Neuropsychopharmacology (2012) 37, 746–758

www.neuropsychopharmacology.org

Developmental Influence of the Serotonin Transporter on the Expression of *Npas4* and GABAergic Markers: Modulation by Antidepressant Treatment

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Alterations of the serotonergic system are involved in the pathophysiology of mood disorders and represent an important target for its pharmacological treatment. Genetic deletion of the serotonin transporter (SERT) in rodents leads to an anxious and depressive phenotype, and is associated with reduced neuronal plasticity as indicated by decreased brain-derived neurotrophic factor (*Bdnf*) expression levels. One of the transcription factors regulating *Bdnf* is the neuronal PAS domain protein 4 (*Npas4*), which regulates activity-dependent genes and neuroprotection, and has a critical role in the development of GABA synapses. On the basis of these premises, we investigated the expression of *Npas4* and GABAergic markers in the hippocampus and prefrontal cortex of homozygous ($SERT^{-/-}$) and heterozygous ($SERT^{+/-}$) knockout rats, and analyzed the effect of long-term duloxetine treatment on the expression of these targets. We found that *Npas4* expression was reduced in both the brain structures of adult $SERT^{+/-}$ and $SERT^{-/-}$ animals. This effect was already present in adolescent $SERT^{-/-}$, and could be mimicked by prenatal exposure to the antidepressant fluoxetine. Moreover, $SERT^{-/-}$ rats showed a strong impairment of the GABAergic system, as indicated by the reduction of several markers, including the vesicular transporter (*Vgat*), glutamic acid decarboxylase-67 (*Gad67*), the receptor subunit GABA A receptor, gamma 2 ($GABA_A$-$\gamma 2$), and calcium-binding proteins that label subgroups of the GABAergic neurons. Interestingly, chronic treatment with the antidepressant duloxetine was able to restore the physiological levels of *Npas4* and GABAergic markers in $SERT^{-/-}$ rats, although some differences in the modulation of GABAergic genes exist between hippocampus and prefrontal cortex. Our results demonstrate that SERT knockout rats, an animal model of mood disorders, have reduced *Npas4* expression that correlates with decreased expression of *Bdnf* exon I and IV. These changes lead to an impairment of the GABAergic system that may contribute to the anxious and depressive phenotype associated with inherited SERT downregulation.

Neuropsychopharmacology (2012) **37,** 746–758; doi:10.1038/npp.2011.252; published online 19 October 2011

Keywords: depression; duloxetine; SERT; transcription factors; GABA; development

INTRODUCTION

It is widely accepted that alterations in the serotonergic system are involved in the pathophysiology and treatment of mood disorders (Neumeister *et al*, 2002). Indeed, serotonin (5-HT) transmission is implicated both in the onset of depression and in the mechanism of action of several antidepressant drugs (Jans *et al*, 2007). Moreover, findings from genetical and pharmacological studies indicate that serotonin signaling during early life is critically involved in the development of brain circuits that modulate adult emotional behavior (Ansorge *et al*, 2008; Ansorge *et al*, 2004).

Gene variants of the 5-HT system have been associated directly to depression and may enhance disease susceptibility, following interaction with stressful life events (Caspi *et al*, 2010; Caspi *et al*, 2003; Karg *et al*, 2011; Munafo *et al*, 2009). In this context, the 5-HT transporter (5-HTT in humans; serotonin transporter (SERT) in rodents) is particularly relevant, due to the presence of a human functional polymorphism within its promoter region that modulates the susceptibility to different neuropsychiatric disorders, and that may also affect antidepressant response

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Received 5 May 2011; revised 30 August 2011; accepted 19 September 2011

(Caspi *et al*, 2003; Huezo-Diaz *et al*, 2009; Serretti *et al*, 2007; Uher and McGuffin, 2008). As the SERT polymorphism is not present in rodents (Caspi *et al*, 2010), its role has been extensively investigated using animal models with a genetic deletion of the transporter, a manipulation that leads to an anxious and depressive phenotype (Homberg and Lesch, 2011; Kalueff *et al*, 2010; Murphy and Lesch, 2008; Olivier *et al*, 2008). Accordingly, we used target-directed mutagenesis to generate SERT-knockout rats (Smits *et al*, 2006), which present an impaired serotonergic system and are characterized by anxiety- and depression-like behavioral alterations (Homberg *et al*, 2007; Kalueff *et al*, 2010; Olivier *et al*, 2008). We have previously demonstrated that SERT knockout ($SERT^{-/-}$) rats have altered neuronal plasticity, as indicated by the reduction of activity-regulated cytoskeleton associated protein (*Arc*) and of brain-derived neurotrophic factor (*Bdnf*) expression levels in the hippocampus and prefrontal cortex (Molteni *et al*, 2009b; Molteni *et al*, 2010). Interestingly, long-term treatment with the antidepressant duloxetine was able to normalize the *Bdnf* expression in both the brain regions (Calabrese *et al*, 2010).

The *Bdnf* gene has a very complex structure that gives rise to multiple transcripts, which are regulated through the cooperation and interaction of different transcription factors (Aid *et al*, 2007; Greer and Greenberg, 2008; Pruunsild *et al*, 2011). Among them is the neuronal PAS domain protein 4 (*Npas4*), which specifically controls the activity-dependent *Bdnf* mRNA levels by acting on promoters I and IV. Indeed, the *Bdnf* expression is reduced by almost two-fold in cultures expressing *Npas4*-RNA interference, when compared with control cultures (Lin *et al*, 2008). *Npas4* belongs to the basic helix-loop-helix-PAS transcription factors family, which is involved in functional regulation of neurons (Ooe *et al*, 2009), in the adaptation of cells to external stimuli, such as environmental stress (Gu *et al*, 2000), and in neuroprotection (Ooe *et al*, 2009). *Npas4* is expressed predominantly in excitatory neurons and is selectively induced by Ca^{2+} influx. Recently, *Npas4* has been shown to control GABAergic synapse development through a program of activity-dependent gene development; in particular, *Npas4*-RNA interference reduced the density of GABA A receptor, gamma 2 ($GABA_A$-$\gamma 2$) and *GABA-β2/3* receptors, and of GABA-producing enzymes glutamic acid decarboxylase (*Gad*)*65* and *Gad67* (Lin *et al*, 2008).

Several clinical and preclinical studies support a central and causal role of the GABAergic system in the etiology of depressive disorders (Luscher *et al*, 2011; Sanacora *et al*, 1999). This led us to hypothesize that the transcription factor *Npas4* may be altered in animal models of depression, and may eventually link alterations of neuroplastic markers, such as *Bdnf*, with an impaired function of the GABAergic system. On this basis, we investigated *Npas4* expression in the hippocampus and prefrontal cortex of $SERT^{+/-}$ and $SERT^{-/-}$ rats, which model depression- and anxiety-related dysfunctions in humans, and we analyzed the expression of different GABAergic markers that may represent downstream targets of *Npas4* and *Bdnf* signaling (Lin *et al*, 2008; Sakata *et al*, 2010). Moreover, we investigated the ability of long-term treatment with the serotonin–norepinephrine reuptake inhibitor (SNRI) duloxetine to normalize molecular defects associated with genetic deletion of SERT.

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), Eurofins MWG-Operon (Ebersberg, Germany), Tebu-bio (Magenta, Italy), GE Healthcare Europe GmbH (Pero, Italy).

