ARTICLE

Behavioural and neuroplastic properties of chronic lurasidone treatment in serotonin transporter knockout rats

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Abstract

Second-generation antipsychotics (SGA) are multi-target agents widely used for the treatment of schizophrenia and bipolar disorder that also hold potential for the treatment of impaired emotional control, thanks to their diverse receptor profiles as well as their potential in modulating neuroadaptive changes in key brain regions. The aim of this study was thus to establish the ability of lurasidone, a novel SGA characterized by a multi-receptor signature, to modulate behavioural and molecular defects associated with a genetic model of impaired emotional control, namely serotonin transporter knockout (SERT KO) rats. At behavioural level, we found that chronic lurasidone treatment significantly increased fear extinction in SERT KO rats, but not in wild-type control animals. Moreover, at molecular level, lurasidone was able to normalize the reduced expression of the neurotrophin brain-derived neurotrophic factor in the prefrontal cortex of SERT KO rats, an effect that occurred through the regulation of specific neurotrophin transcripts (primarily exon VI). Furthermore, chronic lurasidone treatment was also able to restore the reduced expression of different GABAergic markers that is present in these animals. Our results show that lurasidone can improve emotional control in SERT KO rats, with a primary impact on the prefrontal cortex. The adaptive changes set in motion by repeated treatment with lurasidone may in fact contribute to the amelioration of functional capacities, closely associated with neuronal plasticity, which are deteriorated in patients with schizophrenia, bipolar disease and major depression.

Introduction

Second-generation antipsychotics (SGAs) are multi-target agents widely used for the treatment of schizophrenia and bipolar disorders. However, their clinical action and therapeutic utility has also expanded to disorders characterized by impaired emotional control, such as anxiety and depression, as monotherapy or as adjunctive therapy to conventional antidepressants (DeBattista and Hawkins, 2009; McElroy *et al.* 2010; Li *et al.* 2012). One potential mechanism that explains the use of SGAs in improving emotional control is their complex and heterogeneous receptor profile and, in particular, their ability to modulate different neurotransmitter receptors beyond dopamine D_2 (Wong *et al.* 2010). Furthermore, it is widely accepted that, as a difference from classical antipsychotics, SGAs may promote neuroadaptive and

neuroplastic changes, which may contribute to functional recovery (Lieberman *et al.* 2008; Molteni *et al.* 2009*b*). For example, a number of SGAs can increase the expression of neurotrophic molecules under basal conditions (Riva *et al.* 1999; Fumagalli *et al.* 2003; Maragnoli *et al.* 2004), and may also normalize neuroplastic dysfunctions associated with chronic stress exposure (Xu *et al.* 2006; Park *et al.* 2009).

Lurasidone is a novel SGA characterized by potent binding affinities for serotonin (5-HT)$_{2A}$, 5-HT$_7$, 5-HT$_{1A}$, dopamine D_2 and noradrenaline α_{2C} receptors (Ishibashi *et al.* 2010). This multiple target affinity has been associated with emotional control and antidepressant properties, including blockade of 5-HT$_{2A}$ and 5-HT$_7$, as well as activation of 5-HT$_{1A}$ receptors (Ishibashi *et al.* 2010; Wong *et al.* 2010). Moreover, we have recently shown that chronic, but not acute, administration of lurasidone can increase the expression of brain-derived neurotrophic factor (BDNF) and is able to modulate its responsiveness under stressful conditions (Fumagalli *et al.* 2012). This suggests that lurasidone has the potential to boost adaptive mechanisms, which in turn may improve

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symptoms and functions that have deteriorated in patients suffering from impaired emotional control, including major depression (Pittenger and Duman, 2008; Calabrese *et al.* 2009, 2011).

Since limited information is available with regard to the ability of SGAs to improve emotional control in animal models and to correct the molecular deficits associated with such condition, we decided to investigate the effects of chronic lurasidone exposure in serotonin transporter (SERT) knockout rats, an animal model of mood disturbance (Olivier *et al.* 2008; Schipper *et al.* 2011*a*,*b*; Nonkes *et al.* 2012). This model recapitulates defects of the SERT due to the presence of a human functional polymorphism within its promoter region, which modulates the susceptibility to different neuropsychiatric disorders (Caspi *et al.* 2003, 2010). Genetic deletion of SERT in rodents in fact leads to an anxious and depressive phenotype (Homberg *et al.* 2007; Olivier *et al.* 2008; Kalueff *et al.* 2010; Schipper *et al.* 2011*a*,*b*) as well as impaired fear extinction (Schipper *et al.* 2011*a*; Nonkes *et al.* 2012). Also, we have previously demonstrated that SERT knockout (SERT$^{-/-}$) rats exhibit alterations in neuronal plasticity, as indicated by reduced expression of the neurotrophin BDNF in the hippocampus and prefrontal cortex (PFC; Molteni *et al.* 2009*a*, 2010) and that this defect is normalized by long-term administration of the antidepressant duloxetine (Calabrese *et al.* 2010).

In the present study we used SERT$^{-/-}$ rats to specifically address the potential of lurasidone to improve extinction in the fear-conditioning paradigm, a key measure of emotional control that is impaired in post-traumatic stress disorder (PTSD). Moreover, based on the role of the PFC and BDNF in fear extinction (Milad and Quirk, 2002) and considering the impaired expression of the neurotrophin in the PFC of SERT$^{-/-}$ rats (Molteni *et al.* 2010), we also investigated whether chronic lurasidone administration could normalize the changes in the expression of BDNF. Furthermore, we examined whether chronic treatment with the antipsychotic drug could normalize dysregulation of GABAergic markers found in SERT$^{-/-}$ rats (Guidotti *et al.* 2011), which are closely related to BDNF function and may contribute to the aetiology and manifestation of depressive disorders and impaired emotional control (Sanacora *et al.* 1999; Luscher *et al.* 2011).

Method

Materials

General reagents and molecular biology reagents were purchased from Bio-Rad Laboratories (Italy), Eurofins MWG-Operon (Germany), Immuno Biological Laboratories (Germany), Life Technologies (Italy), M-Medical (Italy), Roche (Italy), Sigma-Aldrich (Italy) and Thermo-Scientific (Belgium).

Animals and treatment

SERT knockout rats (Slc6a4^{1Hubr}) were generated in a Wistar background by *N*-ethyl-N-nitrosurea-induced mutagenesis (Smits *et al.* 2006) as described previously (Homberg *et al.* 2007). Experimental animals were derived from crossing heterozygous SERT knockout (SERT$^{+/-}$) rats that were outcrossed for at least 10 generations with wild-type Wistar rats obtained from Harlan Laboratories (The Netherlands). After weaning, aged 21 d, ear cuts were taken for genotyping, which was performed by Kbiosciences (UK). A total of 64 females were used for the experiment and were maintained with food and water available *ad libitum*. A 12-h light–dark cycle was maintained (lights on 08.00 hours). All experiments were carried out in accordance with the current guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC), they were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands and all efforts were made to minimize animal suffering and to reduce the number of animals used.

