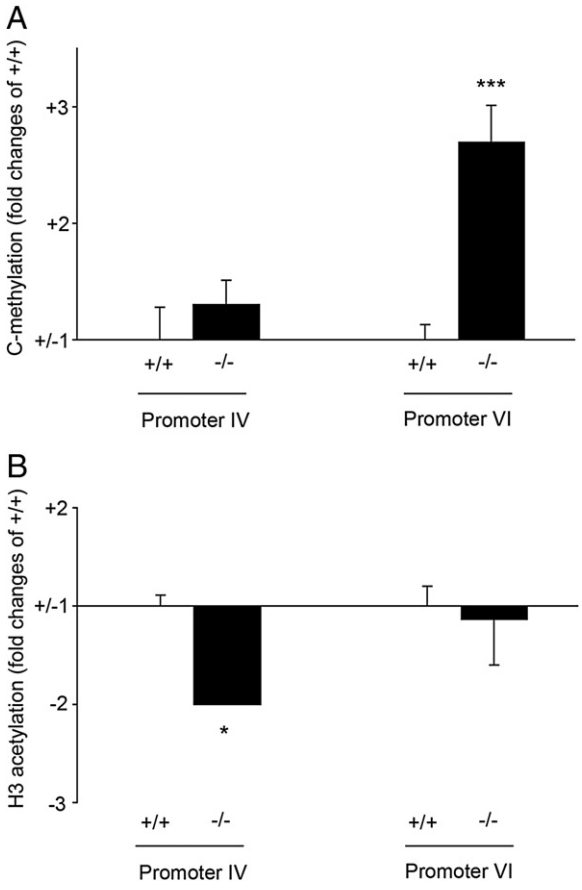

Fig. 5. Epigenetic changes at BDNF promoters occur in the prefrontal cortex of SERT KO rats. (A) Levels of C-methylation at the promoter IV and promoter VI of *Bdnf* in the prefrontal cortex of SERT homozygous (−/−) rats, as compared to their wild-type (+/+) counterparts. (B) Levels of histone H3 acetylation at the promoter IV and promoter VI of *Bdnf* in the prefrontal cortex of SERT homozygous (−/−) rats, as compared to their wild-type (+/+) counterparts. The data, expressed as fold changes of +/+ animals (set at ±1), are the mean ± SEM from at least 4 independent determinations. *$p<0.05$, ***$p<0.001$ vs. +/+ rats (Student's *t* test).

variant in the promoter region upstream of the transcription starting site (Lesch et al., 1996). The S allele leads to reduced transcription and activity of the transporter and is associated with increased susceptibility to mood disorders and unfavorable response to SSRI treatment (Caspi et al., 2003; Serretti et al., 2007). In addition, it has been shown that the L allele can be further subtyped into L_A and L_G alleles, for the presence of the A/G single nucleotide polymorphism (*rs25531*) within the *5-HTTLPR* (Nakamura et al., 2000; Wendland et al., 2006). The L_A variant has been associated with higher SERT mRNA with respect to L_G, whereas the expression levels of the gene containing S or L_G allele are equivalent (Hu et al., 2006).

In order to establish if changes in BDNF expression found in SERT mutant rats can also be observed in humans in association with allelic variants of *SLC6A4* gene, we have investigated the expression levels of the neurotrophin in leukocytes obtained from 50 healthy volunteers genotyped for the *5-HTTLPR/rs25531* polymorphism. Regarding the 5-HTTLPR, the genotype distribution in our study population was 48% for LL, 38% for LS and 14% for SS, and it had no deviation from Hardy–Weinberg equilibrium ($\chi^2=0.99$, df = 1, $p=0.31$). The genotype distribution for the triallelic *5-HTTLPR/rs25531* polymorphism was 40% (20/50) for L_AL_A, 34% (17/50) for L_AS, 8% (4/50) for L_AL_G, 0% (0/50) for L_GL_G, 4% (2/50) for L_GS and 14% (7/50) for SS and had no deviation from Hardy–Weinberg equilibrium ($\chi^2=1.25$, df = 3, $p=0.74$, HWE program, John Ott version 1.10).