Animals And Pharmacological Treatments

Serotonin transporter-knockout rats. SERT-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 Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands. Experimental animals were derived from crossing heterozygous ($SERT^{+/-}$) knockout rats that were out crossed for eight generations. After weaning at the age of 21 days, ear cuts were taken for genotyping. Animals were supplied with food and water *ad libitum* and were kept on a 12 h : 12 h dark–light cycle (lights on at 0600 h).

For basal analysis, a cohort of $SERT^{+/+}$ and $SERT^{-/-}$ rats, and their wild-type controls ($SERT^{+/+}$), were killed around 1100 h at postnatal day (PND) 35, whereas another cohort of $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats were killed at adulthood ($\sim$PND 100).

Drug treatments.

Fluoxetine administration during gestation in wild-type rats: To establish the contribution of SERT during early life in the modulation of *Npas4*, pregnant Wistar rats (Harlan Laboratories, Horst, The Netherlands) were daily orally treated with methylcellulose or fluoxetine (12 mg/kg) from gestation day 11 until birth. Animals were weaned at PND 22, and they were killed at 3 months of age.

Fluoxetine administration at adulthood in wild-type rats: A group of Wistar rats were treated at adulthood with methylcellulose (by gavage) or fluoxetine (12 mg/kg by gavage) for 3 weeks and were killed 24 h after the last injection.

Duloxetine treatment in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats: Adult $SERT^{+/+}$ and $SERT^{-/-}$ rats were treated chronically (21 days) with saline (by gavage) or duloxetine (10 mg/kg by gavage) and killed 24 h after the last injection.

Brain regions of interest (hippocampus, prefrontal cortex) were rapidly dissected. Prefrontal cortex (defined as Cg1, Cg3, and IL sub-regions corresponding to the plates 6 to 10 according to the atlas of Paxinos and Watson (1996)) was 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 $-80\,^{\circ}C$ for further analysis. 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 by a single step of guanidinium isothiocyanate/phenol extraction, using PureZol RNA isolation reagent (Bio-Rad Laboratories s.r.l. Italia) according to the manufacturer's instructions, and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time PCR to assess *Npas4*, aryl hydrocarbon receptor nuclear translocator 2 (*Arnt2*), vesicular GABA transporter (*Vgat*), *Gad67*, *GABA$_A$-γ2*, parvalbumin (*Pvalb*), calbindin (*Calb1*) mRNA levels. An aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories) using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well formats in triplicates as multiplexed reactions with a normalizing internal control (36B4). Probe and primer sequences used (Table 1) were purchased from Eurofins MWG-Operon.

Thermal cycling was initiated with incubation at 50 °C for 10 min (RNA retrotranscription), and then at 95 °C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95 °C for 10 s to enable the melting process, and then for 30 s at 60 °C for the annealing and extension reactions. A comparative cycle threshold (Ct) method was used to calculate the relative target gene expression.

Statistical Analyses

All the analyses were carried out in individual animals (independent determinations). The effect of genotype and/or antidepressant treatment on mRNA levels was analyzed with a two-way analysis of variance (ANOVA) followed by single contrast *post-hoc* test (SCPHT), or with one-way ANOVA, followed by Fisher's protected least significant difference *post-hoc* test or Student's *t*-test. Pearson product moment correlations (*r*) between levels of *Npas4* mRNA and *Bdnf* exon I, *Bdnf* exon IV, and *GABA$_A$-γ2* mRNAs were performed to evaluate the correlation between the expression levels of all the genes in single animals. Significance for all tests was assumed for $p<0.05$. Data are presented as means ± SEM.

RESULTS

Disruption of SERT Affects *Npas4* mRNA Expression Levels

Npas4 is a brain-specific transcription factor with neuroprotective properties (Ooe *et al*, 2009) involved in the formation and/or the maintenance of GABAergic synapses on excitatory neurons (Lin *et al*, 2008). As clinical and preclinical evidence suggest that GABAergic deficits may be relevant for the etiology and manifestation of mood disorders (Luscher *et al*, 2011), we decided to investigate the expression of *Npas4* in the hippocampus of SERT$^{+/-}$ and SERT$^{-/-}$ rats, and their controls, SERT$^{+/+}$ rats. The SERT-knockout rat represents an animal model for anxiety and depression (Olivier *et al*, 2008), and thereby offers an important tool to investigate the mechanisms underlying the pathological condition in humans.

As shown in Figure 1, we found that the expression of the transcription factor *Npas4* was significantly reduced in the hippocampus and prefrontal cortex of adult SERT$^{-/-}$ (−44%, $p<0.001$; −42%, $p<0.001$, respectively), as well as of SERT$^{+/-}$ rats (−33%, $p<0.05$; −26%, $p<0.01$, respectively), when compared with SERT$^{+/+}$ rats (one-way ANOVA with Fisher's protected least significant difference test; Figures 1a–c). *Npas4* mRNA levels were already reduced in both the brain regions of SERT$^{-/-}$ rats at puberty (PND 35, −49%, $p<0.05$ in hippocampus; −51%, $p<0.01$ in prefrontal cortex; Student's *t*–test; Figures 1b–d), suggesting that the reduced expression of the transcription factor in SERT-knockout rats may be due to impaired SERT function during development.

To confirm this possibility, we chronically treated pregnant rats, starting from embryonic day 11, with the antidepressant fluoxetine to mimic the lack of SERT in developing fetuses. We found that the *Npas4* gene expression was significantly decreased in animals that were exposed to chronic fluoxetine during gestation (−31%, $p<0.05$; Student's *t*–test; Figure 2). Conversely, when we treated adult rats with the SSRI antidepressant, we did not observe any significant change in the mRNA levels of the transcription factor (−12%, $p>0.05$; Student's *t*–test; Figure 2). This implies that altered expression of *Npas4* in SERT-knockout rats does not originate from the lack of SERT in adulthood, but is probably due to its impaired function during fetal life.

Table 1 Sequences of Forward and Reverse Primers Used in Real-Time PCR Analysis

Gene	Forward primer	Reverse primer	Probe
Npas4	5′-GGAAGTTGCTATACCTGTCGG-3′	5′-GTCGTAAATACTGTCACCCTGG-3′	5′-CATAGAATGGCCCAGATGCTCGCT-3′
Arnt2	5′-AAGTGCTGTCGGTCATGTAC-3′	5′-GCTGAAGTTGCTTGACGTTG-3′	5′-AGCTTCACCTTCCAGAACCCCTACT-3′
GABA$_A$γ2	5′-ACTCATTGTGGTTCTGTCCTG-3′	5′-GCTGTGACATAGGAGACCTTG-3′	5′-ATGGTGCTGAGAGTGGTCATCGTC-3′
Gad67	5′-ATACTTGGTGTGGCGTAGC-3′	5′-AGGAAAGCAGGTTCTTGGAG-3′	5′-AAAACTGGGCCTGAAGATCTGTGGT-3′
Vgat	5′-ACGACAAACCCAAGATCACG-3′	5′-GTAGACCCAGCACGAACATG-3′	5′-TTCCAGCCCGCTTCCCACG-3′
Pvalb	5′-CTGGACAAAGACAAAAGTGGC-3′	5′-GACAAGTCTCTGGCATCTGAG-3′	5′-CCTTCAGAATGGACCCCAGCTCA-3′
Calb1	5′-AGAACTTGATCCAGGAGCTTC-3′	5′-CTTCGGTGGGTAAGACATGG-3′	5′-TGGGCAGAGAGATGATGGGAAAATAGGA-3′
36B4	5′-TTCCCACTGGCTGAAAAGGT-3′	5′-CGCAGCCGCAAATGC-3′	5′-AAGGCCTTCCTGGCCGATCCATC-3′

Abbreviations: *Arnt2*, aryl hydrocarbon receptor nuclear translocator 2; *Calb1*, calbindin; *GABA$_A$-γ2*, GABA A receptor, gamma 2; *Gad67*, glutamic-acid decarboxylase; *Npas4*, neuronal PAS domain protein 4; *Pvalb*, Parvalbumin; *Vgat*, vesicular GABA transporter.