The experimental groups were as follows: SERT$^{+/+}$ + vehicle; SERT$^{-/-}$ + vehicle; SERT$^{+/+}$ + lurasidone (10 mg/kg.d for 21 d); SERT$^{-/-}$ + lurasidone (10 mg/kg.d for 21 d).

While 'antidepressant' properties of lurasidone may occur at low doses (Ishibashi *et al.* 2010; Hedlund *et al.* 2011), we used 10 mg/kg based on our previous data and in order to establish the neuroplastic properties of the drug at a full regimen, which may produce significant occupancy at several receptors, including dopamine D$_2$ receptors (Ishibashi *et al.* 2010).

Rats were treated with vehicle or lurasidone for 3 wk. Lurasidone (provided by Dainippon Sumitomo Pharma Co. Ltd, Japan) was prepared by suspending the drug at a concentration of 10 mg/ml in a 1% hydroxyethylcellulose solution and was administered *per os* (by gavage) in the amount of 1 ml/kg according to the body weight of the animals. The rats were killed 24 h after the last administration, according to the timing we used in our previous studies. This was followed by the dissection of the PFC [defined as Cg1, Cg3 and IL sub-regions corresponding to the plates 6–10, according to the atlas of Paxinos and Watson (1996)], from 2-mm thick slices. The brain specimens were frozen on dry ice and stored at −80 °C for further analysis.

Fear conditioning

The fear conditioning test was carried out after 2-wk lurasidone administration. Fear conditioning chambers of Med Associates Inc. (USA) were used. The chambers were equipped with metal shock grids, a speaker and a camera, which was mounted in front of the chambers. The chambers were housed in sound attenuated cubicles. The rats were trained to acquire a Pavlovian association

Table 1. Sequences of forward and reverse primers used in the real-time polymerase chain reaction analysis

Gene	Forward primer	Reverse primer	Probe
Total *BDNF*	5'-AAGTCTGCCATTACATTCCTCGA-3'	5'-GTTTTCTGAAAGAGGGACAGTTTAT-3'	5'-TGTGGTTTGTTGCCGTTGCCAAG-3'
BDNF long*			Rn02531967_s1
BDNF exon I	5'-GGGGAGACGAGATTTTAAGACACTG-3'	5'-GTCATCACTCTTCTCACCTGG-3'	5'-TTGTGGCTTTGCTGTCTGGAGA-3'
BDNF exon IV*			Rn01484927_m1
BDNF exon VI*			Rn01484928_m1
Npas4	5'-GGAAGTTGCTATACCTGTCGG-3'	5'-GTCGTAAATACTGTCACCCTGG-3'	5'-CATAGAATGGCCCAGATGCTGCT-3'
Arnt2	5'-AAGTGCTGTCGGTCATGTAC-3'	5'-GCTGAAGTGCTTGACGTTG-3'	5'-AGCTTCACCTTCCAGAACCCTACT-3'
CaRF	5'-GAGATGCACACCATTCCA-3'	5'-GTGTTGGCTCATTGGGTTCT-3'	5'-CAGCCATCCAGCTCTTGTTGAAGA-3'
NF-κB	5'-CTACGAGACCTTCAAGAGACATC-3'	5'-GATGTTGAAAAGGCATAGGGC-3'	5'-AATGGACCAACTGAACCCCGGC-3'
Gadd45β*	5'-GCTGCGACAATGACATTGACATC-3'	5'-CTCGTTTGTGCCTAGAGTCTCT-3'	
Vgat	5'-ACGACAAACCAAGATCACG-3'	5'-GTAGACCCAGCACGAACATG-3'	5'-TTCCAGCCGCCTTCCCACG-3'
Gad67	5'-ATACTTGTGTGGCGTAGC-3'	5'-AGGAAAGCAGGTTCTTGGAG-3'	5'-AAAACTGGGCCTGAAGATCTGTGGT-3'
GABA$_A$γ2	5'-ACTCATTGTGGTTCTGTCCTG-3'	5'-GCTGTGACATAGAGACCTTG-3'	5'-ATGGTGCTGAGAGTGGTCATCGTC-3'
Pv	5'-CTGGACAAAGACAAAAGTGGC-3'	5'-GACAAGTCTCTGGCATCTGAG-3'	5'-CCTTCAGAATGGACCCCAGCTCA-3'
Sst	5'-ACTCCGTCAGTTTCTGCAG-3'	5'-CAGGGCATCGTTCTCTGTC-3'	5'-AGTTCCTGTTTTCCGGTGGCA-3'
Calb I	5'-AGAACTTGATCCAGGAGCTTC3'-	5'-CTTCGGTGGGTAAGACATGG-3'	5'-TGGGCAGAGAGATGATGGGAAAATAGGA-3'
36B4	5'-TTCCCACTGGCTGAAAAGGT-3'	5'-CGCAGCCCGCAAATGC-3'	5'-AAGGCCTTCCTGGCCGATCCATC-3'

* Primers and probes purchased from Life Technologies (Italy), which did not disclose the sequences. All others were purchased from Eurofins MWG-Operon (Germany).

between an auditory stimulus [fear-conditioned stimulus (CS); 30 s, 2 kHz, 85 dB] and a mild footshock (unconditioned stimulus; 0.6 mA, 1 s), which terminated coincidentally with the end of the fear-CS. On day 1 of the test, animals were allowed to habituate 2 min to the chamber, after which a total of five fear-CS–footshock pairings were given with an interval of 1 min. After the last pairing, rats were left in the chamber for another 2 min before they were returned to their home cage. Fear conditioning and extinction were measured respectively 24 and 48 h after conditioning in the same context, to measure both contextual and cue-induced fear responses. After 2 min habituation (measurement of baseline/contextual freezing), a total of eight fear-CS presentations were given with an interval of 2 min ($\pm 50\%$). Notably, during the first eight sessions, fear conditioning without extinction was measured. Extinction was manifested during the second set of eight sessions, 48 h after fear conditioning. Chambers were cleaned with 70% EtOH after each session. Freezing (no visible movement except respiration) was scored using the software package Observer version 5.0 (Noldus Information Technology, The Netherlands).

RNA preparation for real-time PCR and analysis of mRNA levels

Total RNA was isolated by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (RT-PCR) to assess mRNA levels. A 5 μg aliquot of each sample was treated with DNase to avoid DNA contamination.

RNA was analysed 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 triplicate as multiplex reactions with a normalizing internal control (36B4). Probe and primer sequences of all genes are summarized in Table 1 and were purchased from Eurofins MWG-Operon (Germany) and from Life Technologies. Thermal cycling was initiated with an 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 method was used to calculate the relative target gene expression.