We found that BDNF mRNA levels were affected both by *5-HTTLPR* and by *5-HTTLPR/rs25531* ($F_{2,50}=21.541$, $p<0.001$, $F_{4,50}=43.503$, $p<0.001$, respectively). In particular, according to a biallelic classification, we found that BDNF mRNA levels were reduced in leukocytes of carriers of the S allele (−22% in LS, $p<0.001$ and −38% in SS, $p<0.001$) as compared to noncarriers (LL). According to a triallelic classification, as shown in Fig. 7, BDNF expression levels were significantly reduced in individuals with S/S (−42%, $p<0.001$), L_G/S (−40%, $p<0.001$), L_A/L_G (−37%, $p<0.001$) and L_A/S (−21%, $p<0.001$) genotype when compared to L_A/L_A subjects. BDNF mRNA levels in leukocytes were not significantly affected by gender or age ($p=0.523$ and $p=0.129$, respectively).

Discussion

Animals with a target deletion of the serotonin transporter represent a model of anxiety and depressive behavior (Olivier et al., 2008) and can be used to investigate the mechanisms that may underlie such phenotype. In line with the concept that mood disorders are associated with reduced neuronal plasticity and resilience (McClung and Nestler, 2008; Pittenger and Duman, 2008), we demonstrate that, under basal condition, SERT KO rats have decreased expression of the neurotrophin BDNF, a major player in neuronal plasticity.

We found reduced BDNF expression in both hippocampus and prefrontal cortex, two regions that are primarily affected in depressed subjects. The analysis of BDNF different transcripts suggests that the effects observed in the hippocampus are primarily due to a reduction

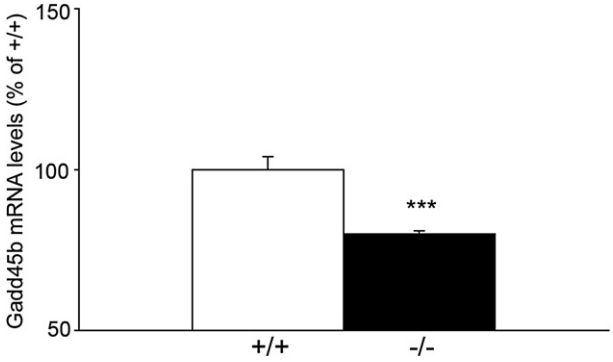

Fig. 6. Reduced Gadd45b gene expression in prefrontal cortex of SERT KO rats. The mRNA levels of Growth arrest and DNA-damage-inducible beta (Gadd45b) were measured by real-time RT-PCR in the prefrontal cortex of SERT homozygous (−/−) rats, as compared to their wild-type (+/+) counterparts. The data, expressed as a percentage of WT rats (set at 100%), are the mean ± SEM from at least 6 independent determinations. ***$p<0.001$ vs. +/+ rats (Student *t* test).

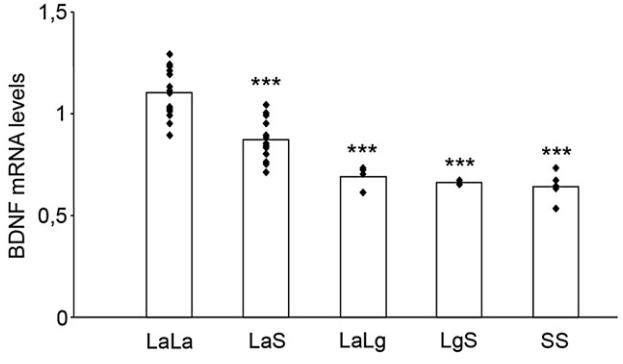

Fig. 7. Total BDNF mRNA levels in human leukocytes are modulated by different *5-HTTLPR/rs25531* genotypes. According to a triallelic classification, human subjects with genotypes L_AL_A ($n=20$), L_AS ($n=17$), L_AL_G, ($n=4$), L_GS ($n=2$) and SS ($n=7$) were identified. BDNF gene expression was measured by real-time RT-PCR and expressed in arbitrary units (AU) as a relative expression ratio between BDNF and the mean of five housekeeping genes (see Materials and methods). ***$p<0.001$ vs. L_AL_A genotype (one-way ANOVA with Fischer's PLSD).

of some isoforms, whereas a more generalized decrease was found in the prefrontal cortex of SERT KO rats. Interestingly, within the prefrontal cortex a partial deletion of the SERT gene, as occurring in SERT+/− rats, is sufficient to determine a significant reduction of BDNF expression, affecting the mRNA levels for exons I, IV and IXa. These results suggest that the expression of BDNF in prefrontal cortex may be particularly "sensitive" to alterations in the function of SERT. The more generalized effect of SERT deletion within the prefrontal cortex may – at least in part – be related to structural alterations in this brain region. Neuroimaging studies have indeed demonstrated that short allele carriers, who have lower SERT expression, showed reduced gray matter volume in limbic regions (Pezawas et al., 2008).