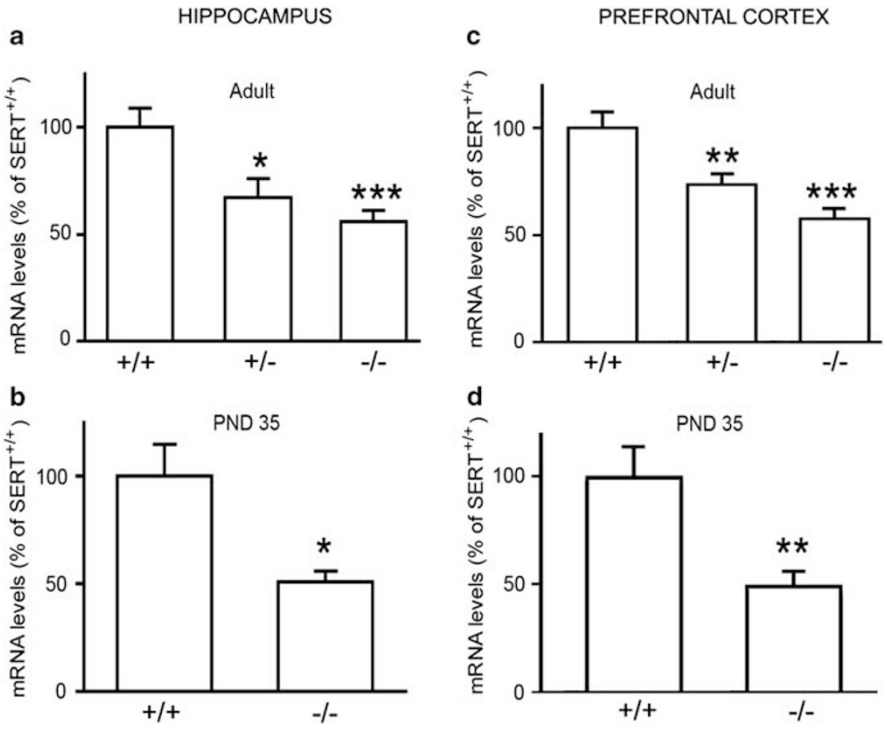

Figure 1 The gene expression of neuronal PAS domain protein 4 (*Npas4*) is altered in rat hippocampus and prefrontal cortex of serotonin transporter (SERT) mutant rats. *Npas4* mRNA levels were measured in the hippocampus (a, b) and prefrontal cortex (c, d) of adult (a, c) and adolescent (b, d) SERT mutant rats (heterozygous, $SERT^{+/-}$; and homozygous, $SERT^{-/-}$), as compared with their wild-type ($SERT^{+/+}$) counterparts. The data, expressed as a percentage of $SERT^{+/+}$ animals (set at 100%), are the mean ± SEM of at least seven independent determinations. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* $SERT^{+/+}$ rats (one-way analysis of variance (ANOVA) with Fischer's protected least significant difference for adult rats; Student *t*-test for postnatal day (PND) 35).

Effect of Chronic Duloxetine Treatment on *Npas4* and *Arnt2* mRNA Levels in $SERT^{-/-}$ Rats

As SERT-knockout rats display anxiety- and depressive-like behaviors (Kalueff *et al*, 2010; Olivier *et al*, 2008), we tested whether antidepressant treatment may restore the expression of *Npas4* in these animals. Therefore, we chronically treated adult $SERT^{-/-}$ rats with the SNRI duloxetine, (Carter and McCormack, 2009), and measured *Npas4* mRNA levels in the hippocampus and prefrontal cortex. In the hippocampus, we found a significant drug treatment effect ($F_{1,36}=9.806$, $p<0.01$) and a significant genotype-X drug-treatment interaction ($F_{1,36}=4.941$, $p<0.05$). Although duloxetine did not change the *Npas4* expression in wild-type animals (+9%, $p>0.05$), long-term administration of the antidepressant was able to normalize the reduced *Npas4* mRNA levels observed in $SERT^{-/-}$ rats (+98% *vs* $SERT^{-/-}$ treated with saline, $p<0.01$; two-way ANOVA with SCPHT; Figure 3a). In the prefrontal cortex, we found a significant drug treatment effect ($F_{1,42}=13.645$, $p<0.001$). Chronic duloxetine treatment normalized the *Npas4* reduction in $SERT^{-/-}$ rats (+69% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.01$), without affecting its levels in wild-type animals (+19%, $p>0.05$; Figure 3c).

To further the analysis of the transcription factors that may contribute to the *Bdnf* changes present in $SERT^{-/-}$ rats, we investigated the expression of *Arnt2*, a member of the Arnt family, that specifically cooperate with *Npas4* in the transcriptional control of *Bdnf* exons (Ooe *et al*, 2009; Pruunsild *et al*, 2011). In the hippocampus, we found a significant genotype effect ($F_{1,24}=13.696$, $p<0.01$) as demonstrated by the reduction of *Arnt2* mRNA levels in $SERT^{-/-}$ rats (−37%, $p<0.05$; two-way ANOVA with SCPHT), although duloxetine treatment was not able to modulate the *Arnt2* expression in wild-type, as well as in $SERT^{-/-}$ rats (Figure 3b). In the prefrontal cortex, we found a significant drug treatment effect ($F_{1,41}=15.955$, $p<0.001$) and a significant genotype-X drug-treatment interaction ($F_{1,41}=6.632$, $p<0.05$). In fact, $SERT^{-/-}$ rats displayed a significant reduction of *Arnt2* mRNA levels (−30%, $p<0.05$), which were significantly upregulated by chronic antidepressant treatment (+94% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.001$; Figure 3d).

The Reduced *Npas4* mRNA Levels are Associated with Impaired Expression of GABAergic Markers in $SERT^{-/-}$ Rats

Given that *Npas4* expression turns on a program of gene expression that triggers the formation and/or maintenance of inhibitory synapses (Lin *et al*, 2008), we investigated the expression of three key elements of the GABAergic synapses, the *Vgat*, the GABA-producing enzyme *Gad67*, and the postsynaptic $GABA_A$-$\gamma 2$, in the hippocampus and prefrontal cortex of $SERT^{-/-}$ rats under basal conditions, as well as after antidepressant treatment.