Preparation of subcellular fractions for protein analysis

PFC tissue from the different experimental groups was homogenized in a glass–glass potter in ice-cold 0.32 M

sucrose buffer (pH 7.4) containing 1 mM HEPES, 0.1 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride, in the presence of commercial cocktails of protease (Roche, Italy) and phosphatase (Sigma-Aldrich) inhibitors. The homogenate was clarified (3000 g; 10 min), obtaining a pellet (P1) enriched in nuclear components, which was resuspended in a buffer (20 mM HEPES, 0.1 mM dithio-threitol, 0.1 mM EGTA) supplemented with protease and phosphatase inhibitors. The supernatant (S1) was then centrifuged (12 500 g; 15 min) to obtain a clarified fraction of cytosolic proteins (S2). The pellet (P2), corresponding to the crude membrane fraction, was resuspended in the same buffer used for the nuclear fraction. Total protein content was measured according to the Bradford protein assay procedure (Bio-Rad, Italy), using bovine serum albumin as calibration standard.

Analysis of glucocorticoid receptor translocation

Protein analysis was performed on nuclear (P1) and cy-tosolic (S2) fractions. Equal amounts of protein (15 μg) were run under reducing conditions on an SDS-poly-acrylamide gel (8% SDS-PAGE) and then electro-phoretically transferred onto polyvinylidene fluoride membranes. Unspecific binding sites were blocked for 1 h in 5% bovine serum albumin in Tris-buffered saline con-taining 0.2% sodium azide. Membranes were then in-cubated with the polyclonal rabbit anti-glucocorticoid receptor primary antibody (1:500; Thermo-Scientific) in a blocking solution at 4 °C overnight. Membranes were washed with Tris-buffered saline containing 0.1% Tween-20 and incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:2000) in Tris-buffered saline. Results were standardized using β-actin as control protein, which was detected by evalu-ating the band density at 43 kDa after blocking the membranes with 10% non-fat dry milk and probing them with a polyclonal antibody (1:20 000, Sigma) followed by a 1:20 000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma). Protein levels were calculated by measuring the optical density of the autoradiographic bands using Quantity One software (Bio-Rad). To ensure that the au-toradiographic bands were in the linear range of inten-sity, different exposure times were used. β-Actin was employed as an internal standard because its expression is not regulated by the experimental paradigm used.

Statistical analyses and presentation of data

The effect of drug treatment or genotype on the mRNA or protein levels was analysed by two-way analysis of vari-ance (ANOVA) followed by single contrast *post hoc* test, in which drug (vehicle *vs.* lurasidone) and genotype (wild-type *vs.* SERT$^{-/-}$) were considered as indepen-dent factors. Significance for all tests was assumed for $p < 0.05$. Data are presented as means ± standard error (S.E.M.) and are expressed as % change of wild-type/vehicle-injected animals.
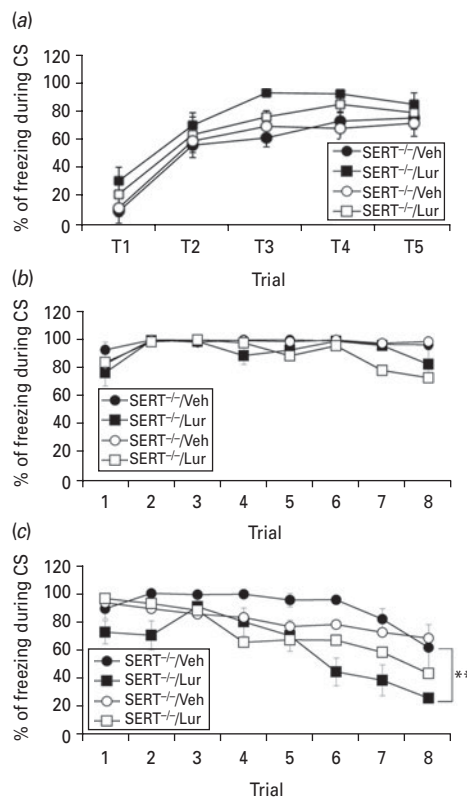

Fig. 1. Effect of chronic lurasidone (Lur) treatment on serotonin transporter knockout (SERT$^{-/-}$) rats in the fear conditioning test. The data show the acquisition curves during acquisition (*a*), extinction (*b*) and extinction recall (*c*). Wild-type (SERT$^{+/+}$) and SERT$^{-/-}$ rats were tested after 14 d subchronic Lur treatment lasting 21 d. The data represent the means ± S.E.M. % freezing during conditioned stimulus (CS) presentation. ** $p < 0.01$, SERT$^{-/-}$/Lur-treated group different from SERT$^{-/-}$/vehicle (Veh)-treated group.

Results

Effect of lurasidone on fear extinction in SERT knockout rats

Pavlovian fear conditioning is a behavioural paradigm with high construct validity. Whereas the fear-associated cue triggers the activation of the PFC, contextual stimuli specifically recruit the hippocampus. Both inhibit – either directly or indirectly – the amygdala (Quirk and Beer, 2006). Since we previously found changes in BDNF ex-pression in both the PFC and hippocampus (Molteni *et al.* 2010), we used the combined cue/contextual fear-con-ditioning test to evaluate the ability of subchronic treat-ment with lurasidone to counteract defects associated with impaired function of SERT.