Limited information exists on the functional role played by each BDNF transcript. It has been postulated that some of them undergo dendritic targeting and therefore may contribute to different pools of BDNF (Chiaruttini et al., 2008; Tongiorgi et al., 2006), whereas there is no evidence that different isoforms can be selectively expressed in specific subtypes of neuronal cells. However, since different intracellular mechanisms may participate to the transcription of specific BDNF exons (Aid et al., 2007; Cohen and Greenberg, 2008), the regulation of their expression may represent a converging point between different signaling mechanisms. Hence, changes in the expression of selected BDNF transcripts, as occurring in SERT mutant rats, may reflect altered function of neurotransmitter systems or circuits involved in the control of BDNF production.

The down-regulation of BDNF mRNA levels is paralleled by a similar reduction of mature BDNF protein levels in the crude synaptosomal fraction of hippocampus, whereas in the prefrontal cortex only the levels of proBDNF are reduced, with a profile that resembles the changes observed in BDNF mRNA levels. These data suggest that despite similar changes at mRNA levels, the regulation of BDNF protein in the hippocampus and the prefrontal cortex can be different. Although further studies are necessary to investigate the anatomical-dependent SERT influence on BDNF protein levels, the alterations found in the synaptic compartment may be relevant for the well-established synaptic function of BDNF, including the modulation of neurotransmitter release and the activation of its high affinity receptor (Bramham and Messaoudi, 2005; Tyler and Pozzo-Miller, 2001).

Deletion of SERT gene in mice (Holmes et al., 2003; Lira et al., 2003) and rats (Olivier et al., 2008) produces anxiety- and depression-related behaviors, an effect that may appear paradoxical given that chronic administration of SSRI antidepressant, which blocks SERT function, results in antidepressant effects. Indeed the anxious and depressed phenotype of SERT KO mice may represent the long-lasting consequence of impaired SERT function during early development (Ansorge et al., 2008; Ansorge et al., 2004; Vogel et al., 1990). As expected, we show that reduced expression of BDNF in SERT KO rats is not due to adult impairment of the transporter. In fact chronic treatment of adult wild-type rats with the SSRI fluoxetine increased BDNF gene expression rather than reduced it. These data are in line with several observations showing that repeated treatment with antidepressant drugs up-regulates BDNF expression in different brain structures, which may normalize defective neuronal plasticity in depressed subjects (Calabrese et al., 2007; Castren et al., 2007; Duman and Monteggia, 2006; Molteni et al., 2009). SERT blockade from postnatal days 4 to 21 (as produced by fluoxetine treatment) may not be sufficient to determine a long-lasting reduction of BDNF expression, although a transient decrease of the neurotrophin can be observed during the second week of postnatal life (Karpova et al., 2009). Hence, the reduction of BDNF levels in SERT KO rats found in our study may not be due to early postnatal impairment of the transporter but may encompass altered SERT function in utero as well as during adolescence. It must be taken into consideration that the effects reported by Karpova et al. (2009) were observed in male mice, which may be somewhat different from SERT female mutant rats used in the present study. Indeed previous studies failed to show significant changes in the levels of BDNF protein in the hippocampus and in the frontal cortex from male SERT knockout mice (Carola et al., 2008; Szapacs et al., 2004), suggesting that species and gender specificity may also be taken into consideration.