We found a significant decrease of *Vgat* expression levels in the hippocampus (−37%, $p<0.05$; two-way ANOVA with

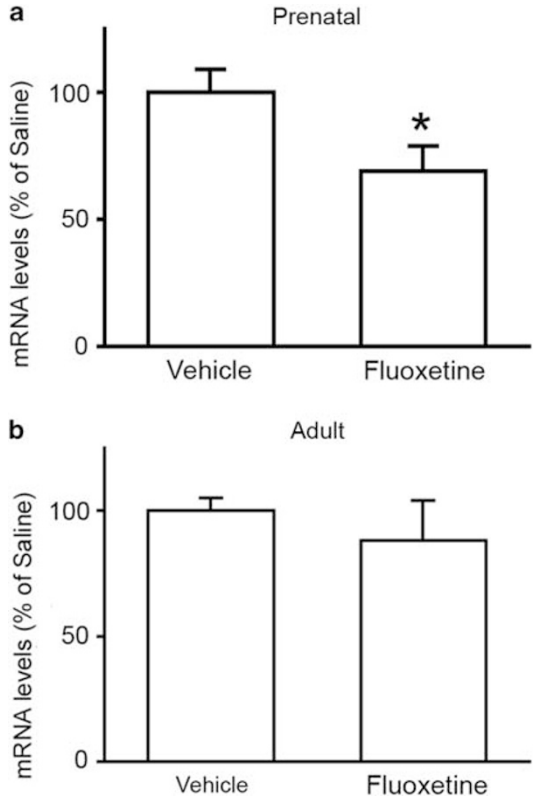

Figure 2 Fluoxetine treatment during gestation, but not at adulthood, influences neuronal PAS domain protein 4 (*Npas4*) gene expression in the hippocampus of adult rats. *Npas4* mRNA levels were measured in the hippocampus of adult rats that were treated chronically with fluoxetine during gestation (a) or at adulthood (b). The data, expressed as a percentage of animals treated with vehicle (set at 100%), are the mean ± SEM from at least seven independent determinations. *$p<0.05$ *vs* vehicle-treated rats (Student's *t*-test).

SCPHT), but not in the prefrontal cortex of $SERT^{-/-}$ rats (Figures 4a–d). Chronic duloxetine treatment was able to restore the physiological expression of *Vgat* in the hippocampus (+52% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.05$; two-way ANOVA with SCPHT; Figure 4a) as demonstrated by the significant genotype-X drug-treatment interaction ($F_{1,25}=5.046$, $p<0.05$), without affecting its levels in the prefrontal cortex (Figure 4d).

Gad67 expression levels were significantly reduced in the hippocampus of $SERT^{-/-}$ rats (−29%, $p<0.05$). Chronic duloxetine treatment had a genotype-specific effect on *Gad67*, as confirmed by the significant genotype-X drug-treatment interaction ($F_{1,26}=6.382$, $p<0.05$). Indeed, chronic duloxetine treatment in $SERT^{-/-}$ rats was able to normalize the reduction of *Gad67* to control levels (+31% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.05$), without affecting its expression in $SERT^{+/+}$ rats (−14%, $p>0.05$; Figure 4b). The expression of *Gad67* was also reduced in the prefrontal cortex (−18%, $p<0.05$) as demonstrated by the significant genotype effect ($F_{1,40}=9.690$, $p<0.01$). However, differently from the hippocampus, duloxetine treatment was not able to normalize the *Gad67* expression in the prefrontal cortex of $SERT^{-/-}$ rats (−4% *vs* $SERT^{-/-}$ treated with vehicle, $p>0.05$; Figure 4e).

To have an indication of inhibitory synapse number in the mutant rat, we also investigated the expression of the $GABA_A$-$\gamma 2$-receptor subunit. In the hippocampus, the receptor expression levels were significantly reduced in $SERT^{-/-}$ rats (−38%, $p<0.05$) and normalized by long-term antidepressant treatment (+80% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.05$; two-way ANOVA with SCPHT; Figure 4c). The effect of duloxetine was limited to $SERT^{-/-}$ rats, as confirmed by the significant genotype-X drug-treatment interaction ($F_{1,26}=8.774$, $p<0.01$). In prefrontal cortex, we found a genotype specific effect ($F_{1,41}=5.996$, $p<0.05$) and a significant drug treatment effect ($F_{1,41}=20.7971$, $p<0.001$). Indeed $SERT^{-/-}$ rats showed a reduction of $GABA_A$-$\gamma 2$ mRNA levels (−35%, $p<0.05$), whereas duloxetine treatment was able to normalize this deficit (+91% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.001$) and to increase the receptor mRNA levels also in wild-type animals (+45%, $p<0.05$; two-way ANOVA with SCPHT; Figure 4f).

The impairment of the GABAergic system in SERT-knockout rats was also associated with a dysfunction of two calcium-binding proteins, parvalbumin and calbindin, which label subgroups of GABAergic interneurons in the hippocampus and prefrontal cortex (Schwaller *et al*, 2002).

Parvalbumin expression was significantly decreased in hippocampus (−42%, $p<0.01$), and to a less extent in the prefrontal cortex (−25%, $p<0.05$) of $SERT^{-/-}$ rats. Duloxetine had a different impact on its expression in the two brain regions, as chronic antidepressant treatment in $SERT^{-/-}$ rats had a significant effect in the hippocampus ($F_{1,26}=8.653$, $p<0.01$), where parvalbumin levels were restored to its physiological levels (+52% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.05$; Figure 5a), but not in prefrontal cortex (+19% *vs* $SERT^{-/-}$ treated with vehicle, $p>0.05$; two-way ANOVA with SCPHT; Figure 5c). With respect to calbindin, its expression was significantly decreased in the hippocampus (−37%, $p<0.01$), but not in the prefrontal cortex of $SERT^{-/-}$ rats. Chronic treatment with the antidepressant duloxetine was able to restore the physiological expression of calbindin in hippocampus (+79% *vs* $SERT^{-/-}$ treated with vehicle, $p<0.05$; two-way ANOVA with SCPHT; Figure 5b) as demonstrated by the significant genotype-X drug-treatment interaction ($F_{1,26}=14.566$, $p<0.001$), without affecting its expression levels in prefrontal cortex (Figure 5d).

Npas4 Expression Changes Correlate With the mRNA Levels for *Bdnf* Exon I, *Bdnf* Exon IV, and $GABA_A$- $\gamma 2$ in Hippocampus and Prefrontal Cortex of Wild-Type and $SERT^{-/-}$ rats

We recently demonstrated that $SERT^{-/-}$ rats have reduced expression of the neurotrophin *Bdnf*, which is sustained by a decrease of several transcripts, including exon I and IV (Molteni *et al*, 2010), and that such changes could be normalized by chronic treatment with duloxetine (Calabrese *et al*, 2010). These alterations were confirmed in the animals investigated in the present study and a summary of the changes for total *Bdnf* mRNA, and for the two transcripts regulated by *Npas4* (exon I and exon IV) are shown in Table 2.

Hence, *Npas4* mRNA data were examined for possible covariation within the gene expression of the two *Bdnf*