During fear conditioning, lurasidone-treated rats showed increased freezing in response to the CS (which ended with a footshock; $F_{3,30} = 7.80$, $p < 0.01$). However, there was no significant genotype effect or genotype × treatment interaction ($F > 0.5 < 1.5$; Fig. 1*a*). During the fear-conditioning test, conducted 24 h after fear
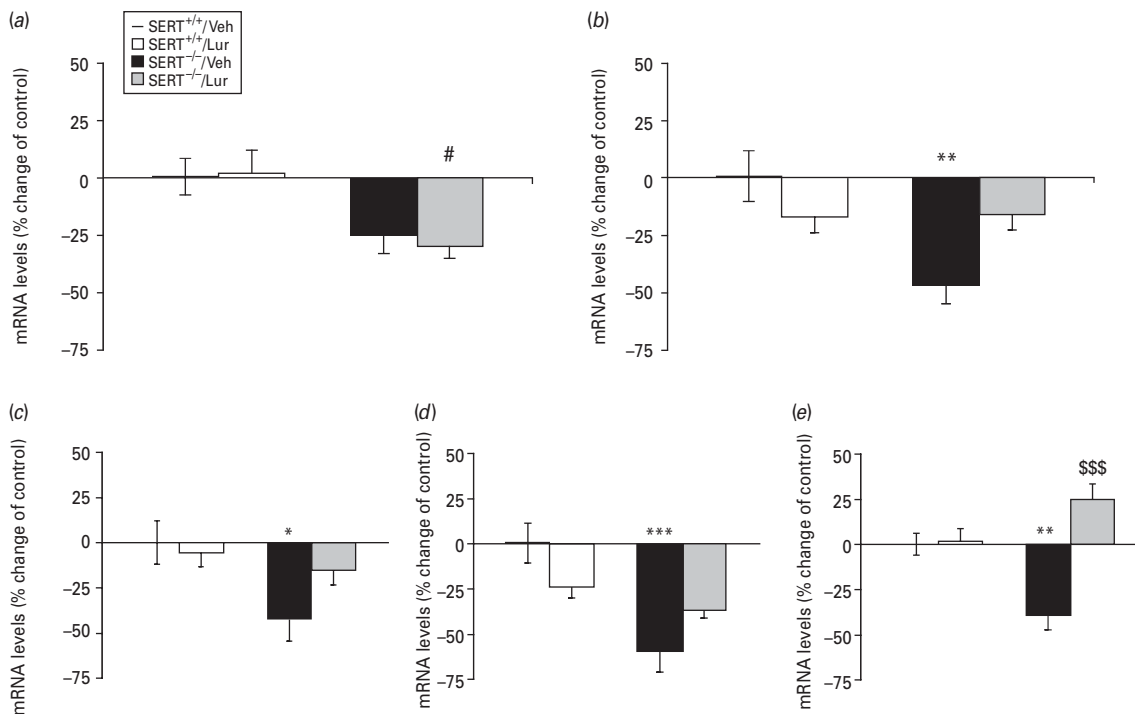
Fig. 2. Chronic lurasidone (Lur) treatment modulates the levels of specific transcripts of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex of serotonin transporter knockout (SERT$^{-/-}$) rats. The mRNA levels for total BDNF (*a*), long 3′-untranslated region BDNF (*b*), BDNF exon I (*c*), IV (*d*) and VI (*e*) were measured in the prefrontal cortex of wild-type (SERT$^{+/+}$) and SERT$^{-/-}$ rats treated for 21 d with vehicle (Veh) or Lur and killed 24 h after the last injection. The data, expressed as a % change over SERT$^{+/+}$/Veh (set at 0%), are the means ± S.E.M. of six to 10 independent determinations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* SERT$^{+/+}$/Veh; # $p < 0.05$ *vs.* SERT$^{+/+}$/Lur; $$$ $p < 0.001$ *vs.* SERT$^{-/-}$/Lur (two-way analysis of variance with single contrast *post hoc* test).

conditioning, no significant differences in freezing were observed during baseline (reflecting contextual freezing), as measured for 2 min before presentation of the first auditory stimulus ($F > 1 < 2$ for all comparisons, data not shown). When freezing during tone presentations was analysed, a near significant treatment effect was noted ($F_{3,30} = 4.15$, $p = 0.053$; Fig. 1*b*). There was no genotype effect ($F_{3,30} = 0.01$, n.s.) and no treatment × genotype interaction ($F_{3,30} = 0.76$, n.s.). Furthermore, none of the interaction terms was significant ($F \leq 1.8$, n.s.). Also, during the first extinction trial no significant effects were found (treatment: $F_{3,30} = 1.23$, n.s.; genotype: $F_{3,30} = 0.030$, n.s.; treatment × genotype interaction: $F_{3,30} = 0.922$, n.s.). Forty-eight hours following the fear-conditioning test, the animals were tested for fear extinction. Again, no significant differences were noted during baseline ($F > 1 < 2$ for all comparisons, data not shown), prior to presentation of the tone. There was an overall significant genotype × treatment interaction ($F_{3,30} = 4.73$, $p < 0.05$), indicating that lurasidone had a differential effect in SERT$^{-/-}$ and wild-type rats. We also found a significant treatment effect ($F_{3,30} = 8.50$, $p < 0.005$), but no overall genotype effect ($F_{3,30} = 0.53$, n.s.; Fig. 1*c*). There was a significant trial × genotype interaction ($F_{3,30} = 5.20$, $p < 0.05$), but the trial × treatment interaction just missed significance ($F_{3,30} = 5.20$, $p = 0.056$). There was no significant trial × treatment × genotype interaction ($F_{3,30} = 0.71$,

n.s.). Subsequent testing revealed that lurasidone significantly increased fear extinction in SERT$^{-/-}$ rats ($F_{2,13} = 11.94$, $p < 0.01$), without a significant trial × treatment interaction ($F_{2,13} = 1.58$, n.s.). In SERT$^{+/+}$ rats, on the other hand, lurasidone did not affect fear extinction ($F_{2,17} = 0.33$, n.s.). Finally, overall fear extinction was not significantly different between vehicle treated SERT$^{+/+}$ and SERT$^{-/-}$ rats ($F_{2,14} = 2.72$, n.s.), although repeated measures ANOVA revealed that SERT$^{-/-}$ rats displayed more freezing than wild-type rats during sessions 2, 3, 4, 5 and 6 ($F_{2,17} = 5.87$, $p < 0.05$). Therefore, our data show that SERT$^{-/-}$, but not wild-type animals, profit from lurasidone treatment.

Restorative properties of lurasidone on alterations of BDNF expression in the prefrontal cortex of SERT$^{-/-}$ rats

It is known that the neurotrophin BDNF within the PFC plays an important role in fear extinction (Peters *et al.* 2010) and we have also previously shown that SERT$^{-/-}$ rats show reduced expression of the neurotrophin in this brain region (Molteni *et al.* 2010). On these bases, we investigated whether BDNF expression in the PFC of SERT$^{-/-}$ rats could be modulated by chronic lurasidone treatment.

As shown in Fig. 2 and in agreement with our previous data (Molteni *et al.* 2010), the expression of BDNF was

significantly reduced in the PFC of SERT$^{-/-}$ rats, alteration that was modulated by chronic lurasidone treatment. In detail, we found that total BDNF mRNA levels were significantly reduced in SERT$^{-/-}$ rats independently from the treatment (genotype effect: $F_{1,36} = 12.79$, $p < 0.01$) and that lurasidone did not produce any significant alteration in the expression of the neurotrophin (treatment effect: $F_{1,36} = 0.02$, $p = 0.879$; Fig. 2a). Conversely, with regard to the mRNA levels for long 3′ untranslated region (UTR) BDNF (Fig. 2b), which identifies a subset of transcripts that may be targeted to dendrites (An et al. 2008), we found a significant genotype effect ($F_{1,33} = 6.46$, $p < 0.05$) and a significant genotype × treatment interaction ($F_{1,33} = 6.63$, $p < 0.05$). Indeed, its mRNA levels were significantly reduced in SERT$^{-/-}$ rats (-47% vs. SERT$^{+/+}$/vehicle, $p < 0.01$) and, while chronic administration of lurasidone did not alter the expression in wild-type animals (-17% vs. SERT$^{+/+}$/vehicle, n.s.), it largely restored the levels of long 3′-UTR BDNF mRNA expression in SERT$^{-/-}$ rats (-16% vs. SERT$^{+/+}$/vehicle, n.s.). These results suggest that lurasidone selectively modulates the neurotrophin in the PFC of SERT$^{-/-}$ rats.