In line with the possibility that reduced BDNF expression in adult SERT mutant rats may represent a long-lasting effect of developmental impairment of the transporter, we demonstrate that epigenetic mechanisms may play a pivotal role. By using methylation-specific real-time PCR we found an increased DNA methylation in the promoter region of exon VI, which can correlate with the repression of its transcription in the prefrontal cortex of SERT KO rats. Such effect may, at least in part, be related to the down-regulation of Gadd45b observed in SERT KO rats (Ma et al., 2009). Conversely, the down-regulation of BDNF exon IV mRNA levels is associated with reduced acetylation of histone H3, a mechanism driving transcription activation. These data are in good agreement with recent findings demonstrating that BDNF gene transcription is under epigenetic control and that stable changes in the expression of specific BDNF transcripts are associated with chromatin remodeling at the corresponding *Bdnf* gene promoters (Tsankova et al., 2006). In particular, such mechanisms may be critical for the phenotypical manifestation observed after exposure to adverse life events early in development. We have previously demonstrated that exposure to stressors during gestation or early postnatal life produces a persistent- and anatomical-selective decrease of BDNF expression (Fumagalli et al., 2004; Roceri et al., 2004). Recent work by Roth and coworkers (2009) has shown that maltreatment of infant rats produce a life-long decrease of BDNF gene expression in prefrontal cortex, which correlates with enhanced methylation at BDNF promoters. Our results provide support to the notion that epigenetic mechanisms may be responsible for persistent changes in the expression of the neurotrophin BDNF. Moreover, we suggest that such epigenetic mechanisms may not only contribute to long-lasting changes in gene transcription following adverse life events but may provide a link between a vulnerable genotype for depression and a major player for neuronal plasticity, which represents an important component for mood disorders (McClung and Nestler, 2008; Mill and Petronis, 2007; Pittenger and Duman, 2008).

Interestingly, we demonstrate for the first time that BDNF expression is significantly reduced also in peripheral cells of control individuals carrying the S allele for the 5-HTTLPR, which leads to lower expression of the transporter expression, suggesting that the changes observed in prefrontal cortex and hippocampus of rats with a genetic defects of SERT may also be present in humans.

A series of studies have shown that changes of BDNF levels in serum may parallel modifications of BDNF expression observed in the rodent brain. A recent meta-analysis has provided strong evidence that serum BDNF levels are reduced in patients suffering from major depression and that neurotrophin levels are elevated following antidepressant treatment, thus resembling the changes observed in animal models (Sen et al., 2008). Although platelets represent the major source of peripheral BDNF, leukocytes can also produce and secrete the neurotrophin in the blood stream. Interestingly, BDNF gene expression is reduced in the leukocytes of depressed patients and correlates with their serum levels (Cattaneo et al., 2009), supporting the usefulness of leukocytes as a peripheral model to identify and investigate biomarkers for depression. Our data suggest that the mechanisms that contribute to the regulation of BDNF in SERT mutant rats may also be operative in human leukocytes and that the analysis of BDNF expression in these cells may be useful to establish a potential correlation with neurotrophin changes in the brain.

An interaction between serotonin and BDNF has been demonstrated at several levels (Martinowich and Lu, 2008). On one end defects in BDNF can alter serotonergic function (Guiard et al., 2008), while the present results suggest that defects in the function of SERT can regulate the expression of the neurotrophin. On the other end,

there might be a reciprocal modulation between SERT and BDNF genes. Loss of BDNF appears to exacerbate neurochemical and behavioral abnormalities observed in SERT mutant mice (Ren-Patterson et al., 2005). Genetic epistasis between the two systems has also been described, although the outcome of such interaction is complex. Depression susceptibility in S/S 5-HTT carriers may be exacerbated by the presence of Met BDNF gene variant, which is associated with reduced sorting and release of the neurotrophin (Kaufman et al., 2006). On the contrary, structural neuroimaging reveals the Met BDNF allele has a protective effect on the impact of *5-HTTLPR* on amygdala-anterior cingulated cortex circuitry, which is important in mood disorders (Pezawas et al., 2008).

In summary, since depression vulnerability can be associated with impaired neuronal plasticity (McClung and Nestler, 2008), we suggest that the reduced expression of BDNF in animals carrying a deletion of the SERT gene may contribute to their pathologic phenotype. When extrapolated to humans, reduced expression of the neurotrophin in carriers of the *5-HTTLPR* short allele may contribute to their increased susceptibility to develop a depressive symptomatology upon exposure to stressful life events.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2009.12.014.

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