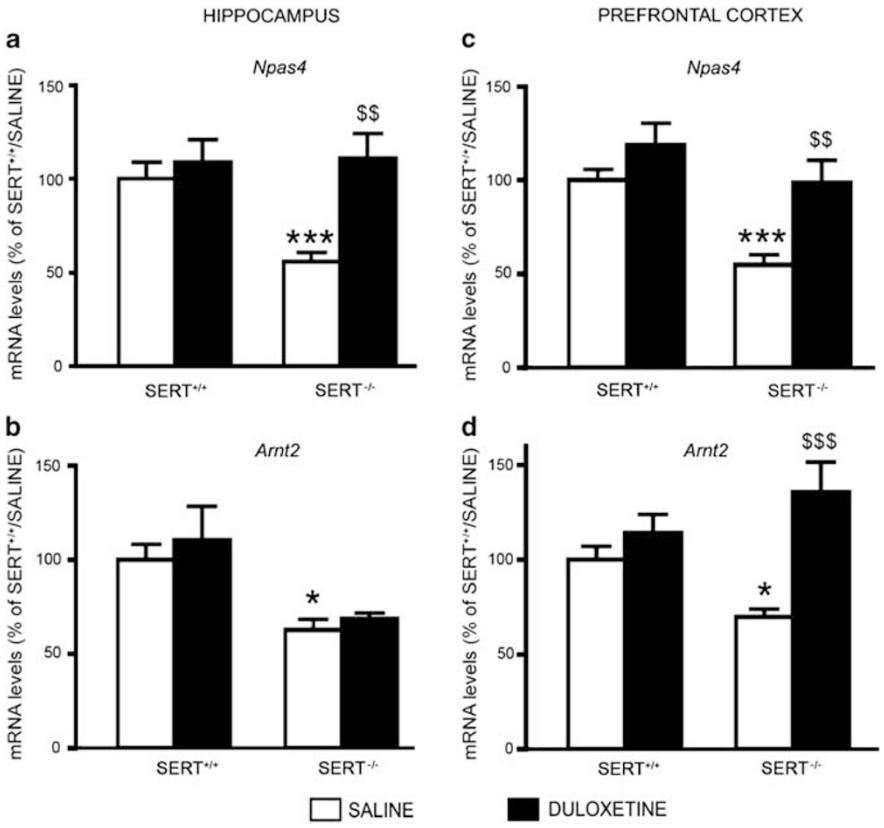

Figure 3 Chronic duloxetine treatment modulates the levels of neuronal PAS domain protein 4 (*Npas4*) and aryl hydrocarbon receptor nuclear translocator 2 (*Arnt2*) gene expression in serotonin transporter (SERT)$^{-/-}$ rats. *Npas4* and *Arnt2* mRNA levels were measured in the hippocampus (a, b) and in prefrontal cortex (c, d) of wild-type (SERT$^{+/+}$), and of SERT$^{-/-}$ rats treated for 21 days with saline or duloxetine, and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/saline (set at 100%), are the mean ± SEM from at least five independent determinations. $^{*}p<0.05$, $^{***}p<0.001$ *vs* SERT$^{+/+}$/saline; $^{\$\$}p<0.01$, $^{\$\$\$}p<0.001$ *vs* SERT$^{-/-}$/saline (two-way analysis of variance (ANOVA) with single contrast *post-hoc* test (SCPHT)).

transcripts in the hippocampus and in the prefrontal cortex. The analyses revealed that, *Npas4* mRNA levels correlated positively with the expression of both *Bdnf* exons in the hippocampus ($r=0.4757$, $n=24$, $p<0.05$; $r=0.4977$, $n=23$, $p<0.05$; exon I and exon IV, respectively; Figures 6a and b), as well as in the prefrontal cortex ($r=0.4863$, $n=26$, $p<0.05$; $r=0.5220$, $n=26$, $p<0.05$; exon I and exon IV, respectively; Figures 6d and e). Moreover, to assess the possible correlation between the changes in *Npas4* gene expression and the alteration in the GABAergic markers, we performed a similar comparison between *Npas4* and the $GABA_A$-$\gamma 2$ mRNA levels. We found a positive correlation with the expression of the two genes in the hippocampus ($r=0.4120$, $n=24$, $p<0.05$), as well as in the prefrontal cortex ($r=0.5900$, $n=24$, $p<0.01$; Figures 6c–f).

DISCUSSION

In this study, we provide evidence that the expression of the transcription factor *Npas4* is significantly reduced in the hippocampus and prefrontal cortex of SERT$^{-/-}$ rats, that such changes are associated with an impairment of the GABAergic system in adulthood, and that these alterations may be normalized by chronic antidepressant treatment.

Npas4 is an activity-regulated transcription factor, whose neuronal expression is selectively induced by the Ca^{2+} influx, and has a critical role in the development of inhibitory synapses by regulating the expression of activity-dependent genes (Lin *et al*, 2008). As SERT$^{-/-}$ rats represent a genetic model of anxiety and depression, our findings raise the possibility that the reduction of *Npas4* expression may be linked, or may contribute to the behavioral alterations found in these animals (Olivier *et al*, 2008). The reduction of *Npas4* expression in SERT$^{-/-}$ rats appears to be the consequence of an impairment of SERT early in life, as it is already present at adolescence (PND 35). Moreover, we show that pharmacological blockade (fluoxetine) of SERT during gestation can mimic the reduced *Npas4* expression found in the hippocampus of SERT$^{-/-}$ rats, providing further support to the developmental origin of the observed changes. During early life, serotonergic neurons are among the earliest to be generated, and 5-HT has an important role in different processes pivotal for neuronal growth and maturation (Gaspar *et al*, 2003). Indeed, blockade of SERT during fetal life of rats can lead to negative outcomes on brain function and behavior, including behavioral despair and anxiety-like behavior (Lisboa *et al*, 2007; Olivier *et al*, 2011). Moreover, pharmacological blockade of SERT during early post-natal life (PND 4–21) results in increased immobility time in the