In order to further characterize this modulatory activity, we investigated the expression of three major BDNF transcripts, which are controlled by separate promoters and may be differentially targeted to dendrites (Chiaruttini et al. 2008). In line with our previous work (Molteni et al. 2010), the mRNA levels of exon I, IV and VI were all significantly down-regulated in SERT$^{-/-}$ rats, although they underwent differential changes in response to chronic lurasidone treatment (Fig. 2). Specifically, the mRNA levels for BDNF exon I were reduced in these animals (-42% vs. SERT$^{+/+}$/vehicle, $p < 0.05$), alteration that was normalized by chronic lurasidone administration (-15% vs. SERT$^{+/+}$/vehicle, n.s.; Fig. 2c). Instead, when considering the expression of exon IV, we found a significant genotype effect ($F_{1,34} = 17.37$, $p < 0.001$) and a significant genotype × treatment interaction ($F_{1,34} = 7.41$, $p < 0.05$). The mRNA levels of exon IV were markedly decreased in SERT$^{-/-}$ rats (-60% vs. SERT$^{+/+}$/vehicle, $p < 0.001$; Fig. 2d), effect that was, to some extent, counteracted by lurasidone.

Finally, we found a significant treatment ($F_{1,31} = 16.84$, $p < 0.001$) and genotype × treatment interaction ($F_{1,31} = 14.84$, $p < 0.001$) when investigating the expression of BDNF exon VI (Fig. 2e), which is the main neurotrophin transcript that undergoes dendritic targeting (Chiaruttini et al. 2008; Baj et al. 2012). Exon VI mRNA levels were in fact significantly down-regulated in SERT$^{-/-}$ animals (-39% vs. SERT$^{+/+}$/vehicle, $p < 0.01$) and chronic lurasidone administration completely normalized these changes, actually leading to a significant up-regulation of its mRNA levels when compared to vehicle-injected/SERT$^{+/+}$ animals ($p < 0.001$ vs. SERT$^{-/-}$/vehicle). Of note, chronic administration of lurasidone to wild-type animals did not produce any significant change of exon I,

Table 2. Chronic lurasidone (Lur) treatment modulates the expression of brain-derived neurotrophic factor-related transcription factors in the prefrontal cortex of serotonin transporter knockout (SERT$^{-/-}$) rats

	SERT$^{+/+}$/ Veh	SERT$^{+/+}$/ Lur	SERT$^{-/-}$/ Veh	SERT$^{-/-}$/ Lur
NPAS4	100 ± 11	74 ± 7	62 ± 7*	76 ± 5
ARNT2	100 ± 9	111 ± 5	111 ± 7	$149 \pm 11$$^{\$/\#}$
CaRF	100 ± 7	94 ± 8	73 ± 6*	$112 \pm 6$$^{\$\$}$
NF-κB	100 ± 9	87 ± 8	69 ± 8*	$97 \pm 6$$^{\$}$

* $p < 0.05$ vs. SERT$^{+/+}$/vehicle [two-way analysis of variance (ANOVA) with single contrast post hoc test (SCPHT)]; $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$ vs. SERT$^{-/-}$/vehicle (two-way ANOVA with SCPHT); $^{\#}$ $p < 0.05$ vs. SERT$^{+/+}$/lurasidone (two-way ANOVA with SCPHT).
The mRNA levels for neuronal PAS domain protein 4 (NPAS4), aryl-hydrocarbon receptor nuclear translocator 2 (ARNT2), calcium-response factor (CaRF) and nuclear factor (NF)-κB were measured in the prefrontal cortex of wild-type (SERT$^{+/+}$) and SERT$^{-/-}$ rats treated for 21 d with vehicle (Veh) or Lur and killed 24 h after the last injection. The data, expressed as a % of SERT$^{+/+}$/VEH (set at 100%), are the means ± S.E.M. of at least five independent determinations.

IV and VI mRNA levels (see Fig. 2c–e), suggesting that the novel antipsychotic exerts a restorative activity specifically in SERT$^{-/-}$ rats.

To better understand how lurasidone may produce different transcriptional effects in SERT$^{-/-}$ animals, we decided to analyse the expression of transcription factors known to regulate BDNF mRNA expression (Tao et al. 2002; Pruunsild et al. 2011). Exon IV represents the best characterized transcript, which is regulated by different factors including neuronal PAS domain protein 4 (NPAS4), nuclear factor-κB, calcium-response factor (CaRF) and aryl-hydrocarbon receptor nuclear translocator 2 (Arnt2), some of which may also cooperate in regulating exon I transcription (Lin et al. 2008; Pruunsild et al. 2011). With regard to NPAS4 (Table 2), we found a significant genotype effect ($F_{1,28} = 4.29$, $p < 0.05$) and a significant genotype × treatment interaction ($F_{1,28} = 5.25$, $p < 0.05$). In line with our previous results (Guidotti et al. 2011), SERT$^{-/-}$ rats show a significant reduction of NPAS4 mRNA levels in the PFC (-38% vs. SERT$^{+/+}$/vehicle, $p < 0.05$), although this effect was minimally affected by chronic lurasidone administration.

The ameliorative effects produced by chronic lurasidone treatment on the expression of BDNF exon I (and to less extent on exon IV) in SERT$^{-/-}$ rats could be sustained by changes of Arnt2 (Table 2), which cooperates with NPAS4 in their transcriptional regulation (Pruunsild et al. 2011). Indeed, when investigating the mRNA levels for Arnt2, there was a significant treatment effect ($F_{1,45} = 7.57$, $p < 0.01$), its expression being
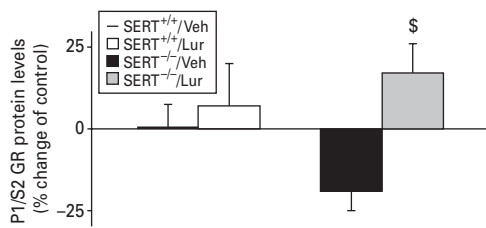
Fig. 3. Chronic lurasidone (Lur) treatment modulates the translocation of glucocorticoid receptor (GR) protein from the cytosolic to the nuclear compartment in the prefrontal cortex of serotonin transporter knockout (SERT$^{-/-}$) rats. The ratio of the GR protein levels between the P1 and S2 fractions were measured in the prefrontal cortex of wild-type (SERT$^{+/+}$) and SERT$^{-/-}$ rats treated for 21 d with vehicle (Veh) or Lur and killed 24 h after the last injection. The data, expressed as a % change over SERT$^{+/+}$/Veh (set at 0%), are the means ± S.E.M. of at least four independent determinations. $ $p<0.05$ *vs.* SERT$^{-/-}$/Veh (two-way analysis of variance with single contrast *post hoc* test).