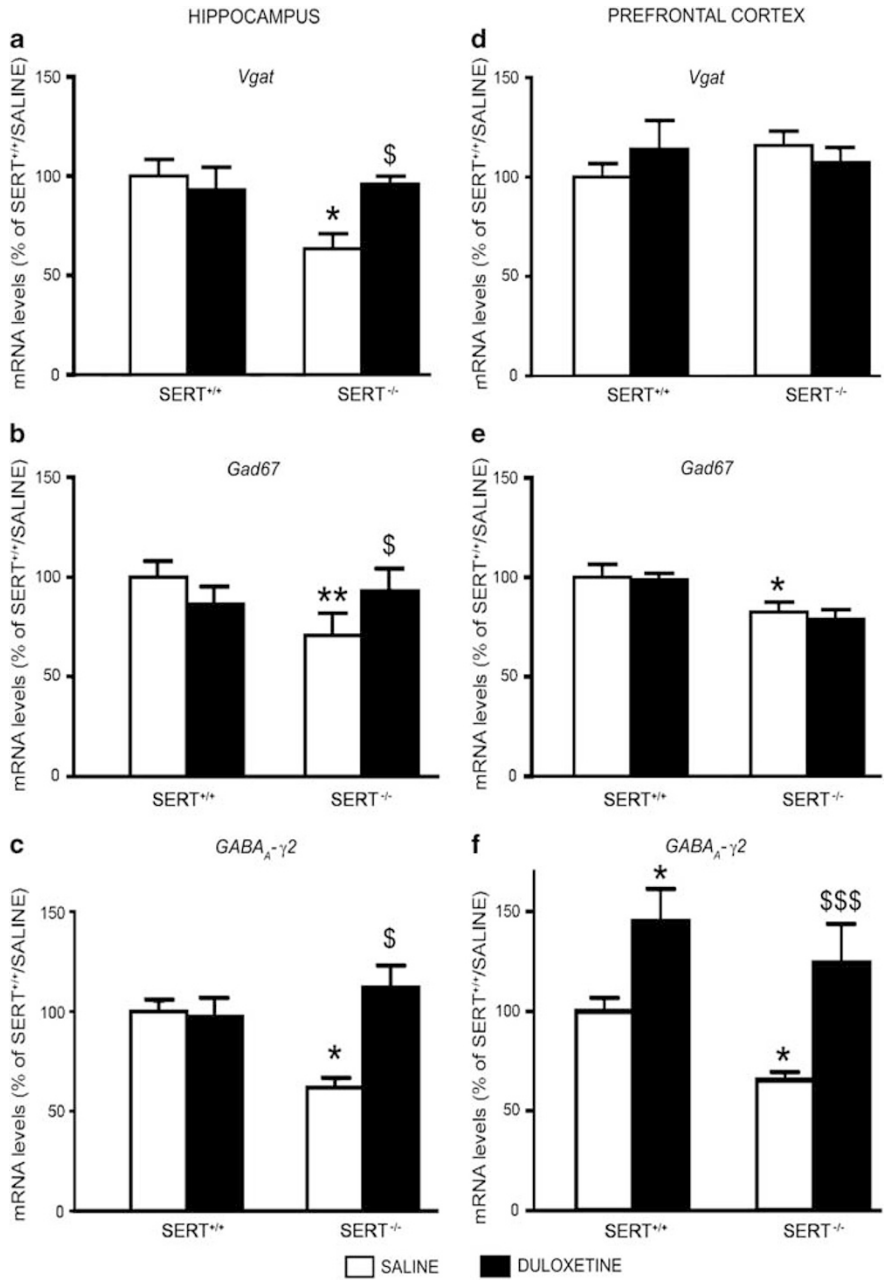

Figure 4 Serotonin transporter (SERT)$^{-/-}$ rats show impaired expression of GABAergic markers, modulation by chronic duloxetine treatment. Vesicular transporter (*Vgat*; a, d), glutamic acid decarboxylase-67 (*Gad67*; b, e), and GABA A receptor, gamma 2 (*GABA$_A$-γ2*; c, f) mRNA levels were measured in the hippocampus (a–c), and prefrontal cortex (d–f) of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with saline or duloxetine, and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/saline (set at 100%), are the mean ± SEM from at least six independent determinations. *$p<0.05$, **$p<0.01$ *vs* SERT$^{+/+}$/saline; $^{\$}p<0.05$, $^{\$\$\$}p<0.001$ *vs* SERT$^{-/-}$/saline (two-way analysis of variance (ANOVA) with single contrast *post-hoc* test (SCPHT)).

forced-swim test (Hansen *et al*, 1997), anxiety-related behavioral disturbances (Ansorge *et al*, 2008; Ansorge *et al*, 2004), and increased REM sleep (Popa *et al*, 2008) at adulthood. Collectively, these data show that SERT blockade exerts age-dependent effects on behavior (for review see Olivier *et al* (2010)), leading to a variety of unfavorable outcomes in rodents, which are opposite with respect to the effects produced by SSRIs during adulthood, but comparable to SERT knockout in rodents (Homberg *et al*, 2011).

It is interesting to note that *Npas4* is a transcription factor associated with promoters I and IV of the *Bdnf* gene (Pruunsild *et al*, 2011), suggesting that it may directly regulate activity-dependent expression of the neurotrophin (Lin *et al*, 2008). Interestingly, we have previously demonstrated that SERT$^{-/-}$ rats have reduced *Bdnf* expression in the hippocampus and prefrontal cortex, and that this occurs through the modulation of different *Bdnf* transcripts, including exon I and exon IV (Molteni *et al*, 2010). A large body of evidence demonstrates that a reduction of *Bdnf* levels is associated with deficits or impairment of neuronal plasticity, which can have a role in anxiety and major depression (Calabrese *et al*, 2009; Calabrese *et al*, 2011;

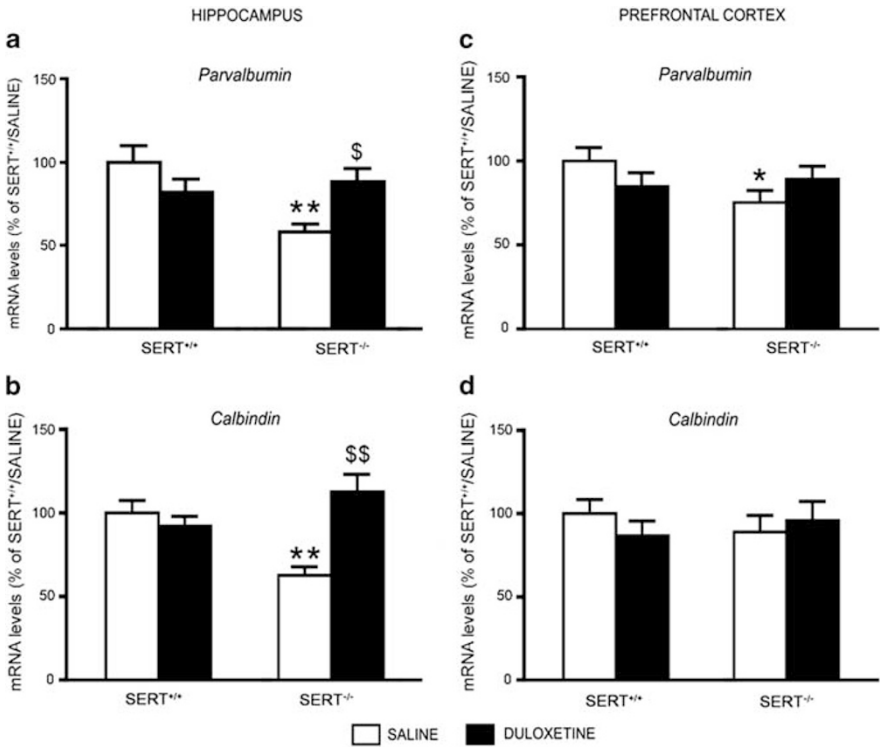

Figure 5 Analysis of *parvalbumin* and *calbindin* gene expression in serotonin transporter (SERT)$^{-/-}$ rats, modulation by chronic duloxetine treatment. *Parvalbumin* (a, c) and *Calbindin* (b, d) mRNA levels were measured in the hippocampus (a, b) and prefrontal cortex (c, d) of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with saline or duloxetine, and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/saline (set at 100%), are the mean ± SEM from at least six independent determinations. *$p<0.05$, **$p<0.01$ *vs* SERT$^{+/+}$/saline; $^{\$}p<0.05$, $^{\$\$}p<0.01$ *vs* SERT$^{-/-}$/saline (two-way analysis of variance (ANOVA) with single contrast *post-hoc* test (SCPHT)).

Table 2 Analysis of the mRNA Levels for *Npas4*, Total *Bdnf*, *Bdnf* Exon I, and *Bdnf* Exon IV in Wild-Type and SERT$^{-/-}$ Rats, and their Modulation by Chronic Duloxetine Treatment

Gene	Hippocampus				Prefrontal cortex			
	+/+/SAL	**+/+/DLX**	**−/−/SAL**	**−/−/DLX**	**+/+/SAL**	**+/+/DLX**	**−/−/SAL**	**−/−/DLX**
Npas4	100 ± 13	109 ± 12	56 ± 5***	111 ± 13$^{\$\$}$	100 ± 6	119 ± 12	55 ± 6***	98 ± 12$^{\$\$}$
Bdnf total	100 ± 4	123 ± 2***	86 ± 2*	117 ± 3$^{\$\$\$}$	100 ± 4	120 ± 4***	72 ± 8***	101 ± 3$^{\$\$\$}$
Bdnf exon I	100 ± 10	105 ± 11	61 ± 5*	138 ± 13$^{\$\$\$}$	100 ± 7	162 ± 16***	63 ± 6**	93 ± 9$^{\$}$
Bdnf exon IV	100 ± 7	95 ± 8	66 ± 8*	138 ± 15$^{\$\$\$}$	100 ± 9	111 ± 19	59 ± 6**	93 ± 9$^{\$}$

Abbreviations: ANOVA, analysis of variance; *Bdnf*, brain-derived neurotrophic factor; DLX, duloxetine; *Npas4*, neuronal PAS domain protein 4; SAL, saline; SCPHT, single-contrast *post-hoc* test; SERT, serotonin transporter; *Vgat*, vesicular GABA transporter.
Npas4, total *Bdnf*, *Bdnf* exon I, and *Bdnf* exon IV mRNA levels were measured in the hippocampus and in the prefrontal cortex of wild-type (+/+) and of SERT$^{-/-}$ (−/−) rats treated for 21 days with SAL or with DLX, and killed 24 h after the last injection. The data, expressed as percentage of +/+/SAL (set at 100%), are the mean ± SEM from at least six independent determinations.
*$p<0.05$.
**$p<0.01$.
***$p<0.001$ *vs* +/+/SAL.
$^{\$}p<0.05$.
$^{\$\$}p<0.01$.
$^{\$\$\$}p<0.001$ *vs* −/−/SAL (two-way ANOVA with SCPHT).