Fig. 4. Chronic lurasidone (Lur) treatment modulates the expression of Gadd45β in the prefrontal cortex of serotonin transporter knockout (SERT$^{-/-}$) rats. The mRNA levels for Gadd45β was measured in the prefrontal cortex of wild-type (SERT$^{+/+}$) and SERT$^{-/-}$ rats treated for 21 d with vehicle (Veh) or Lur and killed 24 h after the last injection. The data, expressed as a % change over SERT$^{+/+}$/Veh (set at 0%), are the means ± S.E.M. of at least nine independent determinations. *** $p<0.001$ *vs.* SERT$^{+/+}$/Veh; $ $p<0.05$ *vs.* SERT$^{-/-}$/Veh (two-way analysis of variance with single contrast *post hoc* test).

up-regulated by chronic lurasidone administration in SERT$^{-/-}$ rats (+49% *vs.* SERT$^{+/+}$/vehicle, $p<0.05$ *vs.* SERT$^{-/-}$/vehicle).

We next investigated the expression of CaRF, another important regulator of BDNF transcription and in particular of exon IV (Tao *et al.* 2002; Zheng *et al.* 2011). Although we did not find significant genotype or treatment effects, there was a statistically significant genotype × treatment interaction ($F_{1,31}=5.35$, $p<0.05$). In fact, while lurasidone did not alter the expression of the transcription factor expression when given to wild-type animals (−6%, *vs.* SERT$^{+/+}$/vehicle, n.s.), CaRF mRNA levels were significantly reduced in SERT$^{-/-}$ rats (−27% *vs.* SERT$^{+/+}$/vehicle, $p<0.05$) and normalized by chronic lurasidone treatment (+12% *vs.* SERT$^{+/+}$/vehicle, $p<0.01$ *vs.* SERT$^{-/-}$/vehicle) (Table 2).

Hereafter, we also observed a significant genotype × treatment effect ($F_{1,34}=6.48$, $p<0.05$) in the regulation of NFκB (Table 2). The expression of the transcription factor was significantly reduced in SERT$^{-/-}$ rats (−31% *vs.* SERT$^{+/+}$/vehicle, $p<0.05$), an effect that was normalized by chronic lurasidone treatment, which had no effect when administered to wild-type animals.

Given that the major modulatory activity of lurasidone in SERT$^{-/-}$ rats was exerted on exon VI and considering that exon VI promoter is the major target for corticosterone-mediated transcriptional regulation of BDNF (Hansson *et al.* 2006), we investigated the possibility that chronic lurasidone treatment could modulate the nuclear translocation of glucocorticoid receptors (GR). We therefore analysed the ratio of GR protein levels between nuclear (P1) and cytosolic (S2) compartment (P1/S2), as a measure of its transcriptional activation and we found a significant treatment effect ($F_{1,19}=6.01$, $p<0.05$; Fig. 3). In particular, GR translocation from the cytosol to the nucleus is reduced in SERT$^{-/-}$ rats, although the effect did not reach statistical significance (−19% *vs.* SERT$^{+/+}$/
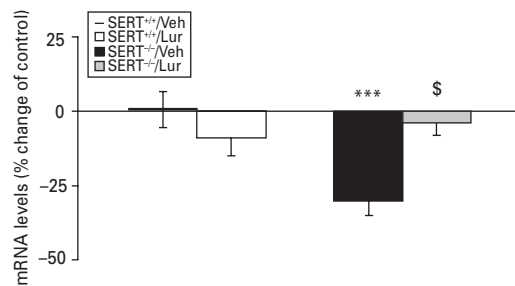
vehicle, n.s.). However, chronic treatment of SERT$^{-/-}$ rats with lurasidone increased GR translocation to the nucleus (+17% *vs.* SERT$^{+/+}$/vehicle, $p<0.05$ *vs.* SERT$^{-/-}$/vehicle), an effect that may contribute to the increased transcription of BDNF exon VI (Fig. 2*e*).

Effect of lurasidone treatment on the expression of the DNA demethylating gene Gadd45β in SERT$^{-/-}$ rats

Several rodent studies suggest that epigenetic mechanisms may account for long-term changes in BDNF expression (Martinowich *et al.* 2003). Indeed, we have previously shown that reduced expression of BDNF exon VI in SERT$^{-/-}$ rats is due to increased methylation of its promoter (Molteni *et al.* 2010). This was sustained by the reduced expression of the mRNA levels of growth arrest and DNA-damage-inducible β (*Gadd45β*), a gene involved in DNA demethylation of several promoters including BDNF (Ma *et al.* 2009). On these bases, we decided to investigate the mRNA levels of *Gadd45β* in response to chronic lurasidone administration in wild-type and SERT$^{-/-}$ rats. We found a significant genotype effect ($F_{1,46}=4.95$, $p<0.05$) and a significant genotype × treatment interaction ($F_{1,46}=8.89$, $p<0.01$). Indeed, as shown in Fig. 4, *Gadd45β* mRNA levels were significantly reduced in SERT$^{-/-}$ rats (−30% *vs.* SERT$^{+/+}$/vehicle, $p<0.001$), whereas chronic lurasidone treatment, which *per se* had no significant effect in wild-type animals (−9% *vs.* SERT$^{+/+}$/vehicle, n.s.), completely restored the mRNA levels of the demethylating gene when given to SERT$^{-/-}$ rats (−4% *vs.* SERT$^{+/+}$/vehicle, n.s. and $p<0.05$ *vs.* vehicle-treated/SERT$^{-/-}$ rats).

Modulation of GABA-related genes in SERT$^{-/-}$ rats after chronic lurasidone administration

We have recently demonstrated that alterations of BDNF expression in SERT$^{-/-}$ rats are paralleled by changes of
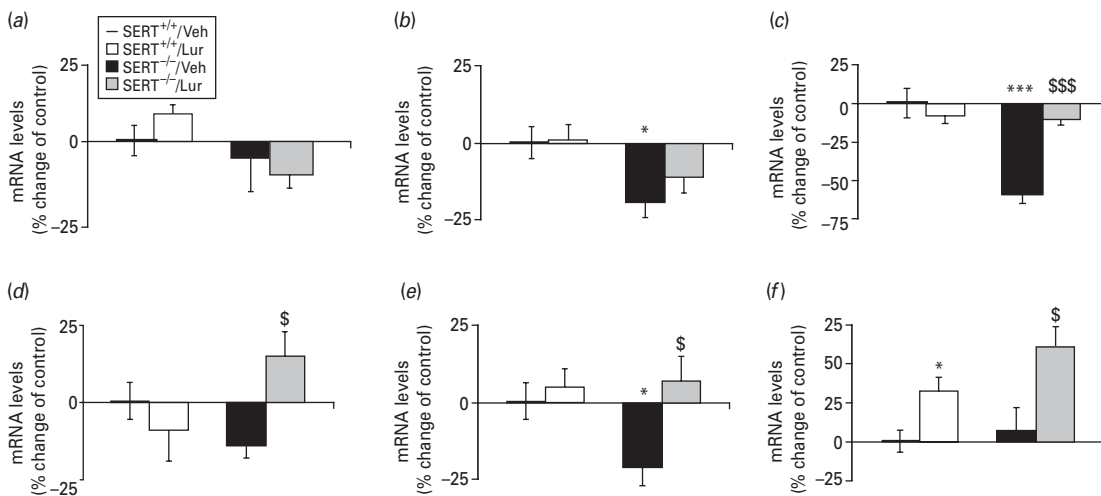
Fig. 5. Chronic lurasidone (Lur) treatment modulates the expression of GABAergic system markers in the prefrontal cortex of serotonin transporter knockout (SERT$^{-/-}$) rats. The mRNA levels for vesicular GABA transporter (*a*), GABA producing enzyme (*b*) and GABA$_A$-γ2 (*c*), parvalbumin (*d*), somatostatin (*e*) and calbindin (*f*) were measured in the prefrontal cortex of wild-type (SERT$^{+/+}$) and SERT$^{-/-}$ rats treated for 21 d with vehicle (Veh) or Lur and killed 24 h after the last injection. The data, expressed as a % change over SERT$^{+/+}$/Veh (set at 0%), are the means $\pm$ S.E.M. of at least six independent determinations. * $p < 0.05$, *** $p < 0.001$ *vs.* SERT$^{+/+}$/Veh; $^\$$ $p < 0.05$, $^{\$\$\$}$ $p < 0.001$ *vs.* SERT$^{-/-}$/Veh (two-way analysis of variance with single contrast *post hoc* test).