Castren, 2005; Krishnan and Nestler, 2008; Martinowich *et al*, 2007; Pittenger and Duman, 2008). Our data demonstrate that there is a significant correlation between the levels of *Npas4* in the hippocampus and prefrontal cortex, and those of *Bdnf* exon I and IV (see Figure 6), suggesting that neurotrophic abnormalities may be causally linked to alterations of the transcription factor. One system lying downstream from *Npas4* and *Bdnf* that may be relevant for the phenotype of SERT mutants is GABA. We demonstrate for the first time that SERT$^{-/-}$ rats show a strong impairment of the GABAergic system in the hippocampus and prefrontal cortex, although some

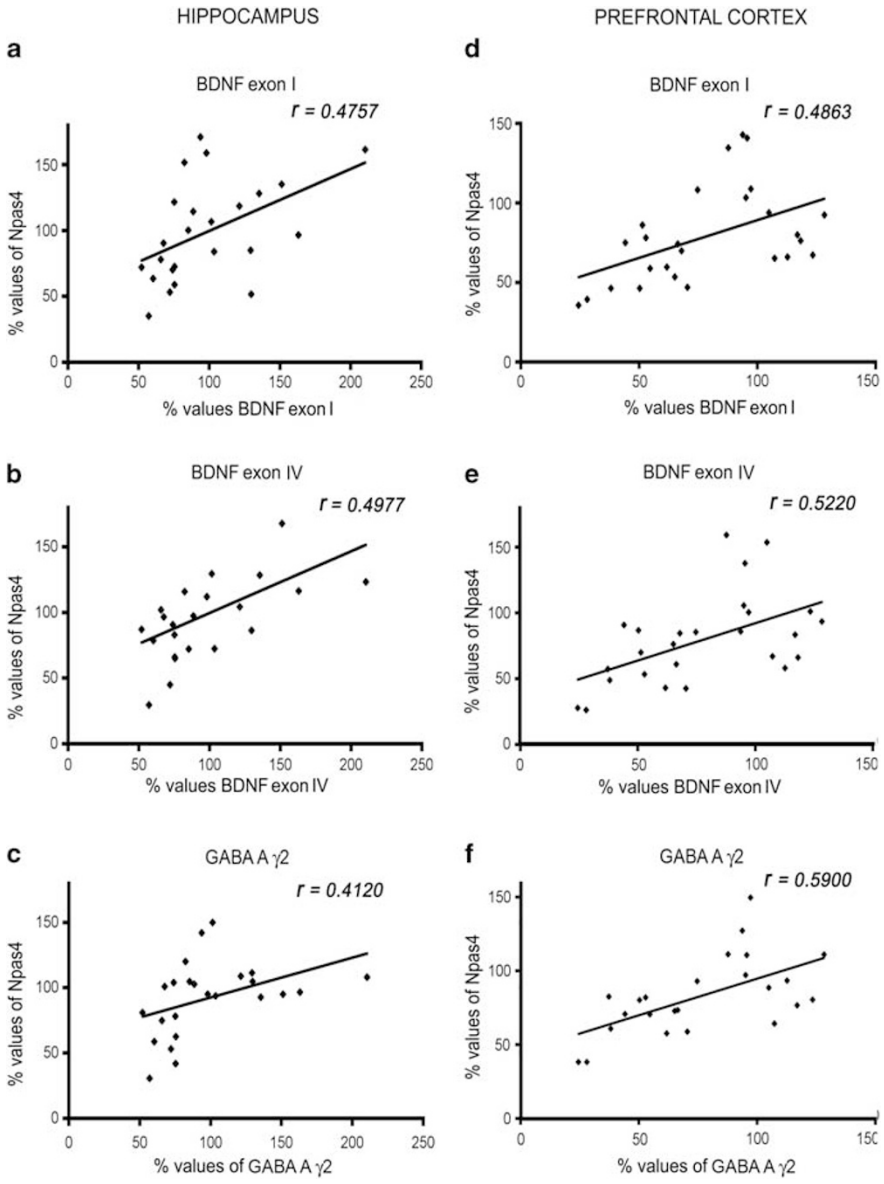

Figure 6 Correlation analyses between neuronal PAS domain protein 4 (*Npas4*), brain-derived neurotrophic factor (*Bdnf*) exon I (a, d), *Bdnf* exon IV (b, e), and GABA A receptor, gamma 2 ($GABA_A$- $\gamma 2$) (c, f) in the hippocampus (a, b, c) and prefrontal cortex (d, e, f) of wild-type and serotonin transporter $(SERT)^{-/-}$ rats. Data points in plots indicate the amount of *Npas4*, *Bdnf* exon I, *Bdnf* exon IV, and $GABA_A$-$\gamma 2$ mRNA levels in single rats. Analyses by Pearson's product–moment correlation (*r*). The data, from at least 11 independent determinations, are expressed as a percentage of $SERT^{+/+}$/ saline (set at 100%).

differences may exist between these two structures. In fact, the expression of *Vgat*, is significantly reduced only in the hippocampus of $SERT^{-/-}$ rats, suggesting that these animals may display a reduced number of GABAergic terminals, whereas the expression of the GABA synthesizing enzyme *Gad67* or the postsynaptic $GABA_A$-$\gamma 2$ was similarly reduced in the hippocampus and prefrontal cortex of mutant rats. Moreover, genetic deletion of SERT may influence the sub-population of GABAergic neurons, as the expression of parvalbumin was decreased in both structures, whereas calbindin, which labels a small sub-group of interneurons, was reduced only in the hippocampus. Parvalbumin alteration in hippocampal interneurons may lead to a loss of perisomatic inhibition of pyramidal neurons, which in turn affects network synchronization and memory formation (Bartos *et al*, 2007; Lewis *et al*, 2005).

Disturbances in the anatomy and function of the GABAergic system have been postulated in animal models of depression and in different stress-related psychiatric disorders (Benes and Berretta, 2001; Brambilla *et al*, 2003; Krystal *et al*, 2002; Luscher *et al*, 2011; Sanacora *et al*, 1999). In fact, $GABA_A$ receptors can be downregulated in different brain regions of rats exposed to the learned helplessness paradigm (Drugan *et al*, 1992), whereas lower CSF and plasma GABA levels have been found in depressed patients, as compared with control subjects (Gerner *et al*, 1984; Gerner and Hare, 1981; Kasa *et al*, 1982; Petty *et al*, 1992). Mice that lack the $GABA_A\gamma 2$ receptor subunit show an anxious, depressive phenotype that is related to HPA axis

hyperactivity (Sen *et al*, 2008). Furthermore, parvalbumin immunoreactive neurons are reduced in the hippocampus of tree shrews after chronic psychosocial stress (Czeh *et al*, 2005), as well as in *Octodon degus* after repeated separation stress (Seidel *et al*, 2008), whereas calbindin immunoreactive neurons were significantly reduced in the CA1 of the hippocampus of mice after inescapable electric foot shocks paradigm (Huang *et al*, 2010). On these bases, the defects of hippocampal GABAergic markers in SERT$^{-/-}$ animals may contribute to the anxious/depressive phenotype observed in these animals (Olivier *et al*, 2008). It remains to be established if different subgroups of interneurons expressing neuropeptides, such as somatostatin, neuropeptide Y, cholecystokinin, or tachykinin-1, which may also be regulated by *Bdnf* (Glorioso *et al*, 2006; Mellios *et al*, 2009), are altered in SERT KO rats, considering that some of these neuropeptides may be altered in depression (Tripp *et al*, 2011).