the GABAergic system in the hippocampus and PFC (Guidotti *et al.* 2011). Based on the close association between BDNF and GABA, we investigated the possibility that chronic lurasidone treatment in SERT$^{-/-}$ rats could be associated with significant changes in the expression of GABAergic markers within the PFC.

First, we investigated the expression of three key elements of the GABAergic synapse, namely the vesicular GABA transporter (VGAT), the GABA-producing enzyme (GAD67) and the post-synaptic GABA$_A$-receptor γ2 subunit (GABA$_A$-γ2) in the PFC of SERT$^{-/-}$ rats. As shown in Fig. 5*a*, we did not find any significant change in the expression of VGAT mRNA levels, whereas the expression of GAD67 was significantly modulated by SERT genotype ($F_{1,45} = 8.77$, $p < 0.01$), although there was no significant genotype × treatment interaction ($F_{1,45} = 0.34$, $p = 0.566$). The mRNA levels of GAD67 were significantly reduced in SERT$^{-/-}$ rats (-19% *vs.* SERT$^{+/+}$/vehicle, $p < 0.05$; Fig. 5*b*), an effect that was not observed when mutant animals were chronically treated with lurasidone (-11% *vs.* SERT$^{+/+}$/vehicle, n.s.). With regard to the mRNA levels encoding for GABA$_A$-γ2 (Fig. 5*c*), a GABA receptor subunit primarily localized at postsynaptic level, we found a significant gene effect ($F_{1,34} = 15.46$, $p < 0.001$), treatment effect ($F_{1,34} = 6.47$, $p < 0.05$) and a significant gene × treatment interaction ($F_{1,34} = 12.94$, $p < 0.01$). Accordingly, GABA$_A$-γ2 mRNA levels were markedly reduced in SERT$^{-/-}$ rats (-56% *vs.* SERT$^{+/+}$/vehicle, $p < 0.001$) and this effect was normalized by chronic lurasidone treatment (-10% *vs.* SERT$^{+/+}$/vehicle, n.s. and $p < 0.001$ *vs.* SERT$^{-/-}$/vehicle).

In order to establish whether the changes observed for the GABAergic system were due to or related with the

modulation of subtypes of GABAergic neurons in the PFC, we investigated the expression of a number of putative markers, including parvalbumin (PV), calbindin (CALB1) and somatostatin (SST). Specifically, with regard to PV we found a significant genotype × treatment interaction ($F_{1,44} = 7.26$, $p < 0.05$; Fig. 5*d*). PV mRNA levels were in fact reduced in vehicle-treated SERT$^{-/-}$ rats (-14% *vs.* SERT$^{+/+}$/vehicle), although the effect did not reach statistical significance, but its expression was increased in SERT$^{-/-}$ rats that received chronic lurasidone ($+15\%$ *vs.* SERT$^{+/+}$/vehicle, $p < 0.05$ *vs.* SERT$^{-/-}$/vehicle). A similar profile was also found for SST, with a significant treatment effect ($F_{1,47} = 6.03$, $p < 0.05$; Fig. 5*e*). The expression of SST was reduced in SERT$^{-/-}$ rats (-21% *vs.* SERT$^{+/+}$/vehicle, $p < 0.05$), an effect that was completely normalized by chronic lurasidone administration ($+7\%$ *vs.* SERT$^{+/+}$/vehicle, n.s.; $p < 0.05$ *vs.* SERT$^{-/-}$/vehicle). A somewhat different pattern of changes was found for CALB1, whose expression was modulated by lurasidone treatment ($F_{1,33} = 14.09$, $p < 0.001$) independently from the genotype [no gene × treatment interaction ($F_{1,33} = 0.21$, $p = 0.650$)]. In fact, while CALB1 mRNA levels were not altered in vehicle-treated SERT$^{-/-}$ rats, chronic administration of lurasidone increased its mRNA levels in wild-type ($+33\%$ *vs.* SERT$^{+/+}$/vehicle, $p < 0.05$) as well as in SERT$^{-/-}$ rats ($+61\%$ *vs.* SERT$^{+/+}$/vehicle, $p < 0.05$ *vs.* SERT$^{-/-}$/vehicle).

We also investigated neuropeptide Y and tachykinin 1, two other markers of GABAergic neurons (Guilloux *et al.* 2011). However, no significant changes were found with regard to the expression of neuropeptide Y and tachykinin 1 as a consequence of SERT deletion or chronic lurasidone treatment (data not shown).

Discussion

In this study we demonstrate that the novel antipsychotic lurasidone increases fear extinction in SERT$^{-/-}$ rats and that this effect may be sustained by BDNF modulation, as well as by changes in the GABAergic system within the PFC.