It is important to point out that *Npas4* can regulate the development of GABAergic synapses, as silencing of *Npas4* gene leads to reduced expression of GABAergic markers, including the presynaptic GABA-producing enzymes *Gad65*, *Gad67*, and the *GABA$_A$-γ2* receptor subunit (Lin *et al*, 2008). Furthermore, a strong relationship exists between *Bdnf* and GABA (Yamada *et al*, 2002). It has recently been demonstrated that promoter IV-driven *Bdnf* transcription has a critical role in GABAergic transmission (Sakata *et al*, 2009), and mice with a selective deficiency of promoter-IV-dependent expression of *Bdnf* show depression-like behavior (Sakata *et al*, 2010). Collectively, these data support the potential link between *Npas4*, *Bdnf*, and GABA in contributing to the phenotypic changes observed in SERT$^{-/-}$ rats.

We also provide evidence that chronic antidepressant treatment may normalize the molecular alterations found in SERT$^{-/-}$ rats. We had previously demonstrated that chronic treatment with the SNRI antidepressant duloxetine is able to normalize *Bdnf* deficits in these animals, also through the modulation of *Bdnf* exon I and IV (Calabrese *et al*, 2010, present results). Such data are in agreement with the possibility that repeated, but not acute, treatment with major classes of antidepressants may improve neuronal plasticity through the modulation of *Bdnf* expression (Calabrese *et al*, 2007; Castren *et al*, 2007; Molteni *et al*, 2009a; Russo-Neustadt and Chen, 2005), and that this effect may contribute to their therapeutic action (Berton and Nestler, 2006; Calabrese *et al*, 2009; Calabrese *et al*, 2011; Groves, 2007; Martinowich *et al*, 2007).

We show here that chronic duloxetine treatment does normalize the reduced expression of *Npas4* in the hippocampus and prefrontal cortex of SERT$^{-/-}$ rats, without altering transcription factor levels in wild-type animals, suggesting that the effect of the antidepressant may be considered a restorative mechanism rather than a general potentiation of *Npas4*-dependent transcription. This effect has strong similarity with the changes produced by duloxetine on the expression of *Bdnf* exon IV in SERT$^{-/-}$ rats, which is significantly upregulated by chronic antidepressant treatment only in SERT$^{-/-}$ rats (Calabrese *et al*, 2010). On the basis of the lack of SERT function in mutant animals, it is feasible to hypothesize that the modulation of *Bdnf* transcripts and *Npas4* by duloxetine may be due to the 'noradrenergic component' of the antidepressant. Because, as mentioned above, *Npas4* can regulate the expression of *Bdnf* exon IV, our data suggest that chronic duloxetine may restore the correct expression of *Bdnf* in SERT$^{-/-}$ rats via modulation of the transcription factor. Moreover, we show that the expression of *Arnt2*, another transcription factor that cooperate with *Npas4* in the regulation of *Bdnf* exon IV (Pruunsild *et al*, 2011) is also significantly reduced in SERT$^{-/-}$ rats, although its expression can be restored by duloxetine treatment in the prefrontal cortex, but not in the hippocampus.

These results provide further support to the notion that transcriptional mechanisms may be an important component of the long-term mechanisms set in motion by antidepressant therapy. It is well established that the cAMP-response element-binding protein (*Creb*) is implicated in depression and in the response to antidepressant treatment (Gass and Riva, 2007), as a decrease in *Creb* phosphorylation was found in the hippocampus of chronically stressed rats (Qi *et al*, 2008), whereas several classes of antidepressants increase the levels of *Creb* expression and function in the rat hippocampus (Nibuya *et al*, 1996; Thome *et al*, 2000). However, *Creb* does not appear to have a role in SERT$^{-/-}$ abnormalities and in the action of duloxetine (data not shown), suggesting that, although *Bdnf* represents a downstream target of several antidepressant drugs, its regulation may occur through different intracellular pathways.

The impairment of the GABAergic system in the hippocampus and in the prefrontal cortex of SERT$^{-/-}$ rats can also be normalized by long-term treatment with the SNRI antidepressant. Duloxetine appears to be highly effective on hippocampal alterations, where all GABAergic abnormalities in SERT$^{-/-}$ rats (*Vgat*, *Gad67*, *GABA$_A$-γ2*, parvalbumin, and calbindin) are normalized by antidepressant treatment, whereas within the prefrontal cortex, duloxetine appears to restore only the expression of *GABA$_A$-γ2*. As *Npas4* changes in SERT$^{-/-}$ rats are normalized by duloxetine in both brain regions, these results suggest that factors, other than *Npas4*, may contribute to GABAergic dysfunction in the prefrontal cortex of SERT mutant animals.

Interestingly, there is recent evidence that treatment with antidepressants can revert the reduction of *Gad67* in human depressed subjects (Karolewicz *et al*, 2010), which parallels our findings. These findings suggest that antidepressant treatment can normalize the dysfunction of the GABAergic system (Luscher *et al*, 2011), which may lead to the increase in GABA release observed in patients (Carter and McCormack, 2009; Sanacora *et al*, 2002). Collectively, animal and clinical studies indicate that a deficit for GABAergic activity may be crucial in the pathophysiology of mood disorders, and that effective modulation of GABAergic transmission may represent another important mechanism through which antidepressant drugs exert their therapeutic activity (Luscher *et al*, 2011).

In summary, our results demonstrate that animals with a genetic deletion of SERT show a reduction of *Npas4* expression and an impairment of the GABAergic system, suggesting that these defects may contribute to SERT$^{-/-}$ behavioral traits, particularly those associated with anxiety and depression. Given that SERT-knockout rodents model

the common SERT promoter polymorphism in humans (Caspi *et al*, 2010; Hariri and Holmes, 2006; Homberg *et al*, 2011), it may be inferred that the pharmacological modulation of *Npas4* may provide a valuable strategy aimed at improving GABAergic function, as well as neuroplastic mechanisms closely related to the neurotrophin *Bdnf*. Furthermore, the characterization of genes downstream from the transcription factor *Npas4* may prove useful to identify novel systems that may be also affected in mood disorders.

ACKNOWLEDGEMENTS

We are grateful to Juliet Richetto for language editing. This publication was made possible by grants to MA Riva from the Ministry of University and Research (PRIN 2007STRNHK), from the Ministry of Health (Ricerca Finalizzata RF 2007 conv/42), from Regione Lombardia (Accordo Quadro 2010), and by a liberal contribution from Eli Lilly Italia.

DISCLOSURE

The authors Guidotti G, Calabrese F, Auletta F, Olivier J, and Homberg J have no financial interest or potential conflict of interest. Racagni G has received compensation as speaker/consultant for Eli Lilly, InnovaPharma, Servier. Riva MA has received compensation as speaker/consultant for AstraZeneca, Bristol-Myers Squibb, Eli Lilly, Servier, Takeda.

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