The failure to extinguish fear is a key feature of anxiety and PTSD and it is a mechanism that can be modulated by gene variants of SERT (Bryant *et al.* 2010). In fact, in analogy with the human 5-HT transporter-linked polymorphic region findings (Pezawas *et al.* 2005; Heinz *et al.* 2007), SERT$^{-/-}$ rats show a failure to extinguish fear after fear conditioning (Nonkes *et al.* 2012). In this study, we observed a significant genotype × treatment interaction, indicating that lurasidone differentially increased fear extinction (as measured 48 h after fear conditioning) in SERT$^{-/-}$ rats. In turn, this suggests that chronic administration of the novel antipsychotic may improve some of the behavioural deficits observed in these animals. This effect of lurasidone is not likely to be due to its effects on the acquisition of fear conditioning, because conditioned freezing during acquisition was increased in lurasidone-treated rats, irrespective of genotype. In fact, due to lurasidone's effect on acquisition, the effect of lurasidone on extinction (recall) effectively may be larger than presented. In contrast to our previous study that measured cue-induced conditional fear (Nonkes *et al.* 2012), we found no significant fear extinction deficit in SERT$^{-/-}$ rats. Most likely, this is because the animals were exposed to the conditional tone in the training context, such that cue- and context fear conditioning were measured simultaneously. The baseline measurement revealed no genotype differences in contextual freezing and the absence of genotype difference in contextual freezing may have weakened the impact of the cue-induced freezing. Yet, there was a tendency towards impaired fear extinction in SERT$^{-/-}$ rats under these conditions, implying a stronger contribution of the PFC rather than the hippocampus to the behavioural effects of lurasidone. Separate contextual and cue-induced fear conditioning tests in future experiments would be helpful to confirm this implication.

Several studies have demonstrated that BDNF affects extinction of conditioned fear memories (Kaplan and Moore, 2011). For example, extinction of conditioned fear leads to an increase in BDNF mRNA expression in the PFC and BDNF micro-infusion into the PFC induces fear extinction (Bredy *et al.* 2007). Thus, the ability of lurasidone to improve fear extinction in SERT$^{-/-}$ rats is in line with the selective modulatory activity exerted by lurasidone on BDNF expression within the PFC. Our data in fact suggest that the restorative properties of lurasidone on the BDNF changes found in the PFC of SERT$^{-/-}$ rats may be primarily related to its ability to regulate exon VI expression, although significant changes were also found for exon I and exon IV. Since BDNF exon VI is the main contributor to BDNF mRNAs that undergo dendritic targeting (Baj *et al.* 2011), these results are in strong agreement with the observation that the main modulatory effect of lurasidone is exerted on the pool of neurotrophin transcripts carrying a long 3′UTR. These transcripts can undergo activity-dependent targeting to the synaptic compartment (An *et al.* 2008) and may be subjected to rapid and robust activation of translation upon neuronal activation (Lau *et al.* 2010), representing a mechanism for the fine tuning of BDNF function and responsiveness.

The mechanisms through which lurasidone regulates BDNF expression may involve different transcription factors that cooperate in the modulation of neurotrophin transcripts (Pruunsild *et al.* 2011). Indeed, lurasidone up-regulates the expression of Arnt2, which cooperates with NPAS4 in promoting the transcription of BDNF exon I and IV. In addition, chronic lurasidone normalizes the reduced expression of NFκB and CaRF in SERT$^{-/-}$ rats, which may also contribute to the transcriptional regulation of BDNF (Pruunsild *et al.* 2011). However, it remains to be established whether similar changes can be found at protein level.

We also hypothesize that the modulation of GR contributes to the changes in BDNF produced by lurasidone, because chronic antipsychotic treatment increased the nuclear translocation of GR in SERT$^{-/-}$ rats. We further suggest that the restorative properties of lurasidone on BDNF expression may be sustained by epigenetic mechanisms, as we have previously shown that reduced expression of some BDNF transcripts is due to epigenetic changes (Molteni *et al.* 2010). In particular, with regard to exon VI, the transcript primarily modulated by lurasidone in SERT$^{-/-}$ rats, we found that its down-regulation in these animals was due to increased methylation of its promoter, an effect that may be sustained by the reduced expression of the DNA demethylating enzyme Gadd45β (Ma *et al.* 2009; Molteni *et al.* 2010). We show that chronic lurasidone treatment normalizes the reduced expression of Gadd45β found in SERT$^{-/-}$ rats and, as a consequence of this, we suggest that the significant up-regulation of BDNF exon VI expression in SERT$^{-/-}$ rats that received chronic lurasidone could be sustained by reduced methylation of its promoter. These results highlight the possibility that Gadd45β can be pharmacologically modulated and provide further support to the notion that epigenetic-related mechanisms could indeed represent an important aspect for the long-term activity of psychotropic drugs, including lurasidone.

Chronic treatment with lurasidone not only restores the reduced expression of BDNF in the PFC of SERT$^{-/-}$ rats, but also ameliorates the impaired expression of GABAergic markers in knockout animals, in particular through a strong effect on the mRNA levels for the post-synaptic GABA$_A$-γ2 receptor subunit, as well as on peptides that co-localize with GABA and that characterize specific subtypes of GABAergic neurons. These results

provide further support to the notion that neuronal defects associated with the SERT genotype can be restored by chronic treatment with lurasidone.

Although an alteration of GABAergic function cannot be considered a hallmark for a specific psychiatric condition, the reduced expression of several GABAergic markers in the PFC of schizophrenic and depressed subjects suggests that these changes may be shared by both disorders (Mellios *et al.* 2009). There is also evidence for a significant correlation between GAD67 and SST (Hashimoto *et al.* 2008), suggesting that GAD67 mRNA expression in SERT$^{-/-}$ rats could be decreased in the subset of GABA neurons that express SST. In the cerebral cortex, SST co-localizes with GABA and has similar inhibitory functions on post-synaptic target neurons, which may summate or synergize with GABA function.

There are some similarities between the effects of lurasidone and those produced by the serotonin–norepinephrine reuptake inhibitor antidepressant duloxetine in the same model (Calabrese *et al.* 2010; Guidotti *et al.* 2011). However, while the modulation of some BDNF transcripts, such as exon VI, could represent a common downstream target for duloxetine and lurasidone, the mechanisms that may lead to such changes might differ, seen by the selective modulation of GADD45β by chronic lurasidone, but not by duloxetine (F. Calabrese and M. A. Riva, unpublished observations).

The neuroplastic properties of lurasidone occur despite the significant antagonism at dopamine D$_2$ receptors (estimated to be about 70–80% at the dose employed) (Ishibashi *et al.* 2010), which is expected to have inhibitory effects on BDNF expression (Fumagalli *et al.* 2003; Park *et al.* 2009). This suggests that other mechanisms, such as 5-HT$_7$ antagonism or 5-HT$_{1A}$ partial agonism, alone or in combination (Ishibashi *et al.* 2010; Li *et al.* 2012), as well as the modulation of glutamate *N*-Methyl-D-aspartate receptors (Yuen *et al.* 2012), may be responsible for the modulatory activity of lurasidone seen on BDNF and the GABAergic system. Future studies will try to establish if lower doses of lurasidone, proven to be effective in animal models of depression (Ishibashi *et al.* 2010; Hedlund *et al.* 2011), will also possess neuroplastic properties in the SERT model.

In summary, these results highlight the strong modulatory activity of lurasidone on neurotrophic mechanisms in the PFC, which have implications in enhancing neuronal plasticity, but may also contribute to the improvement of neurotransmitter-associated dysfunctions and ultimately to the recovery of the functional changes associated with mood disorders.

Acknowledgements

Statement of Interest

None.

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